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Association between Growth Rate and Pathogenicity of Spotted Fever Group *Rickettsia*

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Abstract

***Rickettsia parkeri* and *Rickettsia amblyommatis* are spotted fever group *Rickettsia* (SFGR) associated with *Amblyomma* ticks. *R. parkeri* is a recognized human pathogen that causes an eschar-associated febrile illness, while *R. amblyommatis* has not been confirmed as a causative agent of human disease. We hypothesized that the rate of replication is one of the factors contributing to rickettsial pathogenicity. In this study, growth and infectivity of *R. parkeri* and *R. amblyommatis* in mammalian (Vero E6) and tick-derived (ISE6) cell lines were assessed and compared over a 96-hour time course of infection using quantitative real-time polymerase chain reaction and microscopy. The pathogenic *R. parkeri* displayed a significantly higher level of infection in both Vero E6 and ISE6 cells than *R. amblyommatis* at 72 hours post-inoculation (hpi). Distinct growth profiles between rickettsial species with known and uncertain pathogenicity were identified. *R. parkeri* burdens were significantly greater than those of *R. amblyommatis* from 24 to 96 hpi. The relative fold changes of load were significantly higher in the pathogenic agent than in *R. amblyommatis* from 48 hpi onward and reached the maximum fold increase of ~2002- and ~296-fold in Vero E6 cells and ~1363- and ~161-fold in ISE6 cells, respectively, at 96 hpi. The results from the present study demonstrate that growth rate is associated with the pathogenicity of rickettsiae. Understanding SFGR growth characteristics in mammalian and tick cells will provide insight into rickettsial biology and pathogenesis.**

Keywords: *Rickettsia parkeri, Rickettsia amblyommatis, Amblyomma* spp., growth rate, qPCR

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INTRODUCTION

Rickettsia species are Gram-negative alpha Proteobacteria. Several arthropods, especially ticks and fleas, are important vectors of these obligate intracellular bacteria, as they can be maintained in arthropod populations *via* vertical transmission. Among the diverse genotypes of identified rickettsial species, many of them have been recognized to be agents with variability in pathogenicity to humans.[1,2] Nevertheless, several species of *Rickettsia* with uncertain pathogenicity were recently identified and reported.[2]

Rickettsia parkeri and *Rickettsia amblyommatis* are among several species of *Rickettsia* associated with *Amblyomma* ticks in the United States.[3,4] *R. parkeri* was first isolated in 1937 from the Gulf Coast tick, *Amblyomma maculatum*, and was later identified as a spotted fever group *Rickettsia* (SFGR).[5] *R. parkeri* was reported in 2004 as a causative agent of spotted fever disease in a human.[3] It caused an eschar-associated rash illness, however, with milder symptoms than that of the bioagent that causes Rocky Mountain spotted fever (RMSF), *Rickettsia rickettsii*.[3,6-8] Since then, additional cases of *R. parkeri* rickettsiosis have been reported in many southeastern and southwestern regions of the United States.[9-11] In addition to the primary vector, *A. maculatum, R. parkeri* has also been found in different species of ticks, including *Amblyomma americanum,*[12-14] *Amblyomma triste,*[15] and *Ixodes scapularis.*[14,16] *R. amblyommatis,* formerly named *Rickettsia amblyommii* and *'Candidatus* Rickettsia amblyommii*',* has been detected in *A. americanum* throughout the United States.[17,18] This rickettsial species has been proposed as a possible cause of rickettsiosis in some patients who were initially diagnosed as having RMSF.[19,20] Drexler et al.[21] have also suggested that much of the seroprevalence of human SFG rickettsioses with the very low case fatality rate in the United States is likely caused by infections with mildly pathogenic or non-pathogenic rickettsial species, including *R. amblyommatis*. This agent is also detected in other species of ticks, comprising *A. maculatum,*[4,14] *Amblyomma cajennense* complex,[22] *Amblyomma* nr *maculatum,*[22] and *I. scapularis.*[14,23] The relatively high prevalence of *R. amblyommatis* detected in *A. americanum*[24,25] and its existence in other tick species have been suggested as a potential risk of rickettsial infection to humans; nonetheless, no confirmed cases of human infection by *R. amblyommatis* have been reported to date.

Little is known about the biology and pathogenic determinants of *R. parkeri* and *R. amblyommatis* species. *In vitro* systems are advantageous to investigate the differences between pathogenic and non-pathogenic rickettsiae.[26,27] Among available cell lines, mammalian (Vero E6) and *I. scapularis*-derived (ISE6) cells are commonly used for rickettsial isolation and cultivation.[28,29] According to their frequent use in rickettsial research and the detection of *R. parkeri* and *R. amblyommatis* in *I. scapularis*, Vero E6 and ISE6 cells were thus utilized in the present study as the model cells to study growth characteristics of human pathogen *R. parkeri* (strain Portsmouth) and *R. amblyommatis* (strain Darkwater), a species with uncertain pathogenicity. The growth and infectivity of the two rickettsiae were compared using quantitative real-time polymerase chain reaction (qPCR) and microscopy.

MATERIALS AND METHODS

Cell culture and rickettsiae

African green monkey kidney (Vero E6) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) in a humidified, 5% CO_2 incubator at 34°C. ISE6 cells were maintained in L15B medium (Gibco-BRL, Carlsbad, CA) contained 10% heat-inactivated fetal bovine serum (HyClone) and 10% tryptose phosphate broth (Sigma, St. Louis, MO) at pH 6.8 in a humidified, 5% CO_2 cell culture incubator at 34°C. *R. parkeri* (strain Portsmouth) originally isolated from a skin biopsy specimen[3] and *R. amblyommatis* (strain Darkwater) isolated from a female *A. americanum* tick collected in Wakulla County, Florida in 2006 (CD Paddock, unpublished data) were used throughout this study.

Propagation and semi-purification of *Rickettsia*

Each rickettsial species (passage 4) were propagated using the confluent Vero E6 cells for 4 days. Heavily infected cells, as determined by Diff-Quik (Dade Behring, Deerfield, IL) staining according to the manufacturer's protocol, were washed once with culture medium. Cells were

scraped from the culture flask, collected by centrifugation at 500 × *g* for 8 min at 4°C, and resuspended in culture medium. Semi-purification of rickettsiae was performed as previously described[30] with a slight modification. Briefly, cells were mechanically lysed by forcing 5 mL of cell suspension 4-5 times through a 27-Gauge needle attached to a 10-mL syringe. The remaining intact host cells and cell debris were removed by centrifugation at 500 × *g* for 10 min at 4°C followed by filtration of the obtained supernatant through a 2-µm syringe filter (Whatman, Florham Park, NJ).[31] Rickettsiae in the filtrate were then collected by centrifugation at 9,500 × *g* for 10 min at 4°C. The pellets were washed 3 times and resuspended in culture medium. Partially purified rickettsiae were assessed the viability and counted using LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Carlsbad, CA) as previously described.[32]

Rickettsial infection and replication in cell culture

To compare growth and infectivity of *R. parkeri* and *R. amblyommatis*, Vero E6 and ISE6 cells (10^5 cells per well) were seeded separately, in triplicate, into 48-well cell culture plates (NUNC, Rochester, NY) containing 500 µL of appropriate culture medium. Replicate of eukaryotic cell cultures was also prepared by seeding cells in 48-well plates containing sterile glass coverslips for assessment of the number of *Rickettsia*-infected cells. After incubating the plates in a humidified 5% CO_2 incubator at 34°C for 2 days, each well was gently washed twice with serum-free medium and culture cells were subsequently inoculated with either partially purified *R. parkeri* or *R. amblyommatis* with an estimated multiplicity of infection of 10 in a total volume of 300 µL. Culture plates were briefly spun at 500 × *g* for 1 min to assist the attachment of microorganism to the host cells. After 1 hour of incubation in a humidified 5% CO_2 incubator at 34°C, culture medium was removed, and cells were gently washed 3 times with 300 µL of serum-free culture medium. The appropriate culture medium of 500 µL was then added to each well and culture plates were further incubated in a humidified 5% CO_2 incubator at 34°C. At 1, 6, 12, 24, 48, 72, and 96 hours post-inoculation (hpi), cells were detached by pipetting and collected by centrifugation at 13,000 × *g* for 15 min at 4°C. Individual wells were observed with an inverted light microscope to ensure all cells were removed. Cell pellets were stored at -80°C until used for genomic DNA (gDNA) extraction. To estimate the percentage of *Rickettsia*-infected cells, coverslip cultures of eukaryotic cells at each hpi were stained with Diff-Quik staining and examined with a light microscope using the oil immersion lens. A hundred intact cells of Vero E6 and ISE6 cells were examined and only cells containing rickettsiae were considered to be infected cells. Three independent experiments for growth and infectivity assessments were performed.

DNA extraction and qPCR quantitation of rickettsial load

The gDNA was extracted from the cell pellets using the DNeasy tissue kit (Qiagen, Germantown, MD) according to the manufacturer's recommendation and stored at -20°C until used. The total number of *Rickettsia* in each sample was quantified by qPCR using the primer pair RaRp17.181F and RaRp17.289R that is specific to the highly conserved 17-kDa antigen gene among the species of the genus *Rickettsia.*[33] For relative fold change analysis of rickettsial load, the qPCR for host housekeeping genes that are frequently used as reference genes (beta-actin gene for Vero E6 cells and calreticulin gene for ISE6 cells) were performed using actinF (5'-AACCTTCCTTCCTGGGCAT-3')/actinR (5'-TGATGCTCTCCTTCTGCAT-3') and CRT321F/CRT452R[31] primer pairs, respectively. Plasmids containing single-copy portions of *Rickettsia* 17-kDa antigen and beta-actin gene as well as *Rickettsia* 17-kDa antigen and tick calreticulin gene were constructed as described by Zanettii et al.[34] and Petchampai et al.,[31] respectively, and used as standard templates for quantification. A 35 µL qPCR reaction mixtures were premixed in 96-well plates using 2X iTaq SYBR Green Supermix (BioRad, Hercules, CA), 100 nM of each primer, DNase/RNase-free water, and 5 µL of either gDNA template (samples), serial 10-fold dilutions of a plasmid, or water (negative control). The mixtures were then aliquoted in triplicate 10-µL reactions on 384-well plates. The qPCR was carried out with an ABI 7900HT system (Applied Biosystems, Foster City, CA) using previously optimized conditions.[34] The data were analyzed with the ABI 7900HT sequence detection system (SDS 2.2.3) software. The DNA copies per well of the *Rickettsia* 17-kDa

antigen gene at each time point of infection in the respective cultures were calculated. Relative fold change of rickettsial load, defined as fold changes of the load relative to the first time point (1 hpi), were calculated from cycle threshold (C_T) values for each sample using $2^{-\Delta\Delta CT}$ method.[35]

Statistical Analysis

Data were reported as means ± standard errors of the means (SEM) from at least two independent experiments. T-test was used for a comparison of grouped means at each time point of infection. A P-value of ≤ 0.05 was considered significantly different.

RESULTS

In this study, the growth and infectivity of the human pathogen *R. parkeri* and a *Rickettsia* species of undetermined pathogenicity, *R. amblyommatis*, were compared in Vero E6 (a model of mammalian cells) and the tick-derived ISE6 cells. It was found that rickettsiae were difficult to identify by Diff-Quik staining during the first 24 hpi. The mean percentage of infected cells at 48, 72, and 96 hpi were shown in Fig. 1. The percentage of Vero E6 and ISE6 cells infected with each rickettsial species correlated with an increased duration of infection. At 48 hpi, ~25% of Vero E6 cells were found to be infected by *R. parkeri* and *R. amblyommatis* with no significant difference (P > 0.05). The levels of infection in Vero E6 cells at 72 hpi increased ~3-fold as compared with those at 48 hpi which *R. parkeri* possessed significantly higher infectivity than *R. amblyommatis* (P < 0.05) (Fig. 1A). Although a significant difference in infectivity was not identified at 96 hpi, Vero E6 cells exposed to *R. parkeri* showed a greater density of rickettsiae within the cells than those of *R. amblyommatis*. Cell detachment was not observed, despite the level of infection being close to 100%. ISE6 cells exhibited lower percentages of rickettsial infection than Vero E6 cells. The pathogenic *R. parkeri* displayed a significantly higher level of infection in the ISE6 cells than *R. amblyommatis* at 72 and 96 hpi (P < 0.05). Moreover, infection of ISE6 cells reached nearly 100% by the pathogenic agent within 4 days and partial detachment of cells was observed in some samples. While only ~54% of ISE6 cells were infected by *R. amblyommatis* (Fig. 1B). At the late time point of infection (96 hpi), abundant intracellular and extracellular rickettsiae were identified in both cell lines (Fig. 2).

Growth of *R. parkeri* and *R. amblyommatis* in two types of eukaryotic cell lines, in terms of the total number of *Rickettsia* (17-kDa antigen gene copies) in each sample and the relative fold change of rickettsial load, were assessed by qPCR and compared over a 96-hour time course of infection. In Vero E6 cells, the number of inoculated rickettsiae remaining associated with the host cells at the initial time point (1 hpi) was not significantly different (P > 0.05) between the two rickettsial species and the bacterial loads did not change significantly during the first 12

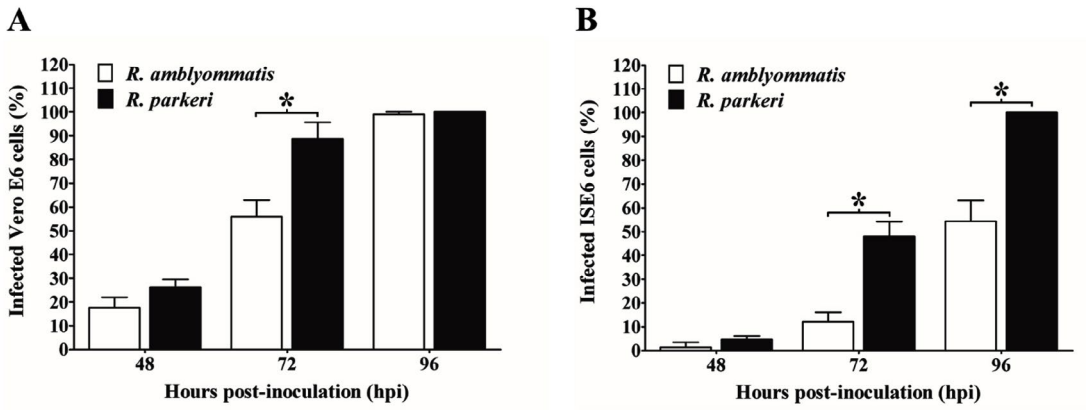

Fig. 1. Percentage of Vero E6 (A) and ISE6 (B) cells infected with *R. parkeri* and *R. amblyommatis* at 48, 72, and 96 hours post-inoculation. Data are presented as means ± standard errors of the means. An asterisk denotes a significant difference between species (P < 0.05; t-test).

hours of the experiment. After 24 hpi, Vero E6 cells displayed a continuous increase in rickettsial burdens for both species that reached the highest number at 96 hpi. Significant differences in growth between species with known and uncertain pathogenicity ($P < 0.05$) were identified from 24 hpi onward; such distinctions were more apparent over time of infection. The numbers of *R. parkeri* elevated from ~5.03×10^5 at 24 hpi to ~4.24×10^9 at 96 hpi with an increase of ~8430 times while *R. amblyommatis* presented only ~670 times increase (from ~3.16×10^5 at 24 hpi to ~2.11×10^8 at 96 hpi) (Fig. 3A). When the relative fold change of rickettsial load for both species was analyzed using $2^{-\Delta\Delta CT}$ method, it was found that the fold change values were in good agreement with their total numbers. *R. parkeri* exhibited ~2-, ~17-, ~142-, and ~2002-fold increase in burden at 24, 48, 72, and 96 hpi, respectively. In contrast to *R. parkeri*, however, *R. amblyommatis* displayed significantly fewer fold changes of load (~0.9-, ~3-, ~39-, and ~296-fold at the same infection period; $P < 0.05$) (Fig. 4A). The results revealed that pathogenic *R. parkeri* propagated in Vero E6 cells faster than *R. amblyommatis*, a species of undetermined pathogenicity.

The growth profile of the two agents was also investigated in ISE6 cells. Comparable quantities ($P > 0.05$) of *R. parkeri* and *R. amblyommatis* that remained associated with ISE6 cells at 1 hpi were identified, however, with lower quantities than those of Vero E6 cells. Following the initial infection, the rickettsial burdens were relatively stable during the first 12 hours, subsequently elevated after 24 hpi, and achieved the greatest number at 96 hpi. From 24 hpi onward, loads of *R. parkeri* were significantly higher than those of *R. amblyommatis* ($P < 0.05$) with an increase of ~368 times (from ~3.37×10^5 at 24 hpi to ~1.24×10^8 at 96 hpi) and ~74 times (from ~1.63×10^5 at 24 hpi to ~1.20×10^7 at 96 hpi), respectively (Fig. 3B). When comparing mean $2^{-\Delta\Delta CT}$ values of the two agents, it was found that *R. parkeri* possessed significantly greater fold

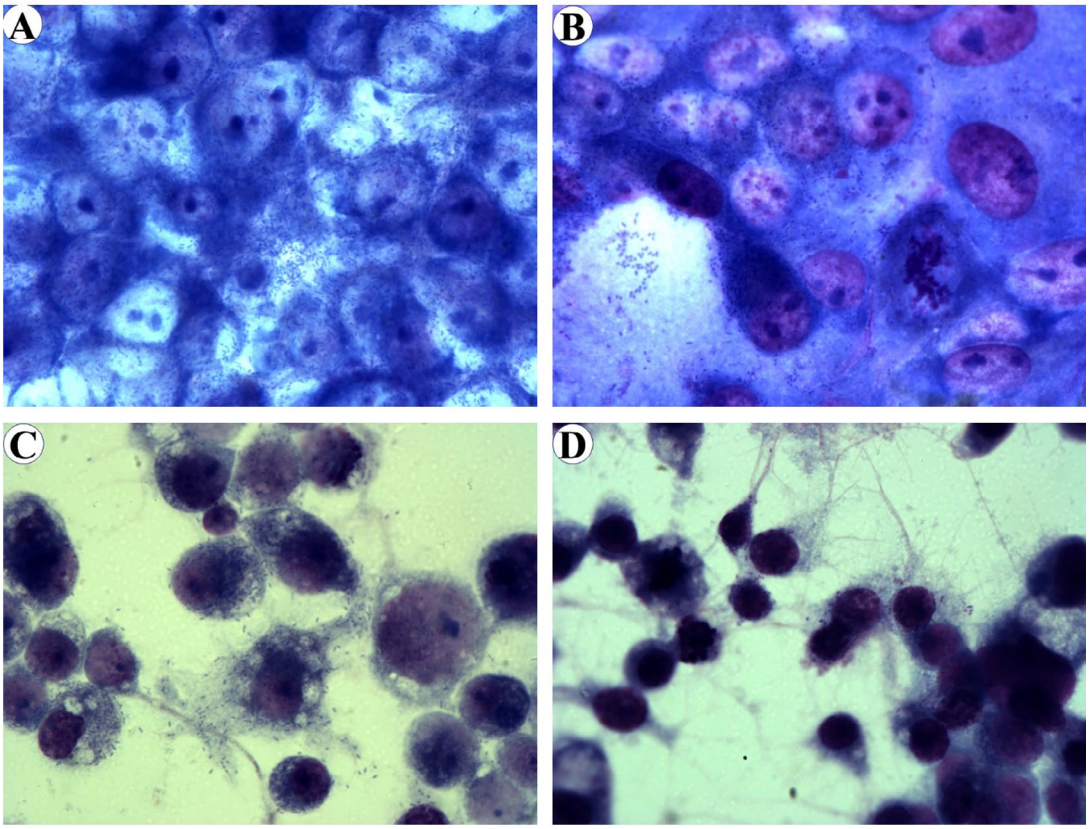

Fig. 2. Diff-Quik stained of Vero E6 cells infected with *R. pakeri* (A) and *R. amblyommatis* (B) and ISE6 cells infected with *R. parkeri* (C) and *R. amblyommatis* (D) at 96 hours post-inoculation. Images are at a magnification of 630.

changes of a burden than *R. amblyommatis* (~20- vs ~10-, ~176- vs ~61-, and ~1363- vs ~161-fold at 48, 72, and 96 hpi, respectively; P < 0.05) (Fig. 4B). These results were consistent with the Vero E6 cell data. Taken together, our results suggested that the human pathogenic *R. parkeri* grew faster than *R. amblyommatis*, a species of uncertain pathogenicity, in both Vero E6 and ISE6 cells.

DISCUSSION

The life cycle of tick-borne SFGR involves both the tick vector and the vertebrate host. To establish an infection, rickettsiae must undertake several stages of the life cycle including adhesion to and internalization into host cells, endosomal escape into the cytoplasm, replication, and dissemination.[36] In this study, the growth and infectivity of *Amblyomma* tick-associated SFGR, the human pathogen *R. parkeri* (strain Portsmouth) and *R. amblyommatis* (strain Darkwater), a species of uncertain pathogenicity, were compared in Vero E6 and ISE6 cells over 96 hours of infection. Quantitative analyses of *R. parkeri* and *R. amblyommatis* in both cell types showed that

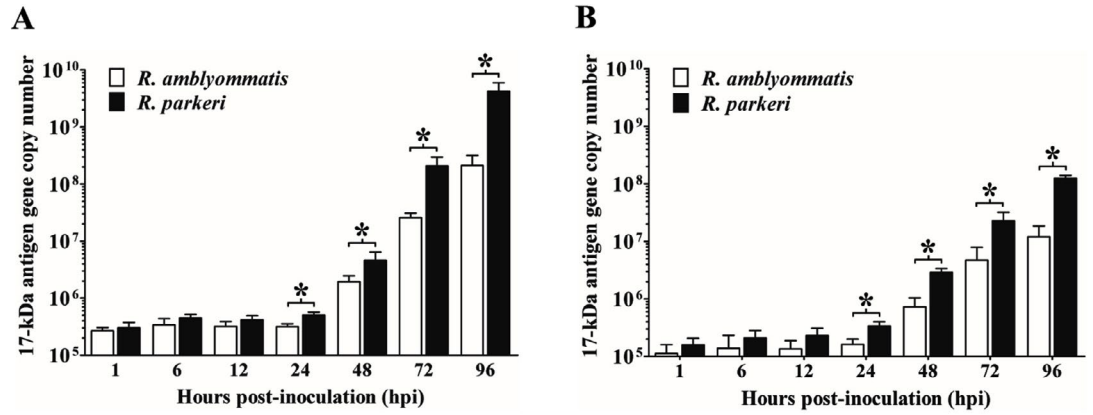

Fig. 3. Quantity of rickettsiae in Vero E6 (A) and ISE6 cells (B) at 1, 6, 12, 24, 48, 72, and 96 hours post-inoculation. The total number of *Rickettsia* (17-kDa antigen gene copy numbers) at each time point of infection was quantified by qPCR using a primer set specific to rickettsial 17-kDa antigen gene. Data are presented as means ± standard errors of the means. An asterisk denotes a significant difference between species (P < 0.05; t-test).

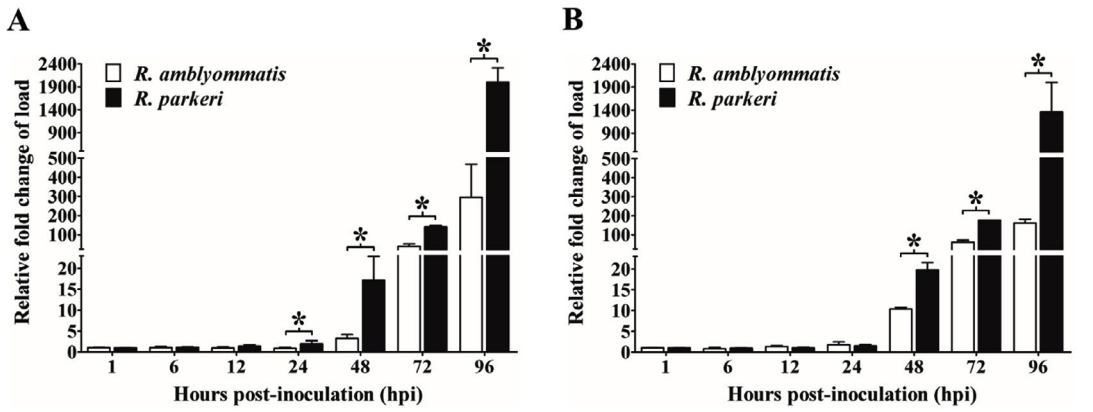

Fig. 4. Relative fold change of rickettsial load in Vero E6 (A) and ISE6 cells (B).
The qPCR analyses for rickettsial 17-kDa antigen gene relative to host gene (beta-actin gene for Vero E6 cells or calreticulin gene for ISE6 cells) at 1, 6, 12, 24, 48, 72, and 96 hours post-inoculation (hpi) were performed. Fold changes of the load relative to the first time point (1 hpi) were calculated from cycle threshold (C_T) values for each sample. Data are presented as means ± standard errors of the means. An asterisk denotes a significant difference between species (P < 0.05; t-test).

rickettsiae levels remained relatively constant for the first 12-24 hpi suggesting the length of the lag phase required for the bacteria to encounter the initial stages of their life cycles before beginning cell division. The lag phase duration of SFGR in cell culture system varied among species, e.g., 2-3 days for *Rickettsia helvetica* cultured in Vero cells,[37] 6-8 days for *Rickettsia felis* grown in S2 and C6/36 cells,[38] except for *Rickettsia slovaca* that showed no lag phase when cultivated in Vero and L929 cells.[39] Other tick-transmitted *Rickettsia* can grow differently in different cell types.[40] The numbers, as well as fold changes in the load of the two rickettsiae, increased rapidly thereafter. Although they both entered the exponential phase of growth, however, distinct profiles for each agent were identified. The pathogenic *R. parkeri* possessed significantly higher numbers and fold changes of a burden than those of *R. amblyommatis* in both mammalian and tick cell lines indicating an association between the growth rate and rickettsial pathogenicity. In addition, the results from infectivity assessment during 72-96 hpi revealed that the levels of infection by *R. parkeri* were greater than those of *R. amblyommatis* implying the ability of the pathogenic species to disseminate more rapidly than species with uncertain pathogenicity. The incubation period of *R. parkeri* rickettsiosis in humans is 2-10 days[8] supporting the growth and infectivity results from this study.

The distinct proliferation rate between rickettsial species with known and uncertain pathogenicity demonstrated by the current study may explain the pathogenic nature of these two *Amblyomma*-associated rickettsiae. *R. parkeri* was recognized as a human pathogen that causes remarkable clinical symptoms such as fever, headache, diffuse myalgia, macular rash, eschars-associated illnesses,[3,7,8,41] while *R. amblyommatis* has occasionally been reported as a possible causative agent associated with rash[19] and mild symptoms.[42] Previous studies have demonstrated that SFGR manipulate vertebrate hosts to establish sufficient loads in the host cells for successful horizontal transfer.[43,44] For other obligate intracellular bacterial pathogens, burdens of bacteria within the host have been shown to be associated with disease severity.[45,46] The greater growth rate of *R. parkeri* in mammalian cells than that of *R. amblyommatis*, resulting in higher bacterial load of the pathogenic agent, may be one of the important factors contributing to increased severity of disease and transmission efficiency to tick vectors.

Tick-to-host transmission and survival in both tick and vertebrate hosts are important for the persistence of rickettsiae.[47,48] To successfully alternate and survive between tick vectors to vertebrate hosts, *Rickettsia* pathogens require effective strategies to evade the host defense system for host cell invasion and causing disease through the obligate intracellular lifestyle. Studies in rickettsial species/strains with different levels of virulence have shown the association of bacterial loads in tick salivary glands and saliva with transmission efficiency to vertebrate hosts.[49,50] Thus, the efficient proliferation of the human pathogen *R. parkeri* over the unknown pathogenic species, *R. amblyommatis*, in tick-derived ISE6 cells found in the current study supports the previous findings that pathogenicity is associated with rickettsial burden and transmission efficiency.

On the other hand, the slow growth rate would rather help promote persistent infections of non-pathogenic rickettsiae in the tick host. It was frequently found that most infected ticks harbor non-pathogenic rickettsial species.[51] *R. amblyommatis* burdens in naturally infected *A. americanum* ticks were shown to be relatively stable in adult tick tissues during feeding and throughout their life cycle stages.[34] Considering as an agent that causes only mild illness[42] together with a slow growth characteristic found in the present study makes *R. amblyommatis* to be the great potential immunostimulant. *R. amblyommatis* has been reported to induce cross-protection against *R. rickettsii* in a guinea pig model.[52,53] This may be due to the fact that *R. amblyommatis* shared immunoreactive proteins with the pathogenic rickettsial species.[54] Further investigation is required to elucidate the mechanisms by which this rickettsial species, sometimes considered as tick symbiont, utilize to persist in the tick host and probably help prevent rickettsial infections. Altogether, the results from the present study demonstrate that the growth of SFGR is associated with pathogenicity. Studying

SFGR growth characteristics in mammalian and tick cells facilitates a better understanding of rickettsial biology and pathogenesis leading to disease prevention and treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AB and WB conceptualized the initial idea, formulated the hypothesis, designed the experiment, curated and analyzed the data. AB conducted the experiment, collected and interpreted data and drafted the manuscript. CDP and KRM supported research materials. KRM supervised the project. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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