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RESEARCH ARTICLE

In vitro propagation of *Candidatus* Rickettsia andeanae isolated from *Amblyomma maculatum*

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Rickettsia; spotted fever group; *Amblyomma*.

Abstract

Candidatus Rickettsia andeanae was identified during an investigation of a febrile outbreak in northwestern Peru (2002). DNA sequencing from two ticks (*Amblyomma maculatum, Ixodes boliviensis*) collected during the investigation revealed a novel *Rickettsia* agent with similarity to the spotted fever group rickettsiae. Since then, *Candidatus* R. andeanae has been detected in *A. maculatum* ticks collected in the southeastern and southcentral United States, Argentina, and Peru. To date, *Candidatus* R. andeanae has not been successfully cultivated in the laboratory. We present evidence for the continuous cultivation in three cell lines of *Candidatus* R. andeanae isolated from an *A. maculatum* tick (Portsmouth, Virginia).

Introduction

Candidatus Rickettsia andeanae is an incompletely characterized spotted fever group rickettsia, first detected in *Amblyomma maculatum* and *Ixodes boliviensis* ticks collected from two domestic horses living in the Coletas and Naranjo areas of Northern Peru (Blair *et al.*, 2004). The ticks were collected during 2002, when a febrile outbreak (which caused two deaths) occurred in the area around the town of Sapillica in northwestern Peru (Blair *et al.*, 2004). Further phylogenetic analysis of the 17-kDa, *gltA*, *ompB*, *ompA*, and *sca4* genes demonstrated strong alignment with other spotted fever group rickettsiae (Jiang *et al.*, 2005). However, the molecular isolates were not found to be identical to any rickettsial agent currently listed in GenBank, and *Candidatus* R. andeanae was determined to be a novel rickettsial agent. Several years later (2010), *Candidatus* R. andeanae was detected in a single *A. maculatum* tick that was obtained in July 2010 during tick collection studies (flagging) in southeastern Virginia, specifically near the Elizabeth River in Portsmouth, Virginia (Wright *et al.*, 2011). The tick was confirmed positive for *Candidatus* R. andeanae by Old Dominion University using a genus-specific quantitative real-time PCR (qPCR) assay and DNA sequencing of a fragment of the *ompB* gene, which revealed a 100% match to *Candidatus* R. andeanae molecular isolate T163 (Wright *et al.*, 2011). Despite molecular characterization of the *Candidatus* R. andeanae, the successful *in vitro* cultivation of this bacterium has remained a challenge because of its slow-growing nature.

Bacterial isolates of rickettsial family members are most often cultured in mammalian cell lines such as Vero and L929. The bacteria have also been shown to grow in monocytes/macrophages (Bechah *et al.*, 2008), tick cells (Pornwiroon *et al.*, 2006), mosquito cells (Sakamoto & Azad, 2007), and *Drosophila melanogaster* cells (Luce-Fedrow *et al.*, 2008). Drawing on this knowledge, we used one-half of the *Candidatus* R. andeanae-positive *A. maculatum* tick collected by Wright *et al.* (2011) to infect cultures of Vero cells (African Green monkey kidney epithelial cells), DH82 cells (canine macrophages), and S2 cells (*Drosophila* hemocytes). Infections were confirmed using qPCR, acridine orange staining, Diff-Quik staining,

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immunocytochemistry, and DNA sequencing. Growth of *Candidatus* R. andeanae is supported by all three types of cell lines, and infections have been continuously maintained in the cell lines since November of 2010, with 23 passages through August of 2011. The successful establishment of *Candidatus* R. andeanae cultures in three distinct cell lines represents a major step toward better characterizing this novel bacterium and understanding the potential virulence of this emerging rickettsial species.

Materials and methods

Maintenance of cell lines

The DH82 cell line (ATCC, Manassas, VA) was maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (ATCC) containing between 5% and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). The Vero cell line (ATCC) was maintained at 35 or 37 °C, 5% CO_2 in DMEM (ATCC) containing between 5% and 10% fetal bovine serum. The S2 cell line (*Drosophila* Genomics Resource Center, Bloomington, IN) was cultivated at 25 °C in Schneider's *Drosophila* medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 5–10% fetal bovine serum. No antibiotics were used for cell culture.

Tick processing

One-half of an *A. maculatum* tick was received from the Department of Biological Sciences, Old Dominion University, Norfolk, VA and stored at −80 °C. In preparation for isolation of *Candidatus* R. andeanae, the tick was disinfected in iodinated alcohol (10 mL of 100% ethanol: 4 mL of 4% iodine) for 10 min, rinsed in sterile RO-water for 5 min, and then triturated into 1 mL of Snyder's I buffer (0.22 M sucrose + 0.0032 M KH_2PO_4 + 0.0086 M Na_2HPO_4 + 0.0049 M glutamic acid L^{-1}, pH 7.4) using a sterile razor blade.

Initial infections and time course infections in DH82, Vero, and S2 cells

DH82, Vero, and S2 cells were plated in six-well tissue culture plates (60 mm; BD Falcon, Franklin Lakes, NJ) at a concentration of 1×10^6 cells per well, 24 h before being infected with tick triturate. Equal volumes of triturate (~333 μL) were added to one well of each six-well plate for each type of cell line. 333 μL of Snyder's I buffer alone was added as a negative control. Four and 5 days following initial infection with the tick triturate, 1 mL of DH82, Vero, and S2 cells were sampled from the infected and uninfected wells. DNA was extracted from each sample using the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD), following manufacturer's protocol for spin column DNA isolation and assessed for the presence of rickettsial DNA using our *Candidatus* R. andeanae qPCR (Rande) assay (Jiang *et al.*, 2011). At 5 days after infection (dpi), all media and any detached cells from the originally infected DH82, Vero, or S2 cells were, respectively, transferred to fresh cultures of DH82, Vero, or S2 cells (the host cells were not removed from the originally infected plates). Fresh media were added to the original cultures for continued maintenance of infections. Six days later, 1 mL of cells was sampled from all of the original cultures and subcultures, and DNA was extracted. All infected samples contained rickettsial DNA as determined by Rande qPCR assay. Infections were then expanded to T25 tissue culture flasks (BD Falcon) by transferring all the media from the infected DH82, Vero, or S2 cells to fresh cultures of DH82, Vero, or S2 cells.

For time course infection studies, DH82, Vero, and S2 cells were plated at a concentration of 1×10^6 cells mL^{-1} in T25 tissue culture flasks for 4–24 h before being infected with *Candidatus* R. andeanae. For infections, the media from the original DH82, Vero, and S2 cells that received tick triturate and from the original subcultures were transferred to the fresh T25 flasks of DH82, Vero, or S2 cells, respectively. Beginning at 1 dpi, 1 mL of cells was sampled from each of the T25 flasks by gently drawing a pipette across the bottom of the flask, then DNA was extracted from the cells, and analyzed by our Rande qPCR assay. Samples were collected and analyzed at selected time points. Duplicate infections created from independent subcultures were monitored during the time courses.

Infections in all three cell lines were also detected at various time points using Diff-Quik stain (Andwin Scientific, Schaumburg, IL) and acridine orange stain (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Cells were prepared on glass slides using a Shandon cytospin. Slides were stained with the Diff-Quik kit according to manufacturer's instructions. For acridine orange staining, slides were dried at room temperature, flooded with acridine orange for 2–3 min, and then rinsed in distilled water. Images were viewed with an Olympus BX43 microscope and DP72 camera (Olympus, Center Valley, PA).

Immunocytochemistry

Immunocytochemistry was performed on *Candidatus* R. andeanae infected DH82, S2, and Vero cells, and on uninfected DH82, S2, and Vero cells. The cells were prepared on glass slides using a Shandon cytospin, fixed with acetone, dried for 10 min, and outlined using a Pap Pen (The Binding Site Inc., San Diego, CA). Samples were placed in PBS containing 0.01% Tween 20 for 5 min and then blocked with PBS containing 10% normal goat

serum for 30 min at 37 °C. Samples were washed in PBS for 5 min and incubated with primary antibody (polyclonal rabbit-anti-spotted fever; 1 : 100 dilution) for 24 h in the dark at 4 °C, or with normal rabbit serum as a control. The slides were washed with PBS for 5 min and were incubated for 1 h in the dark at room temperature with a 1 : 500 dilution of Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Invitrogen/Molecular Probes). Samples were washed in PBS for 5 min and then in distilled water for 5 min before viewing. Images were viewed with an Olympus BX43 microscope and DP72 camera (Olympus).

Determination of infection by qPCR

Infections were assessed by Rande qPCR assay using the Platinum qPCR UDG Supermix kit (Invitrogen) in a Cepheid Smart Cycler. The number of DNA copies of *Candidatus* R. andeanae was normalized to the housekeeping genes *β-actin* (for DH82 and Vero cells) and *Drosophila ribosomal protein 15a* (for S2 cells). *Candidatus* R. andeanae was detected with our Rande qPCR assay, which was designed using a species-specific sequence of *Candidatus* R. andeanae strain T163 *ompB* gene (Jiang *et al.*, 2011). DH82 *β-actin* was detected using forward primer 5′-GGCATCCTGACCCTGAAGTA-3′, reverse primer 5′-ACGTACATGGTTGGG GTGTT-3′, and probe FAM-5′-ACTGGGACGACATGGAGAAG-3′. Vero *β-actin* was detected using forward primer 5′-ACTGGGACGA CATGGAGAAG-3′, reverse primer 5′-GGGGTGTTGAAG GTCTCAAA-3′, and probe FAM-5′-TGGCACCACACCTT CTACAA-3′. *Drosophila ribosomal protein 15a* was detected as previously described (Schneider & Shahabuddin, 2000). Negative controls consisting of uninfected cells and reactions devoid of template were also subjected to the Rande qPCR assay with each qPCR run. No rickettsial copies were detected in our negative controls.

DNA sequencing and analysis of *Candidatus* R. andeanae

DNA from infected and uninfected cells were used in PCR assays targeting segments of the *gltA, sca4*, and *ompB* genes as previously described (Jiang *et al.*, 2005). Following amplification by nested PCR using the Platinum PCR Supermix High-Fidelity kit (Invitrogen), amplicons were purified using the Qiaquick PCR Purification kit (Qiagen). Sequence reactions were performed using the ABI PRISM 3.0 BigDye Terminator Cycle Sequencing kit (Applied BioSystems, Foster City, CA). Sequenced products were purified with Performa DTR Gel Filtration Cartridges (Edge Bio, Gaithersburg, MD). All amplicons were sequenced on an automated ABI Prism 3100 Gene Analyzer (Applied Biosystems). Sequence analysis was completed using Geneious Pro 5.4 software (Drummond *et al.*, 2011).

Results

DH82, S2, and Vero cell lines support the growth of *Candidatus* R. andeanae

To determine whether *Candidatus* R. andeanae could be isolated and propagated in cell culture, we used one-half of a *Candidatus* R. andeanae-positive tick to infect DH82, S2, and Vero cell lines. Infections were assessed by molecular techniques, cytological staining, and immunocytochemistry. Initial isolation of the bacteria in all cell lines was confirmed by qPCR at 4 and 5 days following inoculation of the cells with tick triturate. To confirm that the isolated bacteria were replicating and retained infectiousness, the media from the original three cultures were transferred at 5 dpi to respective uninfected DH82, S2, or Vero cells. Six days later, all subcultures tested positive by Rande qPCR assay for the presence of *Candidatus* R. andeanae DNA. We then compared the kinetics of *Candidatus* R. andeanae infection in all three cell lines (Fig. 1). Time course infection studies in all three cell lines were established by transferring the media from the original cultures and subcultures of DH82, Vero, and S2 cells to fresh flasks of DH82, Vero, or S2 cells, respectively. Duplicate infections created from independent subcultures were monitored during the time courses, which were performed in December of 2010 and February 2011 (Fig. 1). Rickettsial DNA could be detected by qPCR in all cell lines as early as 1 dpi and as late as 17 dpi (Fig. 1). *Candidatus* R. andeanae did not demonstrate sustained, exponential growth in any one particular cell line, but a pattern of increasing and decreasing growth kinetics was generally observed across the time courses in all three cell lines. The maximum number of rickettsial DNA copies observed was 6.4×10^4 mL^{-1} in the S2 cells at 6 dpi (Fig. 1a); 1.6×10^5 mL^{-1} in the DH82 cells at 1 dpi (Fig. 1d); and 5.3×10^5 mL^{-1} in the Vero cells at 2 dpi (Fig. 1e). The minimum number of copies observed was 1 in the S2 cells at 13 dpi and 8 dpi (Fig. 1a and b); 22 in the DH82 cells at 7 dpi (Fig. 1d); and 1 in the Vero cells at 14 dpi (Fig. 1e). At all the instances in which the minimum number of rickettsial DNA copies was detected, there was always a subsequent increase in the number of copies present the following day, indicating that the bacteria were replicating within the indicated cell lines. When comparing the total average number of rickettsial DNA copies present across the time courses, the Vero cells harbored the overall highest average number of rickettsial DNA copies. Consequently, *Candidatus* R. andeanae can be cultivated in DH82, S2, and Vero

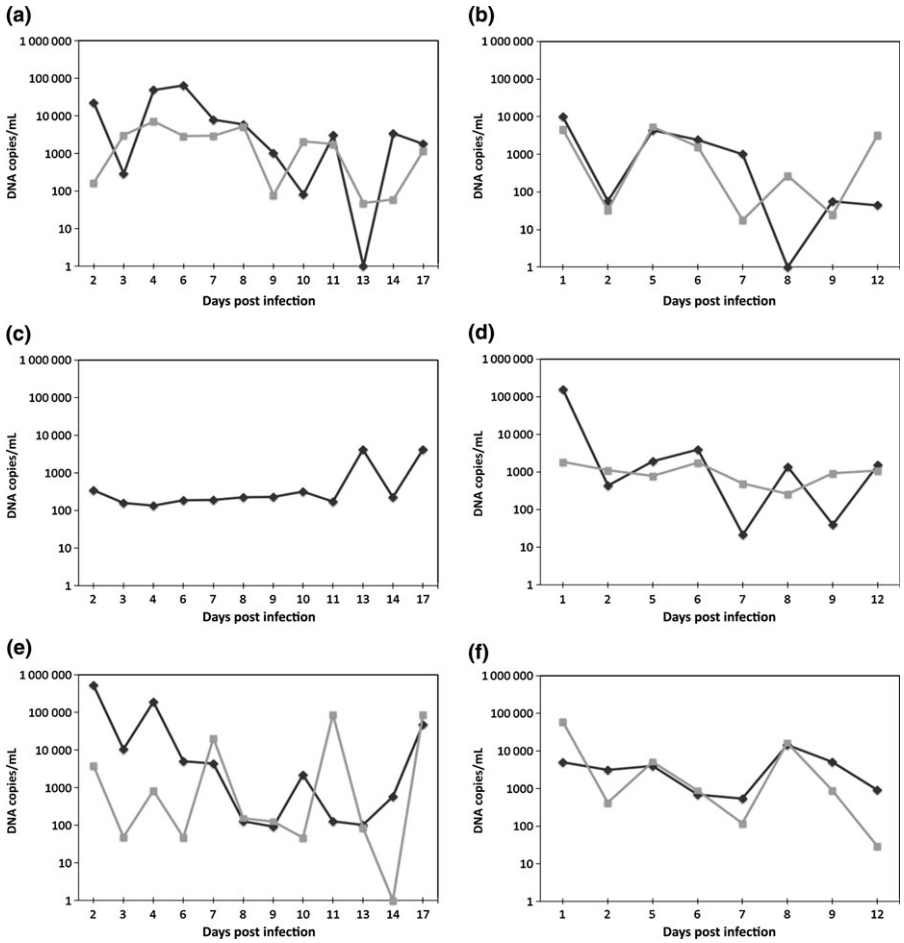

Fig. 1. Growth of *Candidatus* Rickettsia andeanae in three cell lines. Independent time course infections are shown for each cell line: a, b (S2 cells); c, d (DH82 cells); e, f (Vero cells). Each line represents an independent time course sampled from an independent flask: a, c, e were performed in December 2010 and b, d, f were performed in February 2011. The number of DNA copies present at indicated time points was quantitated using Rande qPCR assay. For (c), one of the time course infections became contaminated and was discontinued. No rickettsial copies were detected in uninfected/negative controls (data not shown).

cells lines and completes it replication cycle within each. To date, cultures in all three cell lines have been continuously maintained using infected cell cultures for re-infection of uninfected cells.

To further demonstrate the presence of *Candidatus* R. andeanae growth within our three cell lines, we employed Diff-Quik staining, acridine orange staining, and immunocytochemistry. Cells were monitored by these staining techniques at various time points described for time course infections and at later time points during continuous cultivation. Most commonly, rickettsial organisms were detected starting within 6–9 dpi, and approximately 5–20% of the cells were found to be infected. For both types of staining and when using a polyclonal antibody directed against spotted fever group rickettsiae, rickettsiae could be observed in the cytoplasm of the infected cells and were also found extracellularly in some instances (Figs 2–4). For immunocytochemical staining, the rickettsiae appeared most often in the cytoplasm of the cells (often in groups of organisms) and could also be detected extracellularly (Fig. 4). When normal rabbit serum was used as a primary antibody, no rickettsiae could be detected in infected cells. No rickettsiae could be distinctly identified within the nucleus in any type of infected cells. In addition, the most apparent cytotoxic/cytopathic effect that was observed included an increase in the number of vacuoles in all cell lines during the early stages of infection (1–4 dpi).

DNA sequencing and analysis confirms *Candidatus* R. andeanae

DNA extracted from DH82, S2, and Vero cells that were infected with *Candidatus* R. andeanae was amplified by

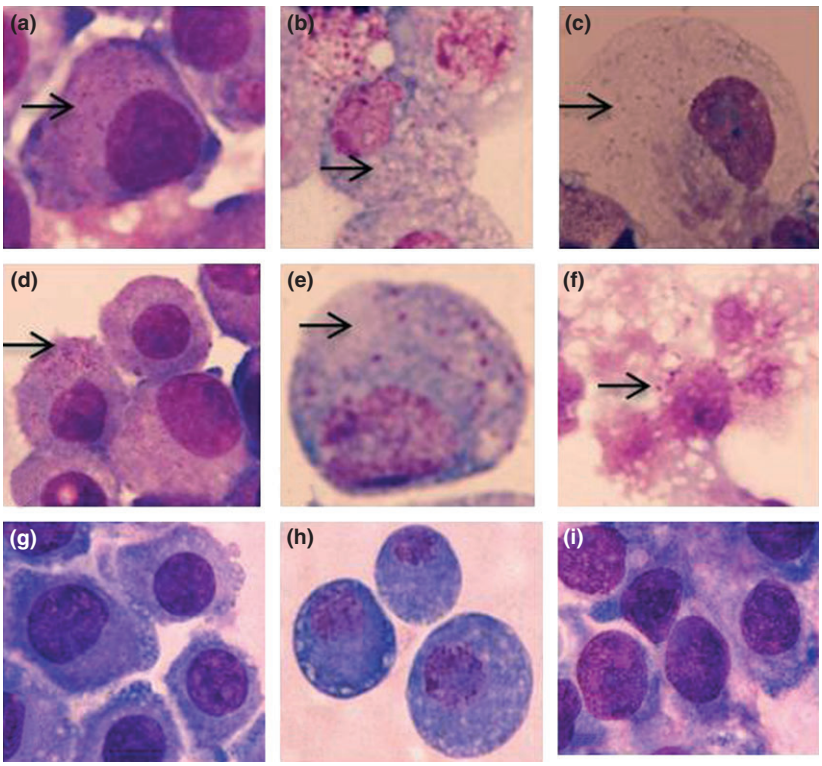

Fig. 2. Diff-Quik staining of rickettsiae in cytoplasm of different cell lines at various time points. Arrow indicates infected cell(s) containing rickettsia in (a, d) DH82 cells (10 dpi); (b, e) S2 cells (7 dpi); and (c, f) Vero cells (9 dpi). No rickettsiae were observed in uninfected cells as shown in (g) DH82; (h) S2; and (i) Vero. Images were captured using an Olympus BX43 microscope and DP72 camera with a 10× ocular and 100× objective.

PCR for fragments of the *sca4*, *gltA*, and *ompB* genes, as previously described (Jiang *et al.*, 2005). The 1653-bp fragment of the *sca4* gene and the 1269-bp fragment of the *ompB* gene both displayed 100% sequence identity with the corresponding homologous gene fragment of *Candidatus* R. andeanae isolate T163 (Table 1). The 1093-bp fragment of the *gltA* gene displayed 100% sequence identity to *Candidatus* R. andeanae isolate T163 and T124 (Table 1). Isolates T163 and T124 were identified in an *Ixodes boliviensis* tick and an *A. maculatum* tick, respectively, collected in northern Peru (Blair *et al.*, 2004) and were the first suggested to be classified as *Candidatus* R. andeanae (Jiang *et al.*, 2005). Additionally, a partial sequence of the *gltA* fragment was found to share 100% homology with *Rickettsia* sp. *'Argentina'* (Table 1). These results established that our three cell lines were infected with *Candidatus* R. andeanae.

Discussion

Herein, we have described the first successful *in vitro* cultivation of *Candidatus* R. andeanae from one-half of a single *Amblyomma maculatum* tick collected in Virginia, USA (Wright *et al.*, 2011). The propagation of *Candidatus* R. andeanae in three distinct cell lines was confirmed using molecular, cytological, and phylogenetic tools. We observed the oscillating presence of rickettsial DNA copies in all three cell lines during a period of 17 days following infection, which may in fact be the result of the random sampling of 1 mL of cells per day. During subsequent continuous cultivation of the bacteria, we have observed the presence of rickettsial DNA (in all three cell types) for longer than 30 days following initial infection of cells and have utilized these infected cultures to start new infections in cell lines. *Candidatus* R. andeanae grew in the highest overall quantity across the time courses in the Vero cells, which are commonly used for the propagation of rickettsial species. In general, *Candidatus* R. andeanae is slow growing in culture, has the ability to persist in low copy numbers for an extended period of time, and can be used continuously for the establishment of new infections in the aforementioned cells lines.

The cytological techniques of Diff-Quik, acridine orange staining and immunocytochemistry, allowed us to observe the *Candidatus* R. andeanae within the host cells. No extreme cytopathic/cytotoxic events were observed in any of the infected cell lines that were assessed. The only notable morphological change in the cells was a large

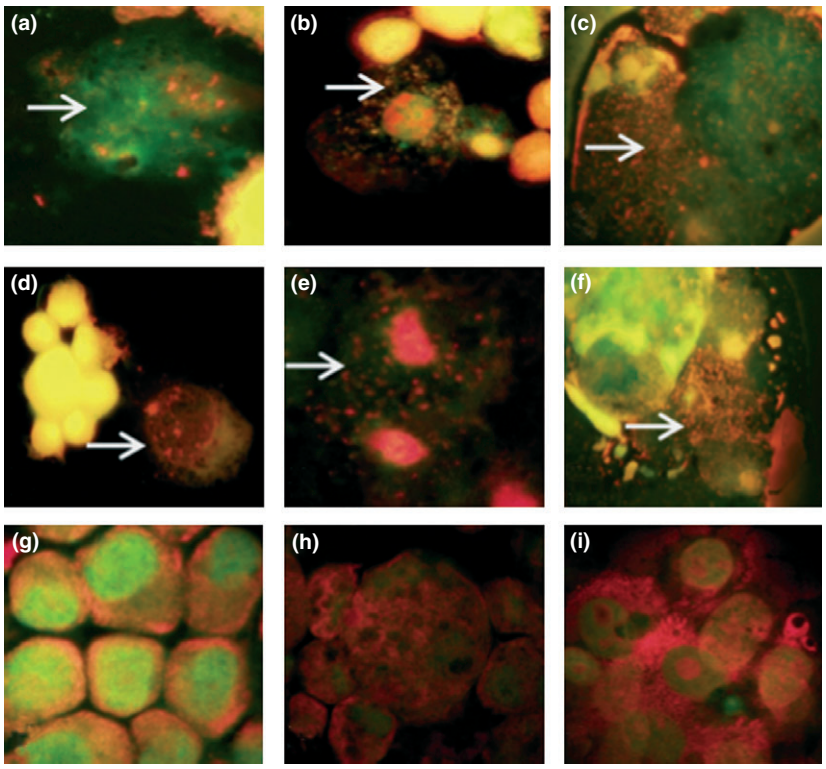

Fig. 3. Acridine orange staining of rickettsiae in different cell lines at various time points. Arrow indicates infected cell containing rickettsia in (a, d) DH82 cells (7 and 5 dpi); (b, e) S2 cells (10 and 5 dpi); and (c, f) Vero cells (6 dpi). No rickettsiae were observed in uninfected cells as shown in (g) DH82; (h) S2; and (i) Vero. Images were captured using an Olympus BX43 microscope and DP72 camera with a 10× ocular and 100× objective.

increase in the number of vacuoles present in the cells during the early time points (1–4 dpi) following infection. The number and size of vacuoles present in the infected cells at these time points varied and may possibly be related to the dose used for the infection. Moreover, bacteria never appeared to be present in these vacuoles at the early time points and the vacuoles lessened in number during the progression of the infection. All rickettsiae identified by staining in this study were found growing the cytoplasm of host cells. *Candidatus* R. andeanae could not be found in the nuclei of any of the infected cell types using the cytological staining techniques or immunocytochemistry. Intranuclear growth has been a characteristic for spotted fever group pathogens such as *R. rickettsii* and *R. japonica* (Burgdorfer *et al.*, 1968; Uchida *et al.*, 1992), but not for those such as *Candidatus* R. amblyommii and R. raoultii (Munderloh *et al.*, 1998; Mediannikov *et al.*, 2008). Therefore, electron microscopy work and nuclear extraction studies will be necessary to establish whether or not intranuclear growth is a characteristic of *Candidatus* R. andeanae.

DNA sequencing analysis confirmed that our cell lines were infected with *Candidatus* R. andeanae. Examination of the *sca4*, *gltA*, and *ompB* genes revealed that our culture isolates were 100% homologous to previously sequenced molecular isolates of *Candidatus* R. andeanae. Moreover, analysis of the *gltA* sequence revealed a 100% match of the partial sequence to the previously isolated, newly discovered, *R.* sp. '*Argentina*'. *R.* sp. '*Argentina*' was first described in *A. parvum* ticks collected in northern Argentina and was found to be closely related to *Candidatus* R. amblyommii and *Candidatus* R. raoultii (now *R. raoultii*) through sequencing of the *ompB* gene (Pacheco *et al.*, 2007). We observed the same close relation of our culture isolate to the aforementioned species upon sequencing of the *ompB* gene. Furthermore, Paddock *et al.* (2010) described a novel *Rickettsia* species in *A. maculatum* ticks collected in Georgia, Florida, and Mississippi. Sequencing analysis revealed that the novel molecular isolate found by Paddock *et al.* (2010) shared identity with *Candidatus* R. andeanae.

In conclusion, we have shown that *Candidatus* R. andeanae can be propagated in three different cells lines and displays characteristics typical of spotted fever group rickettsia. Cytological and genomic studies are underway in our laboratory to finalize the validation of

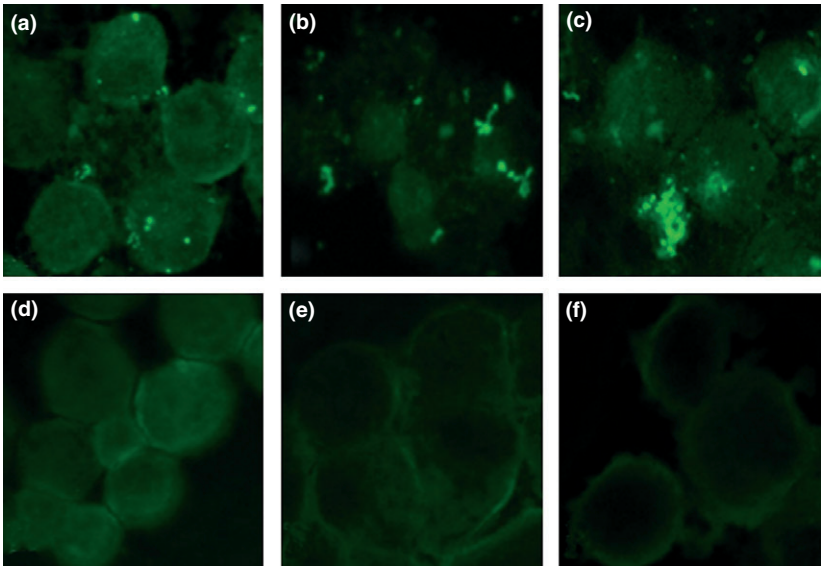

Fig. 4. Immunocytochemical detection of *Candidatus* R. andeanae in different cell lines at 5 days after infection. Rickettsiae could be detected by the incubation of the cells with polyclonal spotted fever group primary antibody and goat anti-rabbit IgG secondary antibody in infected cells as seen in (a) infected DH82; (b) infected S2; and (c) infected Vero cells. No reaction was observed in uninfected cells treated with polyclonal spotted fever group primary antibody and goat anti-rabbit IgG secondary antibody as seen in (d) uninfected DH82; (e) uninfected S2; and (f) uninfected Vero cells. No reaction was observed in either infected or uninfected cells that were treated with normal rabbit serum primary antibody and goat anti-rabbit IgG secondary antibody (data not shown). Images were captured using an Olympus BX43 microscope and DP72 camera with a 10× ocular and 100× objective.

Table 1. Closest relative sequences to gene consensus sequences of *Candidatus* Rickettsia andeanae isolated from *Amblyomma maculatum* and grown in DH82, S2, and Vero cell lines

Gene & GenBank accession number	Species	% Similarity (bp)
sca4		
GU395298.1	*Candidatus* Rickettsia andeanae isolate T163 sca-family protein (*sca4*) gene	100 (1653/1653)
AF155055.1	*Rickettsia japonica* protein PS 120 (*D*) gene	96.4 (1611/1671)
EF219465.1	*Rickettsia* sp. *TwKM01* PS120 (*D*) gene	96.3 (1610/1671)
AY331396.1	*Rickettsia heilongjiangensis* protein PS120 (*D*) gene	96.1 (1606/1671)
gltA		
GU169051.1	*Candidatus* Rickettsia andeanae strain T163 citrate synthase (*gltA*) gene	100 (1093/1093)
GU169050.1	*Candidatus* Rickettsia andeanae strain T124 citrate synthase (*gltA*) gene	100 (1093/1093)
EF451001.1	*Rickettsia* sp. 'Argentina' citrate synthase (*gltA*) gene	100 (1085/1085)
DQ365804.1	*Rickettsia raoultii* strain Khabarovsk citrate synthase (*gltA*) gene	99.5 (1088/1093)
ompB		
GU395297.1	*Candidatus* Rickettsia andeanae isolate T163 outer membrane protein B (*ompB*) gene	100 (1269/1269)
AY652981.1	*Rickettsia andeanae* outer membrane protein B (*ompB*) gene	100 (1269/1269)
FJ455415.1	*Candidatus* Rickettsia amblyommii isolate 85-1084 outer membrane protein B (*ompB*)	98.5 (1250/1269)
HQ232263.1	*Rickettsia raoultii* from *Dermacentor reticulatus* B24 outer membrane protein B (*ompB*) gene	98.3 (1249/1270)

this organism as a new species of *Rickettsia*. More importantly, the human pathogenic potential of newly described species is often undetermined until the pathogens can be isolated in pure culture. As *Candidatus* R. andeanae has been detected in human-biting ticks such as *A. maculatum, Rhipicephalis sanguineus* and *A. parvum* in regions of South America and the United States (Blair *et al.*, 2004; Pacheco *et al.*, 2007; Paddock *et al.*, 2010; Jiang *et al.*, 2011; Wright *et al.*, 2011), the possibility for these novel pathogens to have an impact on human health is a reality. Therefore, the successful cultivation of *Candidatus* R. andeanae is a key step toward future experiments that will determine the pathogenic potential of this novel species.

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