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## Incompetence of the Asian Longhorned Tick (Acari: Ixodidae) in Transmitting the Agent of Human Granulocytic Anaplasmosis in the United States

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### Abstract

The Asian longhorned tick, *Haemaphysalis longicornis* Neumann (Acari: Ixodidae), was recently introduced into the United States and is now established in at least 15 states. Considering its ability for parthenogenetic propagation and propensity for creating high-density populations, there is concern that this tick may become involved in transmission cycles of endemic tick-borne human pathogens. Human granulocytic anaplasmosis (HGA) caused by *Anaplasma phagocytophilum* is one of the more common tick-borne diseases in the United States, especially in the northeastern and midwestern states. There is considerable geographical overlap between HGA cases and the currently known distribution of *H. longicornis*, which creates a potential for this tick to encounter *A. phagocytophilum* while feeding on naturally infected vertebrate hosts. Therefore, we evaluated the ability of *H. longicornis* to acquire and transmit the agent of HGA under laboratory conditions and compared it to the vector competence of *I. scapularis*. *Haemaphysalis longicornis* nymphs acquired the pathogen with the bloodmeal while feeding on infected domestic goats, but transstadial transmission was inefficient and PCR-positive adult ticks were unable to transmit the pathogen to naïve goats. Results of this study indicate that the Asian longhorned tick is not likely to play a significant role in the epidemiology of HGA in the United States.

### Keywords

*Haemaphysalis longicornis* ; Asian longhorned tick; *Anaplasma phagocytophilum* ; human granulocytic anaplasmosis; vector competence

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*Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), is an obligately intracellular bacterium that causes an acute febrile illness known as human granulocytic anaplasmosis (HGA). From a human perspective, it is considered one of the most important species because of its high zoonotic potential (Chen et al. 1994). Symptoms of HGA are nonspecific

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and include fever, chills, headache, myalgia, and malaise; laboratory findings of leukopenia, thrombocytopenia, and elevated liver enzymes are common (Bakken et al. 1994). When left untreated, anaplasmosis can be severe and even fatal (Hardalo et al. 1995). *Anaplasma phagocytophilum* is also the etiological agent of tick-borne fever (TBF) in ruminants as well as of equine and canine granulocytic anaplasmosis (EGA and CGA, respectively) (Dumler et al. 2001, Woldehiwet 2010). Infections with *A. phagocytophilum* in humans and animals are commonly reported on every continent of the Northern hemisphere. Clinical cases of HGA are both more serious and more frequent in the United States, where the human illness was first recognized in the mid-1990s but did not become nationally notifiable until 1999 (as reviewed by Dumler et al. 2003). Since then, the incidence of HGA in the United States has been increasing almost every year with approximately 3,500–6,000 cases reported to CDC annually in 2015–2018 and considerable underreporting of HGA cases is suspected. The incidence and severity of HGA varies by geographic region with most cases occurring in the northeastern and upper Midwest states (CDC 2020).

Based on analysis of the *16S rRNA* gene sequence, several variants of *A. phagocytophilum* have been genetically characterized from various ticks and vertebrate host species around the world. In the United States, there are at least two major variants of the pathogen: Ap-ha and Ap-V1 (Massung et al. 2002, Michalski et al. 2006). The Ap-ha variant is infectious to humans, small rodents, and several medium-sized mammals, whereas the white-tailed deer (*Odocoileus virginianus*) is a major reservoir host for the Ap-V1 variant, but not Ap-ha (Massung et al. 2003, Massung et al. 2005, Reichard et al. 2009). Importantly, the Ap-ha variant is pathogenic for humans, dogs, and horses whereas Ap-V1 has never been implicated in human infections.

Worldwide, the primary vectors of *A. phagocytophilum* are ticks belonging to the *Ixodes ricinus* complex, including *I. Ricinus* Linnaeus in Europe, *I. persulcatus* Schulze in Asia, *I. scapularis* Say in eastern North America, and *I. pacificus* Cooley & Kohls in western North America (Woldehiwet 2010). These ticks acquire the pathogen as either larvae or nymphs with the bloodmeal while feeding on infected vertebrate hosts, maintain infection through the molt into the next life stage, and transmit it to susceptible animals during the next feeding. In addition to the aforementioned tick species, *A. phagocytophilum* has been detected in questing *I. dentatus* Marx, *I. spinipalpis* Hadwen & Nuttall, *Amblyomma americanum* Linnaeus, *Dermacentor variabilis* Say, and *D. occidentalis* Marx in the United States, as well as in questing *I. ovatus* Neumann, *I. nipponensis* Kitaoka & Saito, *I. ventalloi* Gil Collado, *D. silvarum* Olenov, *D. reticulatus* Fabricius, *Haemaphysalis concinna* Koch, *H. megaspinosa* Saito, *H. douglasi* Nuttall & Warburton, *H. longicornis*, and *H. japonica* Warburton in Europe and Asia (as reviewed by Stuen et al. 2013). The vector competence of these tick species as well as their contribution to the endemic cycle of *A. phagocytophilum* (vectorial capacity) remain to be investigated.

Detection of *A. phagocytophilum* DNA in the Asian longhorned tick *H. longicornis* (e.g., Kim et al. 2003, Chen et al. 2014, Dong et al. 2014, Fukui and Inokuma 2019) presents a particular concern for human and veterinary health in the United States because this invasive tick has become established in North America and is currently present in at least 15 eastern states including those with a high incidence of human anaplasmosis (Beard et

al. 2018, United States Department of Agriculture. 2020). Originally native to East Asia (Japan, Korea, and China), *H. longicornis* has spread and become established in the Russian Far East, Australia, New Zealand, New Caledonia, and several island nations in Polynesia (Hoogstraal et al. 1968, Roberts 1970, Steele 1977). In China and Japan, this tick is the primary vector of the severe fever with thrombocytopenia syndrome virus (SFTSV), which causes a human hemorrhagic fever (Luo et al. 2015), and *Rickettsia japonica*, which causes Japanese spotted fever (Uchida et al. 1995, Tabara et al. 2011). In addition, several studies in Asia identified DNA of *Anaplasma*, *Bartonella*, *Borrelia*, and *Ehrlichia* species in *H. longicornis* ticks collected from either vertebrate hosts or vegetation (Luo et al. 2015, Kang et al. 2016, Yang et al. 2018). At least one paper referred to *H. longicornis* as a major vector of *A. phagocytophilum* in East Asian countries, though this claim was not supported by evidence or citations (Tirloni et al. 2015). In all its life stages, *H. longicornis* prefers to feed on medium to large-sized mammals (Heath 2016). In the United States, its host spectrum includes raccoons and gray squirrels (United States Department of Agriculture 2020), both of which are known reservoir species for the variant Ap-ha of *A. phagocytophilum* (Levin et al. 2002, Yabsley et al. 2008). This overlap of ticks, hosts, and the pathogen creates the possibility that *H. longicornis* may be exposed to *A. phagocytophilum* and become involved in its transmission cycle in the United States.

In this study, we evaluated the ability of *H. longicornis* established in the United States to acquire, maintain through the molt, and transmit the local agent of human granulocytic anaplasmosis, and compared it to the vector competence of the primary vector—*I. scapularis*.

## Materials and Methods

### Ticks

*Haemaphysalis longicornis* and *I. scapularis* ticks used in this study were derived from specific-pathogen-free (SPF) colonies maintained at the CDC Medical Entomology Laboratory by feeding on pathogen-free naïve New Zealand white rabbits as previously described (Troughton and Levin 2007, Levin and Schumacher 2016). The *I. scapularis* colony was established in 1999 from adult ticks collected in Connecticut (New Haven County) and has been maintained in the laboratory for numerous generations with periodic supplementation of wild male ticks from the same area to ensure gene flow and prevent inbreeding. The parthenogenetic colony of *H. longicornis* originated from adult ticks collected from vegetation in New York (Westchester County) in 2018 and has been maintained in the laboratory since without supplementation.

Absence of known tick-borne pathogens including *Anaplasma*, *Borrelia*, *Ehrlichia*, and *Rickettsia* species in the laboratory colonies is verified by molecular testing of samples of larvae, nymphs, and adults from each generation as well as serological evaluation of all rabbits used for tick propagation.

## Pathogen

The Dawson isolate of *A. phagocytophilum* originated from *I. scapularis* larvae collected off a white-footed mouse, *Peromyscus leucopus*, caught at Lake Dawson near the town of Woodbridge, Connecticut (Levin and Ross 2004). Following serial propagation in a laboratory tick-mouse transmission cycle, the agent was isolated and cryopreserved in HL60 cells. Passage 9 was used in this study. Previous studies demonstrated that this isolate belongs to the Ap-ha genetic variant of *A. phagocytophilum* and is infectious to domestic goats, which can serve as reservoirs of infection for ticks (Massung et al. 2006).

## Model Animals

Four Boer male goats less than a year-old were used in this study. Throughout the study, the goats were housed indoors and monitored daily for clinical signs of infection including fever (defined as body temperature  $\geq 39.5^{\circ}\text{C}$ ), nasal discharge, lethargy, lameness, and loss of appetite as previously described (Massung et al. 2006). Blood samples from the jugular vein were collected using 22-gauge needles for PCR (three times per week) and IFA (weekly).

## Assessment of Vector Competence

Goats 203 and 204 were each intravenously inoculated with 1 ml of previously cryopreserved HL60 cells containing approximately  $10^7$  gene copies of *A. phagocytophilum*. On day nine postinoculation, each of the two needle-inoculated goats was infested with 150 nymphs of *H. longicornis* and 150 nymphs of *I. scapularis* for acquisition feeding. *H. longicornis* and *I. scapularis* ticks were placed in separate feeding bags attached to the shaven skin on the goats' backs as previously described (Massung et al. 2006, Levin and Schumacher 2016). Due to low attachment rate of *H. longicornis*, an additional 100 nymphs were placed on each goat on day 13 postinoculation. Engorged ticks were collected daily. Representative samples of engorged *H. longicornis* and *I. scapularis* nymphs were tested individually for the presence of *A. phagocytophilum* DNA while the rest of the collected ticks were allowed to molt to the adult stage.

After molting, representative samples of adult ticks of each species were again tested by PCR to assess whether the acquisition-fed ticks can maintain the acquired *A. phagocytophilum* through the molt. For further evaluation of the ability of *H. longicornis* to transmit the infection to susceptible animals, the remaining 58 females that fed as nymphs on the needle-inoculated goats 203 and 204 were placed on a naïve goat 205. Simultaneously, the remaining naïve goat 202 received the remaining *I. scapularis* that also fed as nymphs on the same needle-inoculated goats—15 females and 30 males. The recipient goats were monitored for 28 d post infestation as described above.

## PCR and Serology

DNA extraction and PCR procedures were carried out in separate facilities. DNA was extracted from tick and tissue samples using the Qiagen DNeasy Blood & Tissue kit, whereas blood samples were processed with the Flexigene kit (Qiagen Inc., Valencia, CA) according to manufacturer's protocols and eluted in a final volume of 100  $\mu\text{l}$ . Presence of *A. phagocytophilum* DNA in blood and tick samples was detected by PCR amplifying the *msp2* gene as described previously (Courtney et al. 2004). Genomic DNA extracted from *A.*

*phagocytophilum* cell culture and distilled water were used as positive and negative controls, respectively, and included in each PCR run.

IFA was performed on goat sera to detect IgG antibodies against *A. phagocytophilum*, as previously described (Lennette et al. 1995), using a fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat conjugate (Rockland Immunochemicals, Gilbertsville, PA). Sera samples were tested at the initial dilution of 1/16 and positive samples were titrated by serial two-fold dilutions to determine the endpoint titer. The endpoint antibody titers are expressed as reciprocals of the dilution factor.

The prevalence of infection in cohorts of ticks was compared by chi-squared test at the 95% confidence level (Sokal and Rohlf 2012).

### Ethics Statement

The study was undertaken at a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Care and husbandry of animals have 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 preapproved by the Centers for Disease Control Institutional Animal Care and Use Committee and monitored by a veterinarian stationed on-site. The U.S. Animal Welfare Act and all associated regulations were strictly followed.

## Results

### Pathogen Acquisition by Ticks

Following inoculation with cell-cultured *A. phagocytophilum*, significant temperature increases were not seen in any of the animals during the 4-wk period the observation period. Goat 203 exhibited clear nasal discharge from the third-day post inoculation (DPI) through 20 DPI, while goat 204 had intermittent bouts of nasal discharge and mild anorexia between 7 and 24 DPI. *Anaplasma phagocytophilum* DNA was detected by PCR in the blood of both inoculated goats starting at 10 DPI and they remained bacteremic until euthanasia on day 28. Goats 203 and 204 seroconverted with peak antibody titers of 512 and 1024, respectively.

*Ixodes scapularis* and *H. longicornis* nymphs imbibed pathogen-containing blood from both goats. *H. longicornis* nymphs placed on the goats at different time points were tested separately at first. There was no apparent difference in the proportion of *H. longicornis* nymphs imbibing the pathogen due to the time of tick placement or between the two goats ( $P_{\text{chi-square}} > 0.26$ ). Therefore, all *H. longicornis* ticks were treated as a single cohort for further statistical analysis. Overall, just over 75% of tested engorged *I. scapularis* nymphs contained *A. phagocytophilum* DNA, whereas less than half of tested engorged *H. longicornis* nymphs were PCR positive (Table 1). This difference in pathogen acquisition between tick species was statistically significant ( $P_{\text{chi-square}} = 0.005$ ). Following the molt into the adult stage, *A. phagocytophilum* DNA was detectable in 15 (37.5%) of 40 tested *I. scapularis*, but in only two (3.4%) of 59 tested *H. longicornis*. Thus, approximately one in two *I. scapularis* nymphs acquiring the pathogen during feeding maintained infection

through the molt, whereas less than one in 14 *H. longicornis* were able to do so ( $P_{\text{chi-square}} < 0.0001$ ).

Of the 45 *I. scapularis* (15 females, 30 males) and 58 *H. longicornis* females placed on naïve goats 202 and 205, respectively, 41 *I. scapularis* (11 females, 30 males) and 55 *H. longicornis* survived through feeding and were tested immediately upon collection. The presence of pathogen DNA was detected in 38 engorged *I. scapularis* and in only one *H. longicornis* (Table 1). The overall prevalence of *A. phagocytophilum* in fed adult *H. longicornis* was more than 50 times lower than in *I. scapularis* and the difference was statistically significant ( $P_{\text{chi-square}} \ll 0.0001$ ). Notably, the prevalence of *A. phagocytophilum* in adult *I. scapularis* more than doubled during adult feeding (from 37.5% to 92.7%;  $P_{\text{chi-square}} \ll 0.0001$ ) due to either transmission of the pathogen between co-feeding ticks, acquisition of pathogen from a systemically infected host, or pathogen amplification in feeding ticks. On the other hand, there was no amplification of infection during adult feeding of *H. longicornis* as the percent of PCR-positive engorged females was similar to that of unfed ticks ( $P_{\text{chi-square}} = 0.6$ ). This provides additional evidence of the Asian longhorned tick's inability to transmit the North American Ap-ha variant of *A. phagocytophilum*.

Goat 202, which was fed upon by infected *I. scapularis* adults remained afebrile throughout the observation period, but became bacteriemic on day 7 postinfestation, and blood-PCR remained consistently positive through day 21. This goat also seroconverted by 14 DPI and the peak antibody titer of 1024 was recorded at 21 DPI. Conversely, goat 205 infested with *H. longicornis* remained blood-PCR negative throughout the observation period and did not display any clinical signs of infection. It also remained sero-negative throughout the 28-day observation period despite being fed upon by at least one PCR-positive tick for 10 d.

## Discussion

Within the last decade, the Asian longhorned tick *H. longicornis* invaded the United States and, so far, has spread to at least 15 eastern states. Because of its ability for parthenogenetic reproduction, this tick is capable of establishing very large local populations vastly outnumbering indigenous tick species. Although not overly aggressive toward humans, it feeds on a wide range of North American vertebrate hosts including those known as reservoirs for the agent of HGA—the Ap-ha variant of *A. phagocytophilum*—such as raccoons and gray squirrels. Therefore, there is a possibility that this invasive tick may feed on animals systemically infected with *A. phagocytophilum* and become involved in its transmission cycle. In this study, we assessed the vector competence of *H. longicornis* collected in the United States for the agent of HGA using an Ap-ha isolate, *A. phagocytophilum* Dawson.

Results of this study demonstrate that the Asian longhorned tick can acquire the Ap-ha variant of *A. phagocytophilum* with the bloodmeal while feeding on bacteremic hosts but is inefficient in maintaining the infection through the molt to the next life stage. Furthermore, PCR-positive adult *H. longicornis* did not transmit *A. phagocytophilum* to a susceptible goat during feeding as revealed by the lack of bacteremia or seroconversion in the animal host.

In contrast, *I. scapularis* ticks, tested in parallel under the same conditions, successfully transmitted the infection to their host, which became bacteremic for an extended period of time and developed high antibody titers. This indicates that *H. longicornis* is unlikely to contribute toward transmission of the agent of human anaplasmosis in the United States.

Our observation that *H. longicornis* was imbibing pathogen-containing blood from experimentally infected goats corresponds with reports from China and Korea documenting identification of *Anaplasma* DNA in blood-fed *H. longicornis* collected from domestic and wild animals (e.g., Chen et al. 2014, Dong et al. 2014). This, however, does not indicate an ability of the tick to either maintain the infection transstadially or transmit it to susceptible animals and cannot be interpreted with regard to ticks' vector competence.

In this study, a naïve goat fed upon by at least one PCR-positive *H. longicornis* remained uninfected as it was both PCR- and IFA-negative. Additionally, there was no evidence of cofeeding transmission between the infected and uninfected ticks that fed side-by-side for extended periods of time. It is unknown if the observed lack of pathogen transmission by a PCR-positive *H. longicornis* adult to a susceptible goat was due to insufficient quantity of *A. phagocytophilum* in the tick, inability of the agent to penetrate and establish infection in the salivary glands of this tick species, or to detection of only DNA fragments still remaining in a tick after the molt. Regardless of the reason, this absence of pathogen transmission by a PCR-positive tick serves as yet another proof that PCR-positivity of ticks by itself must not be interpreted as evidence of their vector competence (Moraes-Filho et al. 2018, Ramírez-Hernández et al. 2020).

Our results demonstrate the stark difference between vector competence of the native blacklegged tick *I. scapularis* and the low to nonexistent propensity of the Asian longhorned ticks for maintenance and transmission of the agent of human granulocytic anaplasmosis in the United States. However, genetic diversity is being increasingly recognized among strains and isolates of *A. phagocytophilum* worldwide (as reviewed by Stuenkel et al. 2013, Dugat et al. 2015, Matei et al. 2019). Although only ticks of the genus *Ixodes*, and more specifically those belonging to the *I. ricinus* complex, have been confirmed as the primary vectors of *A. phagocytophilum* to humans, it is plausible that some of the genetic variants of this bacterial species or closely related species may adapt to transmission by non-*Ixodes* tick vectors. However, vector competence of those ticks and their vectorial capacity in enzootic cycles of respective agents are yet to be investigated.

The North American strains of *A. phagocytophilum* primarily belong to two variants (Ap-ha and Ap-V1), of which only Ap-ha has been detected in humans (de la Fuente et al. 2005, Stuenkel et al. 2013). It remains unknown whether *H. longicornis* present in the United States may become involved in the natural transmission cycles of these nonhuman variants. Yet, the results of the present study demonstrate that it is unlikely that the Asian longhorned tick would play a role in the epidemiology of human granulocytic anaplasmosis in the United States.

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Efficiency of acquisition and transstadial maintenance of *Anaplasma phagocytophilum* by *Haemaphysalis longicornis* and *Ixodes scapularis*

**Table 1.**

	Prevalence of infection: pos/tested (% ± 95% conf. interval)		
	Engorged nymphs	Molted adults	Engorged adults
<i>I. scapularis</i>	34/45 (75.6 ± 12.5) <sup>a</sup>	15/40 (37.5 ± 15.0) <sup>c</sup>	38/41 (92.7 ± 8.0) <sup>e</sup>
<i>H. longicornis</i>	26/54 (48.1 ± 13.3) <sup>b</sup>	2/59 (3.4 ± 4.6) <sup>d</sup>	1/55 (1.8 ± 3.2) <sup>f</sup>

$P_{\text{chi-square}}^{a/b} = 0.005$ ;  $P_{\text{chi-square}}^{c/d} < 0.0001$ ;  $P_{\text{chi-square}}^{e/f} \ll 0.0001$ .

$P_{\text{chi-square}}^{a/c} = 0.0002$ ;  $P_{\text{chi-square}}^{c/e} \ll 0.0001$ ;  $P_{\text{chi-square}}^{b/d} \ll 0.0001$ ;  $P_{\text{chi-square}}^{df} = 0.6$ .