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Original article

Pathogenic potential of a Costa Rican strain of '*Candidatus* Rickettsia amblyommii' in guinea pigs (*Cavia porcellus*) and protective immunity against *Rickettsia rickettsii*

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ABSTRACT

'*Candidatus* Rickettsia amblyommii' is a spotted fever group rickettsia that is not considered pathogenic, although there is serologic evidence of possible infection in animals and humans. The aim of this study was to evaluate the pathogenic potential of a Costa Rican strain of '*Candidatus* R. amblyommii' in guinea pigs and determine its capacity to generate protective immunity against a subsequent infection with a local strain of *Rickettsia rickettsii* isolated from a human case. Six guinea pigs were inoculated with '*Candidatus* R. amblyommii' strain 9-CC-3-1 and two controls with cell culture medium. Health status was evaluated, and necropsies were executed at days 2, 4, and 13. Blood and tissues were processed by PCR to detect the *gltA* gene, and end titers of anti-'*Candidatus* R. amblyommii' IgG were determined by indirect immunofluorescence. To evaluate protective immunity, another 5 guinea pigs were infected with '*Candidatus* R. amblyommii' (IGPs). After 4 weeks, these 5 IGPs and 3 controls (CGPs) were inoculated with pathogenic *R. rickettsii*. Clinical signs and titers of anti-*Rickettsia* IgG were determined. IgG titers reached 1:512 at day 13 post-infection with '*Candidatus* R. amblyommii'. On day 2 after inoculation, two guinea pigs had enlarged testicles and '*Candidatus* R. amblyommii' DNA was detected in testicles. Histopathology confirmed piogranulomatous orchitis with perivascular inflammatory infiltrate in the epididymis. In the protective immunity assay, anti-*Rickettsia* IgG end titers after *R. rickettsii* infection were lower in IGPs than in CGPs. IGPs exhibited only transient fever, while CGP showed signs of severe disease and mortality. *R. rickettsii* was detected in testicles and blood of CGPs. Results show that the strain 9-CC-3-1 of '*Candidatus* R. amblyommii' was able to generate pathology and an antibody response in guinea pigs. Moreover, its capacity to generate protective immunity against *R. rickettsii* may modulate the epidemiology and severity of Rocky Mountain spotted fever in areas where both species circulate.

1. Introduction

'*Candidatus* R. amblyommii' was first detected in *Amblyomma americanum* and referred to as the WB-8-2 *Rickettsia* (Burgdorfer et al., 1981). In these studies, Burgdorfer et al. concluded that WB-8-2 *Rickettsia* was probably not pathogenic for humans because it did not generate an immune response or disease in guinea pigs, although an antibody response was evidenced in field mice (*Microtus pennsylvanicus*) (Burgdorfer et al., 1981). '*Candidatus* R. amblyommii' has since been identified as a common *Rickettsia* in ticks of North and South America, especially those of the genus *Amblyomma* (Mixson et al., 2006; Labruna et al., 2011). In some areas, its prevalence in ticks can be higher than 50% (Bermúdez et al., 2009; Jiang et al., 2010; Zhang et al., 2012; Blanton et al., 2014; Nadolny et al., 2014).

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The pathogenic potential of '*Candidatus* R. amblyommii' has been debated. For example, a localized rash was attributed to '*Candidatus* R. amblyommii' after a tick bite (Billeter et al., 2007), and seroconversion with a fourfold or greater rise in IgG titers to '*Candidatus* R. amblyommii', but not to *Rickettsia rickettsii*, was demonstrated in patients with a presumptive clinical diagnosis of Rocky Mountain spotted fever (RMSF) in North Carolina, USA (Apperson et al., 2008). In Tennessee, where cases of RMSF are frequently reported, a study failed to find *R. rickettsii* in ticks, but found a high prevalence of '*Candidatus* R. amblyommii' in *A. americanum* (Moncayo et al., 2010). There is also molecular and serological evidence of '*Candidatus* R. amblyommii' infection in dogs following exposure to tick bites (Barrett et al., 2014), while other seroprevalence studies also suggest that there may be infection in dogs and horses (Labruna et al., 2007; Bermúdez et al., 2011). However, a recent study failed to detect signs of disease in guinea pigs infected with a North American strain of '*Candidatus* R. amblyommii', confirming Burgdorfer's initial finding (Blanton et al., 2014).

Several rickettsiae are known to elicit an immune response that may later protect its host from a more pathogenic species. This has been demonstrated for species such as *Rickettsia montanensis*, *Rickettsia australis*, *Rickettsia conorii*, *Rickettsia typhi*, and more recently for '*Candidatus* R. amblyommii' (Feng and Waner, 1980; Walker et al., 1984; Feng and Walker, 2003; Blanton et al., 2014). Considering that strains of rickettsiae may show differences in virulence and that '*Candidatus* R. amblyommii' and *R. rickettsii* are present in many areas of Central and South America (Ellison et al., 2008; Parola et al., 2013), the purpose of this study was to evaluate the pathogenic potential of a Costa Rican strain of '*Candidatus* R. amblyommii' in guinea pigs and confirm its capacity to generate cross-protective immunity against a local virulent strain of *R. rickettsii*.

2. Materials and methods

2.1. Animals

Male guinea pigs, *Cavia porcellus*, 200–280 g body weight, were used at the beginning of all experiments. They were maintained in separate cages at the Animal Research Laboratory of Universidad de Costa Rica with vitamin C supplement, and food and water *ad libitum*. When indicated for each experiment, animals were anesthetized with an intramuscular injection (dosage 25 mg/kg) of Zoletil® 50 (Virbac), which is a mixture of tiletamine and zolazepam (25 mg/mL of each). To euthanize animals, an overdose of these anesthetics was applied, followed by an intracardiac injection of magnesium sulfate. All experiments and procedures were performed or supervised by a veterinarian, were approved by Universidad de Costa Rica's Institutional Committee for the Use and Care of Laboratory Animals (number CICUA-35-10), and follow the International Guiding Principles for Biomedical Research Involving Animals (CIOMS and ICLAS, 2012).

2.2. Rickettsia isolates

'*Candidatus* R. amblyommii' strain 9-CC-3-1 was isolated in Costa Rica from *Amblyomma cajennense* sensu lato (Hun et al., 2011). The pathogenic isolate of *R. rickettsii* employed was obtained from a human case (Arguello et al., 2012). Both rickettsiae were cultured separately in confluent monolayers of Vero E6 cells in Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum and maintained at 28 °C and 5% CO_2. The second passage of '*Candidatus* R. amblyommii' strain 9-CC-3-1 and the third passage of *R. rickettsii* were used. Unless otherwise stated, the concentration of bacteria was determined by flow cytometry using an acridine orange staining (0.01 μg/mL) with a Guava EasyCyte™ cytometer (Silverman et al., 1979; Luce-Fedrow et al., 2012).

2.3. Pathogenic potential of 'Candidatus *R. amblyommii*'

A total of eight guinea pigs were used to assess pathogenic potential. The method for infection and evaluation of guinea pigs was adapted from those described previously with other rickettsiae (Feng and Waner, 1980). On day 0, all animals were anesthetized, and 6 guinea pigs were inoculated intraperitoneally with 1 mL of 4×10^6 '*Candidatus* R. amblyommii' bacteria suspended in MEM. The approximate concentration of bacteria was determined with Breed's method by performing dilutions and counting bacteria stained with Giménez stain under a light microscope (1000× magnification) (Giménez, 1964) and by acridine orange staining with a flow cytometer (see above). Two other guinea pigs served as controls and were inoculated in the same manner with only MEM.

Weight, temperature, and clinical signs of disease were evaluated for all guinea pigs at days 0, 1, 2, 3, 4, 7, 9, and 11. Animals were anesthetized and a 0.1 mL blood sample was drawn by cardiac puncture on days 0, 1, 2, 3, 4, 7, 9, 11, and 13. Serum and blood clot were separated and stored at −20 °C for immunofluorescence and PCR analyses, respectively. Necropsies of infected guinea pigs were performed in duplicate on days 2, 4, and 13. Necropsies of both controls were done on day 13. The last day of the experiment was determined based on Feng and Waner (1980) and when no increase was detected in IgG end point titers by immunofluorescence, as well as by continuous negative PCR results on previous days. Tissue samples from brain, heart, lungs, spleen, liver, intestines, kidneys, and testicles of each guinea pig were stored frozen at −20 °C for PCR analyses or preserved in 10% buffered formalin for histopathological studies. Organs were processed by standard histopathological protocols, and organ sections were stained with hematoxylin–eosin (H&E).

2.4. Cross-protective immunity

A total of eight guinea pigs were used to assess cross-protective immunity. The method for evaluating cross-protective immunity in guinea pigs was adapted from the one described by Feng and Waner (1980). Five guinea pigs (IGPs) were inoculated intraperitoneally on day 0 with 6×10^6 '*Candidatus* R. amblyommii' bacteria suspended in 1 mL of MEM. Another 3 guinea pigs were inoculated with MEM and used as non-immune controls (CGPs). At day 0 and for the following three weeks, temperature, weight, and signs of disease were evaluated twice weekly. A 0.1 mL blood sample was drawn once a week by cardiac puncture to detect anti-*Rickettsia* IgG by immunofluorescence.

One month later (day 32 after initial infection), IGPs and CGPs were infected intraperitoneally with 1 mL of 1×10^6 *R. rickettsii*, which is equal to the 50% tissue culture infectious dose ($TCID_{50}$). The $TCID_{50}$ for the pathogenic strain of *R. rickettsii* was determined in Vero E6 cells with the Dulbecco plaque assay, according to methods described previously (Wike et al., 1972). Temperature, weight, and signs of disease were evaluated daily until the end of the experiment on day 11 after *R. rickettsii* infection, when all animals were euthanized simultaneously. The end of the experiment was determined by the need to euthanize 2 CGPs following the recommendation of the veterinarian in charge, who established that the animals were moribund.

Two sample *t*-tests were used to determine the statistical significance of differences in mean temperature and weight changes between IGPs and CGPs on the last day of the experiment (0.05 level). The variation introduced due to differences in the initial weight of guinea pigs was corrected by subtracting the weight of