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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

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each guinea pig on day 0 from the values obtained in the following evaluations.

Blood samples (0.1 mL) were drawn from all guinea pigs by cardiac puncture on the day of *R. rickettsii* inoculation and during necropsies. Sera and blood clots were separated and stored at −20 °C for immunofluorescence and PCR analyses, respectively. Necropsies were performed, and tissue samples from brain, heart, lungs, spleen, liver, intestines, kidneys and testicles of each guinea pig were frozen at −20 °C until analyzed by PCR.

2.5. PCR analyses and indirect immunofluorescence

For PCR analyses, genomic DNA was extracted from blood clots and tissue samples using the NucleoSpin® Tissue kit (Macherey-Nagel), following the manufacturer's instructions. *Rickettsia*-specific DNA was detected by single-step PCR using primers CS-78 and CS-323 of the *gltA* gene (401 bp product) (Labruna et al., 2004). Presence of the *ompA* gene was determined with primers Rr190-70 and Rr190-701 and a subsequent semi-inested step with primers Rr190-70 and Rr190-602 that yield a 532 bp product (Regnery et al., 1991; Roux et al., 1996). The *R. rickettsii* isolate was used as a positive control and C6/36 or Vero E6 cells as a negative control. Products were visualized under ultraviolet light after being run in a 2% agarose gel.

The presence of anti-*Rickettsia* sp. IgG antibodies in guinea pig sera was determined by indirect immunofluorescence using '*Candidatus* R. amblyommii' and *R. rickettsii* antigen, according to methods described previously (Walker et al., 1977). Anti-guinea pig IgG produced in goat and conjugated to fluorescein isothiocyanate (FITC) (Sigma–Aldrich) was used as secondary antibody at a dilution of 1:128. Each sample was first evaluated at a dilution of 1:32, and an end point titer was determined in two-fold serial dilutions of positive samples. Controls included were anti-*Rickettsia* sp. IgG positive and negative guinea pig sera.

3. Results

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

Two days after inoculation with '*Candidatus* R. amblyommii' strain 9-CC-3-1, two of six guinea pigs showed bilateral enlargement of the testicles upon clinical examination. These were the two guinea pigs euthanized on day 2. None of the other infected animals presented clinical signs of disease when compared to control guinea pigs. Infected and uninfected animals gained weight in a similar manner throughout the experiment, and temperature stayed within the normal range (37.5–39.5 °C) (data not shown).

During the necropsies, both of the guinea pigs euthanized on day 2 had enlarged testicles, as did one of the guinea pigs euthanized on day 4. Histologically, the testicles of one guinea pig sacrificed on day 2 showed an inflammatory process around testis with a perivascular infiltrate of polymorphonuclear cells (predominant) and some mononuclear cells (Fig. 1A). In addition, the epididymis showed a perivascular inflammatory infiltrate with mainly mononuclear cells (Fig. 1B). The testicles of the second guinea pig euthanized on day 2 presented a severe inflammatory process into testis with an infiltration consisting mainly of polymorphonuclear cells and some mononuclear cells (Fig. 1C); there was also vascular congestion and extravasation of erythrocytes. The epididymis showed a perivascular inflammatory infiltrate mainly with mononuclear cells (Fig. 1D). The guinea pig with enlarged testicles euthanized on day 4 had a light infiltration of polymorphonuclear cells and some mononuclear cells in the tunica albuginea without testicular lesions (Fig. 1E, 1F). No other significant gross or microscopic alterations that differed between control and infected animals were observed after necropsies and pathologic evaluations of all guinea pigs analyzed.

Of all the organs analyzed by PCR, the presence of '*Candidatus* R. amblyommii' was evidenced by detection of the *gltA* and *ompA* fragments only in the testicles of the two guinea pigs euthanized on day 2.

Anti-'*Candidatus* R. amblyommii' IgG was detected in all infected guinea pigs on day 7 with an end point titer of 1:32. Antibody titer reached a maximum of 1:512 on day 11 in all guinea pigs evaluated, and remained constant until the end of the experiment on day 13 (Fig. 2). Cross-reactivity with *R. rickettsii* antigen was detected on day 9 (1:32 titer), and the end point titer increased to a maximum of 1:128 (Fig. 2). All guinea pigs showed the same end point titers on each day evaluated.

3.2. Cross-protective immunity

All guinea pigs infected with '*Candidatus* R. amblyommii' strain 9-CC-3-1 (IGPs) prior to their inoculation with *R. rickettsii* developed IgG antibodies to antigen of '*Candidatus* R. amblyommii' with a maximum end point titer of 1:512 (Fig. 3). IGPs also had cross-reacting IgG end point titers of 1:128 to antigen of *R. rickettsii*. No weight loss, fever, or apparent disease was evident in IGPs after inoculation with '*Candidatus* R. amblyommii' or in any of the guinea pigs before inoculation with *R. rickettsii*.

Three days after *R. rickettsii* inoculation, one IGP showed moderately enlarged testicles, while all control guinea pigs (CGPs) presented severe bilateral orchitis. Guinea pigs from both groups decreased or stopped weight gain after *R. rickettsii* inoculation; IGPs continued weight gain after 4 to 5 days, but CGPs began losing weight and continued weight loss until the end of the experiment on day 11 after *R. rickettsii* infection (Fig. 4A). On the last day of evaluation, the mean weight change in IGPs and CGPs was 272 g (95% CI: 227–317 g) and 148 g (95% CI: 49–247 g), respectively, and this difference was statistically significant ($T = 4.53$; $p = 0.004$). With only one exception, IGPs showed fever (>39.5 °C) that began 3–5 days after *R. rickettsii* infection (Fig. 4B) and reached maximum temperatures that ranged from 39.8 °C to 40.5 °C. Fever lasted 1 day in two IGPs and 5 days in the other two IGPs. All CGPs presented high fever that began on day 4 after *R. rickettsii* inoculation and lasted until the end of the experiment (Fig. 4B). Maximum temperatures in CGPs were between 40.5 °C and 41.2 °C. On the last day, mean temperature of IGPs and CGPs was 38.5 °C (95% CI: 38.0–39.0 °C) and 40.4 °C (95% CI: 39.9–41.0 °C), respectively, and this difference was statistically significant ($T = 8.2$; $p = 0.0002$).

All CGPs but no IGPs exhibited behavioral alterations including loss of appetite, increased aggression, weakness, and decreased response to auditory and mechanical stimuli. According to ethical considerations and following the veterinarian's recommendation, the experiment ended on day 11 after infection with *R. rickettsii* because it was advised to euthanize two of the CGPs.

At the end of the experiment, none of the IGPs showed behavioral or macroscopic alterations of the organs that could be attributed to a rickettsial infection, with the exception of one guinea pig that had slightly enlarged testicles. In contrast, the CGPs had severe cachexia, orchitis with dilated blood vessels, abnormal testicular color, and vasodilation in lungs and spleen, as well as several other organs. The presence of *R. rickettsii* was demonstrated by PCR only in testicles of all CGPs. Other organs of CGPs were PCR-negative, as were all organs from IGPs.

In IGPs, the end point titer of IgG antibodies at the end of the experiment was 1:2048 for *R. rickettsii* and 1:1024 for '*Candidatus* R. amblyommii'. In the control group, IgG end point titers to *R. rickettsii* were 1:4096 and only 1:512 to *R. amblyommii* (Fig. 3). As with the previous experiment, all guinea pigs within the same group showed the same end point titers on each day evaluated.

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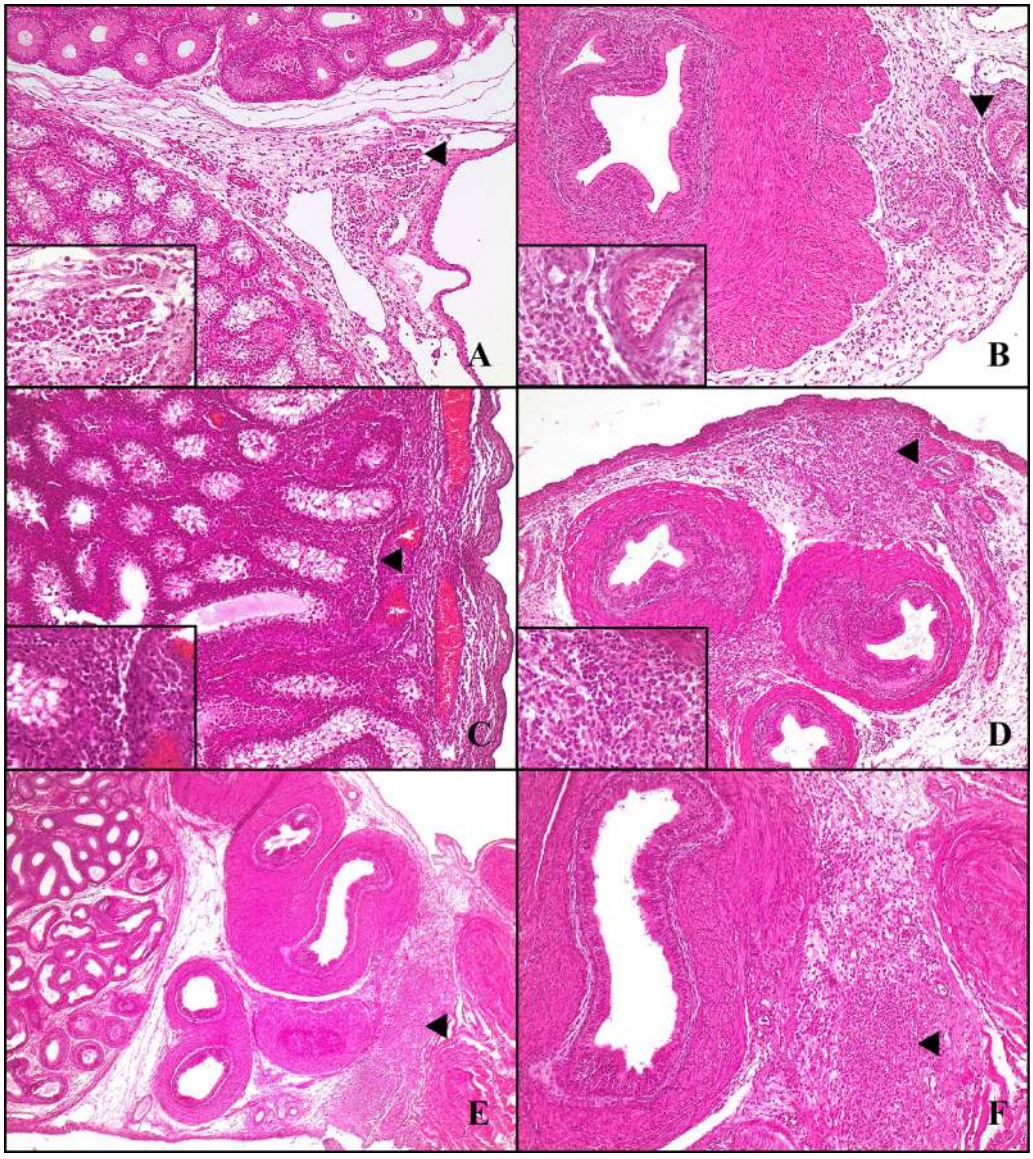

Fig. 1. Histopathological alterations in guinea pigs inoculated with '*Candidatus* Rickettsia amblyommii' strain 9-CC-3-1. (A) Periorchitis with a perivascular infiltrate of polymorphonuclear cells (predominant) and mononuclear cells (arrowhead; magnification: 100×); detail of the inflammatory infiltrate in the inset (magnification: 600×). (B) Epididymis with a perivascular inflammatory infiltrate of mainly mononuclear cells (arrowhead; magnification: 100×); detail of the inflammatory infiltrate in the inset (magnification: 600×). (C) Testis with inflammatory infiltrate of polymorphonuclear cells (predominant) and mononuclear cells (magnification: 100×); detail of the inflammatory infiltrate in the inset (magnification: 600×). (D) Epididymis with perivascular inflammatory infiltrate of mononuclear cells (arrowhead; magnification: 100×); detail of the inflammatory infiltrate in the inset (magnification: 600×). (E) Testis without inflammatory process (arrow) and tunica albuginea with a light infiltration of polymorphonuclear cells and some mononuclear cells (arrowhead; magnification: 100×). (F) Detail of the inflammatory infiltrate in the albuginea (arrowhead; magnification: 400×).

4. Discussion and conclusions

In this study, two of the five guinea pigs inoculated with '*Candidatus* R. amblyommii' strain 9-CC-3-1 showed signs of testicular alterations early after infection, which was confirmed during necropsies and in the histopathological analysis. In addition, the detection of '*Candidatus* R. amblyommii' DNA confirmed that rickettsiae were present in the testicles on day 2, but probably not in other organs. This tropism is common in *R. rickettsii* and other rickettsiae of the spotted fever group (Walker et al., 1977), but had not been demonstrated for '*Candidatus* R. amblyommii'. Moreover, it is likely that '*Candidatus* R. amblyommii' was responsible for the periorchitis and orchitis, especially considering the presence of a perivascular inflammatory infiltrate in the epididymis inflammatory process. In contrast, no clinical manifestations were observed in guinea pigs independent of the inoculation method in the first studies by Burgdorfer et al. (1981), although rickettsial infection was noted occasionally in tunica vaginalis of meadow voles (*M. pennsylvanicus*). Similarly in a more recent experiment with guinea pigs, there was no evidence of illness after intradermal and intraperitoneal inoculation with '*Candidatus* R. amblyommii' (North Texas isolate), but a histopathological analysis was not performed (Blanton et al., 2014). The pathology observed in the present study may be due to a four times greater amount of bacteria inoculated (4×10^6), when compared to the inoculum used by Blanton et al. (2014) (1×10^6), although both were in the same order of magnitude. While intraperitoneal inoculation was also evaluated by Blanton et al. (2014), it is possible that this infection route may facilitate the entry of bacteria into the testicular tunica vaginalis (Quezada Domínguez, 1997; Pham et al., 2005).

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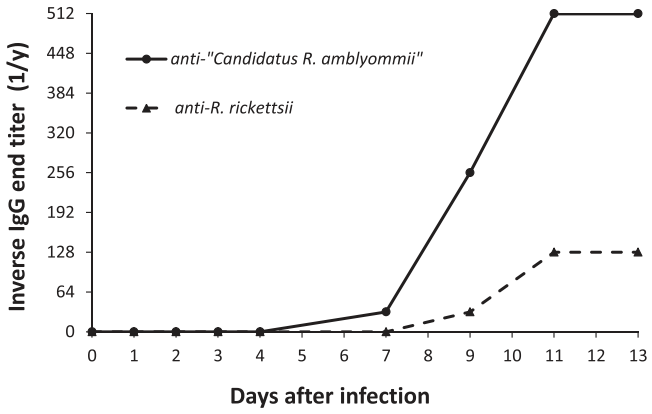

Fig. 2. Anti-*Rickettsia* IgG inverse end point titers after inoculation of guinea pigs with '*Candidatus* Rickettsia amblyommii' strain 9-CC-3-1.

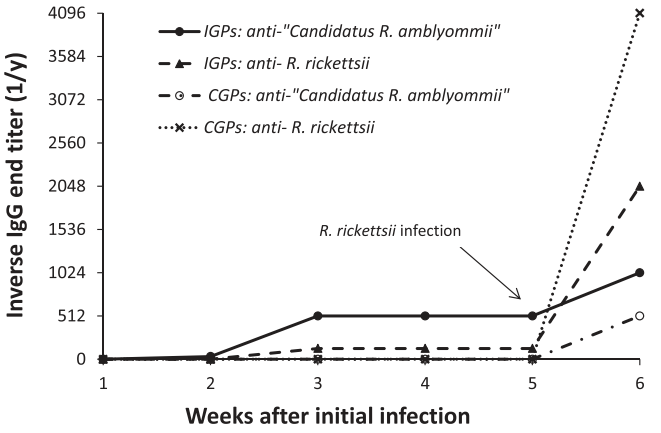

Fig. 3. Anti-*Rickettsia* IgG inverse end point titers of guinea pigs immunized with '*Candidatus* Rickettsia amblyommii' strain 9-CC-3-1 (IGPs) and controls (CGPs) during the assay of cross-protective immunity against *Rickettsia rickettsii*.

In spite of developing orchitis, guinea pigs inoculated with '*Candidatus* R. amblyommii' strain 9-CC-3-1 did not present other signs of disease such as high fever, weight loss, or behavioral abnormalities. Infection with spotted fever group rickettsiae can range from asymptomatic to severe disease, depending on the efficiency of host's immune response and/or the ability of bacteria to generating disease, which may depend on the strain (Walker, 2007). In concordance with Blanton et al. (2014), guinea pigs in this study developed an immune response evidenced by production

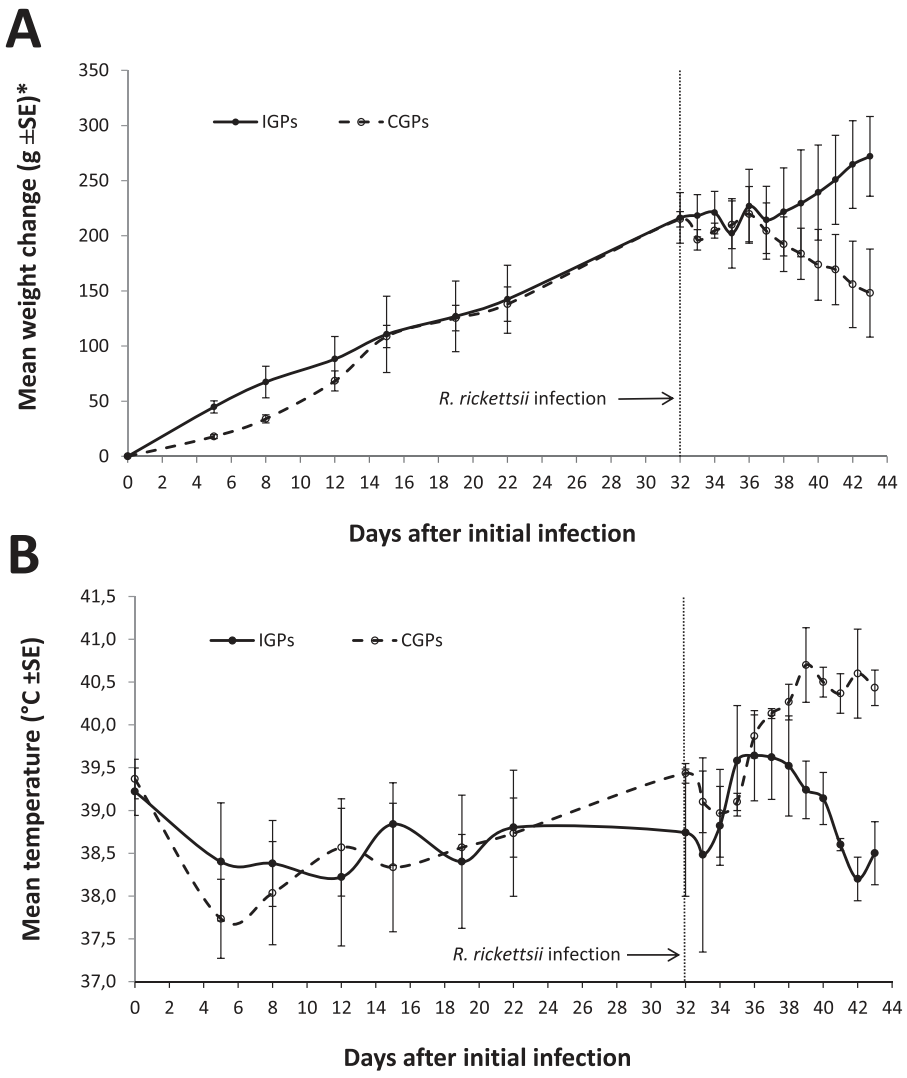

Fig. 4. Mean weight change (A) and mean temperature (B) of guinea pigs during the protective immunity assay. IGPs: animals immunized with '*Candidatus* Rickettsia amblyommii' strain 9-CC-3-1; CGPs: Control animals not immunized with '*Candidatus* R. amblyommii'; SE: standard error.

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of IgG antibodies, which indicates a possible infection. Although IgG antibodies were detected as early as day 7 after inoculation with '*Candidatus* R. amblyommii' strain 9-CC-3-1, maximum IgG end point titers (1:512) were lower than those reported with the North Texas isolate (≥1:4096 at day 14) (Blanton et al., 2014). This may be associated with differences in the secondary antibody conjugate used, inoculum size, inoculation route, *Rickettsia* strain, and characteristics of the guinea pigs. Seroprevalence studies in dogs and horses have shown that IgG end point titers specifically to '*Candidatus* R. amblyommii' are variable, but can be as high as 1:4096 (Labruna et al., 2007; Bermúdez et al., 2011). In a prospective study carried out in Oklahoma, USA, dogs that were naturally exposed to ticks seroconverted to '*Candidatus* R. amblyommii' without signs of disease and reached maximum IgG end point titers of 1:4096 or greater (Barrett et al., 2014). Therefore, all the recent available evidence suggests that '*Candidatus* R. amblyommii' has the capacity to infect and elicit an immune response in guinea pigs, and possibly other animals, although this results in an unapparent or only mild disease.

The assays to determine cross-protective immunity confirmed that a previous infection with '*Candidatus* R. amblyommii' strain 9-CC-3-1 generates an immune response that protects against severe disease by *R. rickettsii*. This was evaluated only recently with '*Candidatus* R. amblyommii' North Texas isolate, but it has been studied further in other species to understand protective immune responses and possible candidates for vaccine development (Feng and Waner, 1980; Walker et al., 1984; Feng and Walker, 2003; Valbuena et al., 2004; Blanton et al., 2014). All guinea pigs that had been infected previously with '*Candidatus* R. amblyommii' survived developing transient disease after inoculation of *R. rickettsii*, while animals infected only with *R. rickettsii* showed all clinical signs and organ damage that is characteristic of severe disease (Walker et al., 1977; Walker and Henderson, 1978; Arguello et al., 2012). The negative PCR result in most organs of control animals despite their moribund condition suggests a fulminant infection, in which bacteria may have concentrated in the testicles and vascular endothelium but may not have had time to directly infect other organs. Previous studies reported bacteria in spleen, lymph nodes, and testicles (especially in vascular endothelium and smooth muscle) of moribund guinea pigs, but not in other organs (Walker et al., 1977). In addition, there were more advanced lesions and more bacteria when supportive therapy was given as opposed to untreated infections. Considering that endothelial cells are recognized as key immunoreactive cells involved in host defense and inflammation (Sahni et al., 2013), some of the alterations observed in the various organs may be the result of a strong immune response.

The protection observed in immunized guinea pigs demonstrates that there was indeed infection with '*Candidatus* R. amblyommii', since previous studies have shown that the immune response obtained from inoculating dead rickettsiae does not prevent animals from dying, independent of antibody production (Kenyon et al., 1979). Moreover, stimulation through the intracellular route that activates T cells and induces cytokines like IFN-γ, TNF-α, IL-1B, and CCL5 seem to be important in eliminating pathogenic rickettsiae (Feng and Walker, 2000; Valbuena et al., 2004; Sahni et al., 2013). Infection with *R. rickettsii* probably occurred in guinea pigs previously infected with '*Candidatus* R. amblyommii', given transient temperature increase, antibody production, and visible orchitis (one animal), but the resulting immune response efficiently eliminated *R. rickettsii* to levels undetectable by PCR.

In spite of the limitations of this study such as the small number of guinea pigs used and the inoculation dose and route, results show that '*Candidatus* R. amblyommii' strain 9-CC-3-1 was able to cause some degree of pathology in guinea pigs, indicating that it should not be excluded as a possible cause of mild disease in these and other animals without further investigation. Indirect evidence has suggested that '*Candidatus* R. amblyommii' may be responsible for disease in humans (Billeter et al., 2007; Apperson et al., 2008), thus specific conditions of the host, such as compromised immune system and the presence of other diseases, should be evaluated. Results also demonstrate the immune response generated after infection with '*Candidatus* R. amblyommii' strain 9-CC-3-1 from Costa Rica protects guinea pigs from severe disease by a virulent strain of *R. rickettsii* from the same country, which indicates that '*Candidatus* R. amblyommii' may modulate local transmission dynamics and the epidemiology of Rocky Mountain Spotted fever in regions where both species are present.

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