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EVALUACIÓN DEL POTENCIAL PATOGÉNICO DE *BRUCELLA* SP. ST27 AISLADA DE UN “CACHALOTE ENANO” (*KOGIA SIMA*) USANDO MODELOS ANIMALES Y CELULARES

Tesis sometida a la consideración de la Comisión del Programa de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología con énfasis en Genética y Biología Molecular

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DEDICATORIA

A Dios, mi creador, a quien le debo absolutamente todo.

A mi esposo, mis papás, mis hermanos, y mis pastores Salazar y Umanzor.

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RESUMEN EN ESPAÑOL

Los miembros del género *Brucella* son bacterias patogénicas que causan pérdidas económicas significativas, con una creciente preocupación por los mamíferos marinos debido a su potencial impacto zoonótico en la conservación. En esta investigación, evaluamos el grado de patogenicidad de *Brucella* sp. ST27, aislada de la *Kogia sima*, en modelos de laboratorio, en comparación con las cepas de referencia *Brucella abortus*, *Brucella ceti* y *Brucella pinnipedalis*. La resistencia a la polimixina B y al complemento de *Brucella* sp. ST27 fue similar a la de *B. abortus*, *B. ceti* y *B. pinnipedalis*. En el bazo de ratones, *Brucella* sp. ST27 se replicó en mayor medida que *B. ceti*, similar a *B. pinnipedalis*, y en menor número que *B. abortus*. Asimismo, la respuesta inflamatoria inducida en el bazo por *Brucella* sp. ST27 no difirió significativamente de la inducida por *B. ceti* y *B. pinnipedalis*, pero fue considerablemente menor que la producida por *B. abortus* 2308W. Los perfiles hematológicos y las lesiones en el bazo de ratones causadas por *Brucella* sp. ST27 fueron menos severos que las de *B. abortus* 2308W. La replicación intracelular de *Brucella* sp. ST27 en células HeLa fue significativamente menor que la de *B. ceti* y *B. abortus* 2308W, pero similar a la *de B. pinnipedalis*. En general, *Brucella* sp. ST27 muestra rasgos patogénicos similares a otras cepas que infectan mamíferos marinos, pero es más atenuada en modelos de laboratorio que *B. abortus* 2308W.

ABSTRACT

Members of the genus *Brucella* are bacterial pathogens causing significant economic losses and human suffering, with increasing concern over marine mammals due to its potential impact on conservation zoonotic risk. We evaluated the pathogenic potential of *Brucella* sp. ST27 from the dwarf whale *Kogia sima* compared to *Brucella abortus, Brucella ceti* and *Brucella pinnipedalis* in laboratory models. The resistance to polymyxin B and serum complement of *Brucella* sp. ST27 was similar to *B. abortus*, *B. ceti* and *B. pinnipedalis.* In the spleen of mice, *Brucella* sp. ST27 replicated to a greater extent than *B. ceti*, to a similar level than *B. pinnipedalis,* and at a lower number than *B. abortus.* Likewise, the inflammatory response induced in the spleen by *Brucella* sp. ST27 was not significantly different from that induced by *B. ceti* and *B. pinnipedalis* but considerably lower than that produced by *B. abortus* 2308W. The hematological profiles and spleen lesions caused *Brucella* sp. ST27 were less severe than *B. abortus* 2308W. The replication kinetics of *Brucella* sp. ST27 in HeLa cells was significantly lower than *B.ceti* and *B. abortus* 2308W but similar to *B. pinnipedalis*. In general, *Brucella* sp. ST27 displays similar virulent behavior to other strains infecting marine mammals but shows less virulent potential in laboratory models than pathogenic *B. abortus* infecting cattle.

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CFU: Por sus siglas en inglés: Colony-Forming Unit

CICUA: Comité Institucional para el Cuidado y Uso de los Animales de la Universidad de Costa Rica (CICUA)

DMEM: Por sus siglas en inglés: Dulbecco’s Modified Eagle Medium

SENASA: Servicio Nacional de Salud Animal

I.P.: Por sus siglas en inglés: Intraperitoneally

LPS: Por sus siglas en inglés: Lipopolysaccharide

MAG: Ministerio de Agricultura y Ganadería

MIC: Por sus siglas en inglés: Minimum Inhibitory Concentration

MLST: Por sus siglas en inglés: Multilocus Sequence Analysis

PBS: Por sus siglas en inglés: Phosphate-buffered saline

P.I.: Por sus siglas en inglés: Post-infection

RPM: Por sus siglas: Revolutions per minute

ST27: Por sus siglas en inglés: Sequence-type 27

TSA: Por sus siglas en inglés: Trypticase Soy Agar

TSB: Por sus siglas en inglés: Trypticase Soy Br

INTRODUCCIÓN

La brucelosis es una enfermedad zoonótica de distribución mundial con implicaciones económicas importantes en animales de producción. El agente causal es *Brucella*, un género de bacterias intracelulares Gram negativas que afecta a una variedad de mamíferos, incluyendo al humano (Moreno & Moriyón et al., 2006). Las pérdidas económicas y morbilidad humana que genera la brucelosis se asocian, generalmente, al consumo de productos lácteos no pasteurizados y al contacto con animales infectados (Franc et al., 2018).

Hasta el momento se han descrito cerca de 24 diferentes especies de brucelas (Osterman & Moriyón, 2006; Moreno, 2021). A partir de 1994, se realizaron aislamientos de *Brucella* en mamíferos marinos, revelando diferencias bioquímicas y genéticas con las seis especies tradicionales (Ewalt et al.,1994; Ross et al., 1994). Por lo tanto, en 2007 se establecen formalmente las cepas *B. ceti* y *B. pinnipedialis*, obtenidas de cetáceos y pinnípedos, respectivamente (Foster et al., 2007).

La patogenicidad de *Brucella* dependen en gran medida de su capacidad de invadir, sobrevivir y multiplicarse dentro de compartimentos intracelulares de células fagocíticas y células no fagocíticas del hospedero (Roop et al., 2009). La composición de su membrana celular externa les confiere resistencia a las sustancias bactericidas de la respuestas inmunes del hospedero y constituye un factor de virulencia importante para el establecimiento de la infección (Gorvel & Moreno, 2002). Por lo tanto, cabe señalar que, aunque se ha investigado ampliamente la patogenicidad de las brucelas terrestres, los estudios relacionados en brucelas marinas son limitados (Bingham et al., 2008; Maquart et al., 2009; Larsen et al., 2013a; Larsen et al., 2013b; Nymo et al., 2016).

Las brucelas marinas se han aislado de múltiples órganos de mamíferos marinos infectados, siendo el sistema nervioso central y los órganos del sistema reproductor de las hembras los más afectados, causándoles graves lesiones e incluso su muerte (Guzmán-Verri et al., 2012). Se han reportado además tres casos de infección en humanos relacionados con cepas de *Brucella* de origen marino en Perú y Nueva Zelanda (Sohn et al., 2003; McDonald et al., 2006), sin embargo, la fuente de infección no ha sido bien establecida. Los pacientes presentaban afectaciones en el sistema nervioso central y parasitismo en la médula ósea. El análisis de estas bacterias mediante la técnica

"Multilocus sequence typing" (MLST) empleado para la caracterización taxonómica de bacterias, confirmó que dichos aislamientos compartían un genotipo único clasificado como ST27 ("Sequence-type 27" por sus siglas en inglés) (Whatmore et al., 2007). Hasta ese momento, este genotipo ST27 solo había sido reportado en un delfín nariz de botella en la costa occidental de los Estados Unidos, denominado *Brucella* sp. F5/99 (Ewalt et al., 1994). Por lo tanto, la cepa *Brucella* sp. ST27 se volvió de particular interés debido a su asociación con mamíferos marinos y su posible capacidad para infectar a humanos (Whatmore et al., 2008), aunque esto aún no se ha probado.

En la región del Pacífico Central de Costa Rica, se aisló *Brucella* sp. en un cachalote enano hembra preñada de la especie *Kogia sima*, que encalló en la Playa de Herradura en marzo de 2018. Las infecciones causaron aborto y eventualmente la muerte del cetáceo como consecuencia de lesiones en la placenta y en órganos del feto (Hernández-Mora et al., 2021). Algunos de estos aislamientos se sometieron a secuenciación del genoma y se compararon contra *B. ceti* del tipo delfín, marsopa y con *B. pinnipedialis*. Los resultados mostraron relación filogenética estrecha con *Brucella* sp. ST27 (Hernández-Mora et al., 2021), conocida por su relación con infecciones humanas (Whatmore et al., 2008). En este sentido, surge la necesidad de investigar la capacidad de las brucelas con genotipo ST27 para establecer una infección persistente y evaluar los posibles riesgos zoonóticos.

Con el fin de ampliar el conocimiento sobre las especies de *Brucella* que infectan a animales marinos, formulamos la siguiente pregunta: ¿Cuál es el grado de patogenicidad en modelos murinos y modelos celulares de las brucelas con genotipo ST27 aisladas de la *K. sima*? La hipótesis central sostiene que el grado de patogenicidad de las brucelas ST27 aisladas de la *K. sima* es menor para los ratones y cultivos celulares en comparación con *B. abortus* como referencia, y similar a otras especies que infectan animales marinos como *B. ceti* y *B. pinnipedialis*.

El objetivo general de esta tesis fue determinar el potencial patogénico de *Brucella* sp. ST27 obtenidos de *K. sima*, utilizando modelos *ex vivo* (cultivos celulares) y modelos murinos. Como objetivos específicos me propuse: (i) evaluar la capacidad de esta bacteria de penetrar y replicarse en células *ex vivo*; (i) caracterizar la resistencia de la envoltura celular de esta bacteria frente a agentes bactericidas y (iii) determinar la capacidad de multiplicación y persistencia de *Brucella* sp ST27 en órganos blanco de ratones. Los resultados fueron comparados contra *B. ceti* y *B. pinnipedialis* y con la cepa virulenta de referencia *B. abortus* 2308W.

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ARTÍCULO CIENTÍFICO

Pathogenicity of *Brucella* sp. sequence-type 27 isolated from a dwarf sperm whale *Kogia sima*

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ABSTRACT

Members of the genus *Brucella* are bacterial pathogens causing significant economic losses and human suffering, with increasing concern over marine mammals due to its potential impact on conservation zoonotic risk. We evaluated the pathogenic potential of *Brucella* sp. ST27 from the dwarf whale *Kogia sima* compared to *Brucella abortus, Brucella ceti* and *Brucella pinnipedalis* in laboratory models. The resistance to polymyxin B and serum complement of *Brucella* sp. ST27 was similar to *B. abortus*, *B. ceti* and *B. pinnipedalis.* In the spleen of mice, *Brucella* sp. ST27 replicated to a greater extent than *B. ceti*, to a similar level than *B. pinnipedalis,* and at a lower number than *B. abortus.* Likewise, the inflammatory response induced in the spleen by *Brucella* sp. ST27 was not significantly different from that induced by *B. ceti* and *B. pinnipedalis* but considerably lower than that produced by *B. abortus* 2308W. The hematological profiles and spleen lesions caused *Brucella* sp. ST27 were less severe than *B. abortus* 2308W. The replication kinetics of *Brucella* sp. ST27 in HeLa cells was significantly lower than *B.ceti* and *B. abortus* 2308W but similar to *B. pinnipedalis*. In general, *Brucella* sp. ST27 displays similar virulent behavior to other strains infecting marine mammals but shows less virulent potential in laboratory models than pathogenic *B. abortus* infecting cattle.

Keywords

Brucella, Brucella sp. ST27, *Brucella ceti, Brucella pinnipedalis, brucellosis,* marine mammals, zoonosis, pathogenicity, intracellular replication, mice.

INTRODUCTION

Members of the genus *Brucella* are bacterial pathogens of vertebrates with a global distribution that causes significant economic losses and considerable human suffering. All members of the genus, such as *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*, are facultative extracellular-intracellular pathogens with the ability to replicate extensively in the reticuloendothelial and reproductive systems, as well as surviving and replicating within nonphagocytic and phagocytic cells, promoting chronic infections in mammals [1-3].

The presence of *Brucella* organisms in marine mammals has drawn increasing attention due to its potential impact on wildlife conservation and zoonotic risk [4,5]. Numerous cases of *Brucella* spp. infections in marine mammals have been described worldwide since the first isolation in a bottle-nose dolphin in 1994 [6,7]. For now, two species of *Brucella* have been described in marine mammals: *B. ceti*, a pathogen of cetaceans, and *B. pinnipedialis*, primarily infecting pinnipeds [8]. *B. ceti* infections have been linked to a broad spectrum of pathologies, including meningoencephalitis, discospondylitis, subcutaneous abscesses, endometritis, and myocarditis, often leading to stranded marine mammals and, in some cases, fatal outcomes. In contrast, detailed reports on the pathological findings of *B. pinnipedialis* infections remain limited [4,9,10].

Furthermore, three reports of human infections and one laboratory-acquired infection with brucellae strains closely related to *Brucella* species infecting marine mammals and whose source remains unknown have been documented [11-13]. A multilocus sequence analysis (MLST) revealed that these strains isolated from humans shared a genotype classified as sequence type 27 (ST27) [14,15], which is present in *Brucella* strains isolated from marine mammals suffering fatal brucellosis [6,16-19]. For that, *Brucella* sp. ST27 strains became particularly interesting due to their potential zoonotic [15].

Here, we have explored the pathogenic potential of *Brucella* sp. ST27 isolated from a dwarf sperm whale, *Kogia sima,* that aborted and died due to brucellosis in the shorelands of Costa Rica [18]. We demonstrated that *Brucella* sp. ST27 can infect and replicate in mice and epithelial cells like other *Brucella* species isolated from dolphins and seals but to a lower extent than in *B. abortus* infecting cattle.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Bacterial cultures were performed as previously described [8,20-22]. *Brucella* sp. (bmarCR39b) and *Brucella* sp. (bmarCR42b) isolates [18] were grown and maintained in standard trypticase soy broth (TSB) (BD) or agar (TSA) (BD) at 37°C supplemented with 5% CO_2. Bacteria were stored at −80°C prepared in skim milk (BD) aliquots with 20% glycerol (Sigma).

Table 1. *Brucella* strains studied.

Strain	Characteristics	Source/reference
B. abortus (2308W)	Wild-type, virulent, biotype 1, Nal^r spontaneous mutant of strain 2308	Suárez-Esquivel (2016)
Brucella canis (bcanCR12)	Wild-type, virulent	Suárez-Esquivel (2021)
B. abortus (2.13)	2308 Nal^r *bvrS*::Tn*5*	Sola-Landa (1998)
Brucella ceti (B14/94)	Wild-type, virulent, Atlantic dolphin type	Foster (2007)
Brucella pinnipedialis (B2/94)	Wild-type, virulent, seal type	Foster (2007)
Brucella sp. (bmarCR39b)	Wild-type, virulent, isolated from mother	Hernández-Mora (2021)
Brucella sp. (bmarCR42b)	Wild-type, virulent, isolated from fetus	Hernández-Mora (2021)

Susceptibility assays

Determination of the minimum inhibitory concentration to polymyxin B

Brucella strains were cultured overnight in TSB at 37°C with shaking at 200 rpm. A bacterial suspension of 1 McFarland was then prepared in sterile saline solution (0.85%) and diluted 1:100 in TSB. Polymyxin B sulfate (Sigma) was dissolved in sterile water to obtain a final concentration of 500 µg/mL, and two-fold serial dilutions were made in 96-well microtiter-type plates (100 µL per well) using TSB.

To each well containing the polymyxin B dilutions, 100 µL of the bacterial suspension was dispensed into duplicate rows and then incubated at 37°C for 48 to 72 hours. After incubation, the growth of the bacterial culture in each well of the plate was evaluated visually, and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration of polymyxin B that inhibited visible bacterial growth. The results were expressed as the mean of MIC ± standard deviation of at least three experiments performed.

Serum bactericidal activity

Bacterial suspensions containing 10^4 colony-forming units (CFUs) of late-exponentially growing bacteria per mL of phosphate-buffered saline (PBS) were dispensed (200 µL) in Eppendorf tubes containing non-immune human serum (400 µL). As a control, we used a fresh human serum that was decomplemented by adding a pinch of dehydrated yeast *Saccharomyces cerevisiae* and heating at 56°C for 45 minutes. The serum control was then centrifugated at 5000 rpm for 5 min. After 90 minutes of incubation at 37°C, we plated 80 µL of the inoculum in triplicate on TSA plates using the Drigalsky spread plate method. The results were expressed as the mean of CFU survival percentage ± standard deviation of one representative experiment.

Mice infections

CD1 mice obtained from the Servicio Nacional de Salud Animal (SENASA), Ministry of Agriculture, were housed in the biotery of the Veterinary School of the National University, Costa Rica, until use. Mice were kept under biosafety containment conditions with water and food *ad libitum* on a 12:12 h light:dark cycle. Protocols for experimentation with mice were revised and approved by the Comité Institucional para el Cuidado y Uso de los Animales of the Universidad de Costa Rica (CICUA) and in agreement with the corresponding Costa Rican law ("Ley de Bienestar de los Animales No. 7451").

Eight-week-old female and male CD1 mice (18-22 g) were arranged in five groups of 12 mice each. Mice were infected with *B. abortus* 2308W, *B. ceti* B14/94, *B. pinnipedialis* B2/94, *Brucella* sp. (bmarCR39b), or *Brucella* sp. (bmarCR42b), respectively. Each mouse was inoculated intraperitoneally (i.p.) with 0.1 mL sterile PBS holding 10^6 CFUs of the corresponding *Brucella* strain, as determined by serial dilutions and CFU evaluation. At days 8 and 30 post-inoculation, spleen and blood samples were collected, and the bacterial load in the spleen (CFU/gram) was determined as described elsewhere [23].

Histopathology and hematology profiles

For the histopathological studies, the spleen was fixed in 10% neutral buffered formalin, processed, and stained with hematoxylin and eosin stain as described [24]. The spleen lesions were determined by semiquantitative analysis and scored as normal (0) to severe (4), as previously described [25]. The hematological analyses were performed using the VetScan HM5 Hematology Analyzer, following the manufacturer's instructions.

Cell infections

Human HeLa cells (American type culture collection No. CCL-2) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum, 2.5% sodium bicarbonate, and 1% glutamine. Two days before infection, cells were seeded in 24-well tissue culture plates to obtain a final concentration of 5 X 10^5 cells per well and maintained at 37°C under 5% CO_2. Each bacterial strain was grown to a late exponential or stationary phase to infect HeLa cells and diluted in DMEM to reach a multiplicity of infection (MOI) of 500. Plates were then centrifuged at room temperature for 5 min at 1500 revolutions per minute (rpm) and incubated for 45 min under a 5% CO_2 atmosphere at 37°C. After incubation, wells were washed three times with PBS and then incubated for one hour with DMEM supplemented with 100 µg/ml gentamicin (Sigma) to kill the extracellular bacteria. Cells were then incubated with DMEM supplemented with 5 µg/ml gentamicin as a maintenance medium at 0-, 4-, 24- and 48 hours post-infection. Plates were washed twice with PBS and were treated for 10 minutes with 0.5 ml of sterile water to lyse the cells at the indicated times. Lysates were serially diluted in TSA plates and incubated at 37°C under a 5% CO_2 atmosphere to determine CFU.

Statistics

GraphPad Prism software (version 10.0.2) (https://www.graphpad.com) and R software (https://www.r-project.org) (version 4.3.1) were used for statistical analysis. Unless otherwise stated, all results are presented as means ± standard deviation from at least three independent experiments. ANOVA and Kruskal-Wallis tests were used for multiple comparisons to determine statistical significance. P values of <0.05 and <0.01 were considered statistically significant for all tests.

RESULTS

Resistance to serum complement and polymyxin B

Given the relationship between the virulence of *Brucella* organisms and its resistance to the bactericidal activity of serum complement and polycationic peptides [22,26], we evaluated the susceptibility of *Brucella* sp. ST27 to these substances and compared to other *Brucella* species. No significant differences in the resistance against the serum complement of *Brucella* sp. ST27 strains (bmarCR39b and bmarCR42b) were observed with respect to the survival of *B. abortus* 2308W, *B. canis*, *B. ceti* B14/94 and *B. pinnipedialis* B2/94 (Fig. 1A). Likewise, no significant difference of *Brucella* sp. ST27 strains against polymyxin B were observed with *B. abortus* 2308W and the other strains (Fig 1B). The *B. canis* strain (bcanCR12), included as control [27], was resistant to polymyxin B and displayed significant differences when compared to *B. pinnipedialis* B2/94 strain. As serum susceptible control, we included the mutant *B. abortus* 2.13 (bvrS mutant) [22], and as a high polymyxin B-resistant strain, we included *B. canis* [27].

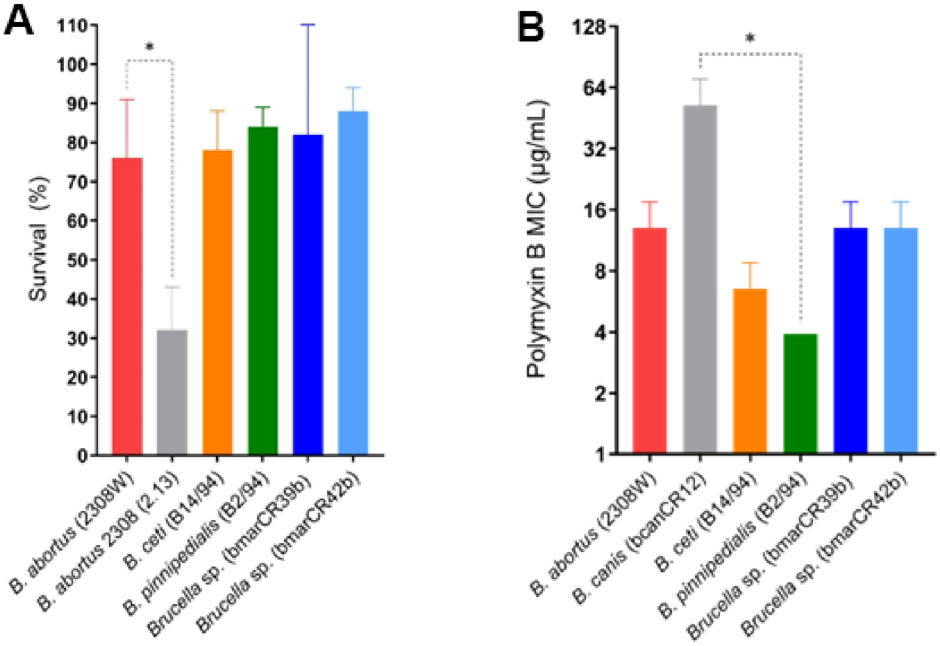

Figure 1. Survival percentage of ***Brucella*** **strains after incubation in non-immune serum and polymyxin B.** (A) All strains were incubated in non-immune human serum for 90 min, and the survival percentage was determined as the average number of CFUs remaining. Each bar graph shows the mean ± standard deviation percent survival of one representative experiment performed in triplicate from at least three independent assays. (B) Bacteria were exposed to serial dilutions of polymyxin B for 48 h at 37°C.

Each bar graph shows the mean ± standard deviation of MIC values resulting from three independent assays performed in duplicate. Significant differences are indicated as * ($p \leq 0.05$).

Brucella replication in the mouse spleen

We quantified the bacterial loads of *Brucella* sp. ST27 isolates in the spleen of infected mice at 8- (acute phase) and 30 days (chronic phase) post-infection (p.i.) [27]. At day 8 p.i., the spleen bacterial loads of *Brucella* sp. ST27 strains were comparable to those of mice infected with *B. abortus* 2308W (Fig. 2). Only *B. ceti* B14/94 and *B. pinnipedialis* B2/94 showed fewer bacterial loads at this time, with a significant difference between them and bacterial load of *Brucella* sp. ST27 bmarCR42b isolate. At day 30 p.i., the bacterial loads of all brucellae from marine mammals declined when compared to *B. abortus* 2308W, with a significant difference in the bacterial loads between mice infected with marine mammal strains and *B. abortus* 2308W, indicating that, at least in this system, the strains from marine mammals are more attenuated than *B. abortus*.

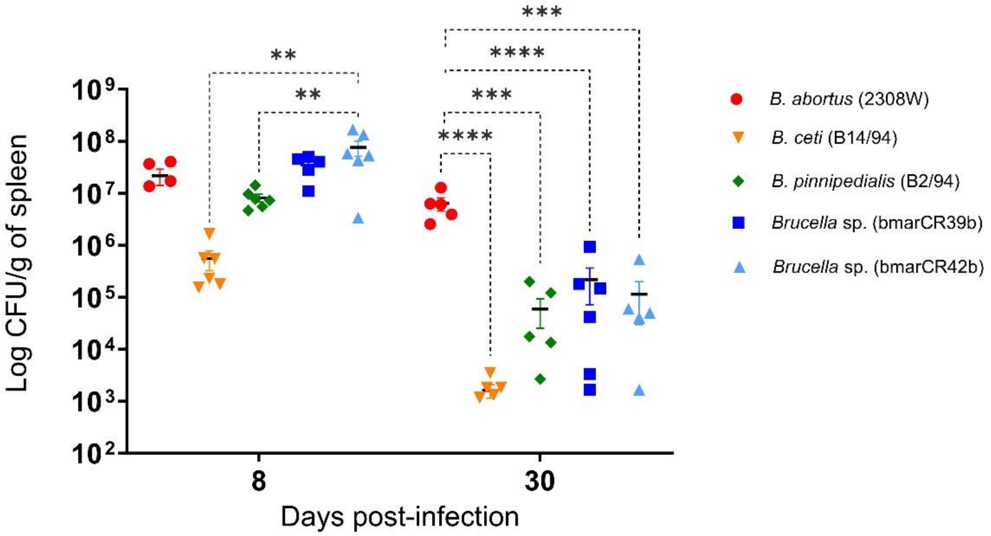

Figure 2. Bacterial counts in the spleen. CD1 mice were infected intraperitoneally with 10^6 bacteria colony-forming units (CFU). Mice were sacrificed eight- and thirty days post-infection, and spleen bacterial load was determined. The results are expressed as the mean ± standard error of individual Log_{10} CFU per spleen. The p-values ≤0.01 (**), ≤0.001 (***), and ≤0.0001 (****) are indicated.

Hematological and histopathological profiles

The hematological results at eight days post-infection (Table 2) show that the percentage of lymphocytes and neutrophils and platelets count were the only hematological parameters that displayed significant differences between *B. ceti* B14/94 and *B. pinnipedialis* B2/94 strains compared to *B. abortus* 2308W. Notably, no significant differences were observed between mice infected with *Brucella* sp. ST27 strains and those infected with *B. abortus* 2308W. In general, the hematological profiles of the *Brucella* sp. ST27 strains were highly similar to *B. ceti* B14/94 and *B. pinnipedialis* B2/94. At 30 days post-infection, we did observe significant differences in the hematological parameters in all marine brucellae strains in some lymphocytes and neutrophils parameters compared to *B. abortus* 2308W (Table 3). This difference was probably due to the changes in the bacterial load. Overall, the strain that displayed the most differences compared to *B. abortus* 2308W at 8- and 30-days p.i. was *B. ceti* B14/94, probably due to the lowest colonization of all strains.

Table 2. Hematological parameters of CD1 mice (n=6) at eight days post-infection with *Brucella* strains.

Parameter	**Unit**	Mice non-infected	*B. abortus* (2308)	*B. ceti* (B14/94)	*B. pinnipedialis* (B2/94)	*Brucella* sp. ST27 (bmarCR39b)	*Brucella* sp. ST27 (bmarCR42b)
		Mean	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Leucocytes	10^9/L	10.6	5.6	7.3	8.1	5.1	10.3
Lymphocytes	10^9/L	8.3	3.4	5.6	6.4	3.5	7.1
Monocytes	10^9/L	0.2	0.3	0.3	0.2	0.2	0.2
Neutrophils	10^9/L	1.6	1.9	1.4	1.5	1.3	3.0
Lymphocytes	%	80.4	62.0	76.9(*)	77.5(*)	69.8	68.8
Monocytes	%	2.3	6.3	3.6	2.1	4.7	2.1
Neutrophils	%	15.1	31.7	19.5(*)	20.4	25.5	29.1
Red blood cells	10^{12}/L	11.2	6.8	7.9	8.5	7.5	8.4
Hemoglobin	g/dL	17.0	10.7	12.6	13.1	10.8	13.3
Hematocrit	%	64.0	37.6	44.3	48.1	42.5	45.1
Platelets	10^9/L	-	613.2	382.5(*)	455.2	716.2	497.8

(*) Significant differences compared to *B. abortus* (2308W) reference strain ($p \leq 0.05$).

Table 3. Hematological parameters of CD1 mice (n=6) at thirty days post-infection with *Brucella* strains.

Parameter	Unit	Mice non-infected	*B. abortus* (2308)	*B. ceti* (B14/94)	*B. pinnipedialis* (B2/94)	*Brucella* sp. ST27 (bmarCR39b)	*Brucella* sp. ST27 (bmarCR42b)
		Mean	**Mean**	**Mean**	**Mean**	**Mean**	**Mean**
Leucocytes	10^9/L	10.6	8.2	9.6	9.5	7.4	7.4
Lymphocytes	10^9/L	8.3	3.8	7.9(*)	7.7	5.8	5.5
Monocytes	10^9/L	0.2	0.3	0.4	0.2	0.2	0.2
Neutrophils	10^9/L	1.6	3.3	1.4(*)	1.8	1.4(*)	1.6(*)
Lymphocytes	%	80.4	50.2	81.8(*)	79.2(*)	79.6(*)	77.2
Monocytes	%	2.3	4.2	4.3	2.0	2.6	2.6
Neutrophils	%	15.1	46.7	13.9(*)	18.8	17.8	20.2
Red blood cells	10^{12}/L	11.2	9.2	9.2	9.0	8.2	9.5
Hemoglobin	g/dL	17.0	12.2	14.1	13.8	12.1	13.8
Hematocrit	%	64.0	42.0	50.3	48.0	42.6	47.6
Platelets	10^9/L	-	531.5	495.5	395.5	395.8	436.8

(*) Significant differences compared to the *B. abortus* (2308W) reference strain ($p \leq 0.05$).

The histopathological examination of the spleen showed that all *Brucella* strains induced the inflammation described in *Brucella* infections in the mouse model, including the classical granuloma formation [27,28]. However, compared to *B. abortus* 2308W, lesions were less conspicuous in *Brucella* sp. ST27 strains (Fig. 3) and in *B. ceti* B14/94 and *B. pinnipedialis* B2/94 strains (not shown). Regarding the granulomatous inflammation, we observed significant differences in the histopathological score between *B. abortus* 2308W and *Brucella* sp. ST27 strains at 8 days p.i., with a less severe response of the latter during the early stage of infection (Fig. 4A). This difference is remarkable since the bacterial loads in the spleen at this time point were very similar to *B. abortus* 2308W. Mice infected with *B. ceti* B14/94 and *B. pinnipedialis* B2/94 also displayed a similar granulomatous inflammatory response to *Brucella* sp. ST27 strains, but these differences did not reach statistical significance. A similar trend was observed at 30 days p.i. Significant differences existed between *B. abortus* 2308W and *B. ceti* B14/94, *B. pinnipedialis* B2/94, and *Brucella* sp. ST27 (bmarCR42b) strains (Fig. 4B). No significant difference was observed with bmarCR39b strain at this time, suggesting some *Brucella* sp. ST27 strain-specific differences in their pathogenicity potential.

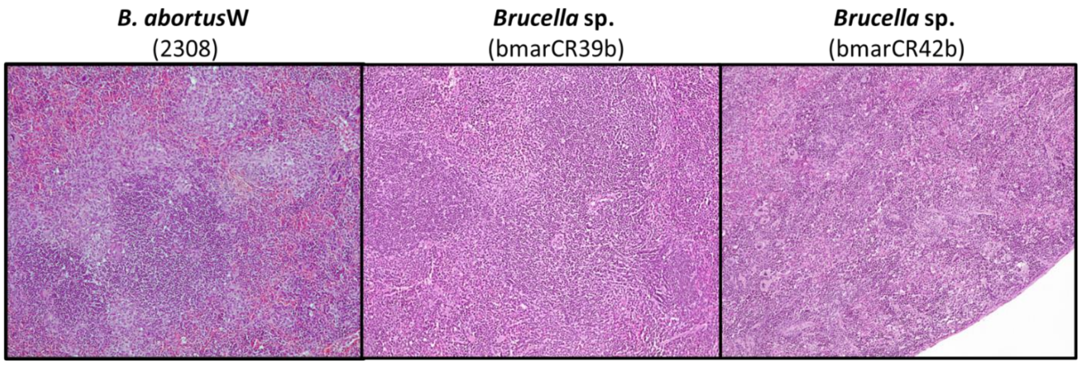

Figure 3. *Brucella* sp. **ST27 strains induce a low granulomatous inflammation in the spleen.** CD-1 mice were infected i.p. with 10^6 CFU of bacteria. Mice were then sacrificed at 8 days post-infection, and sections of the spleen were analyzed by histopathological examination after hematoxylin-eosin staining.

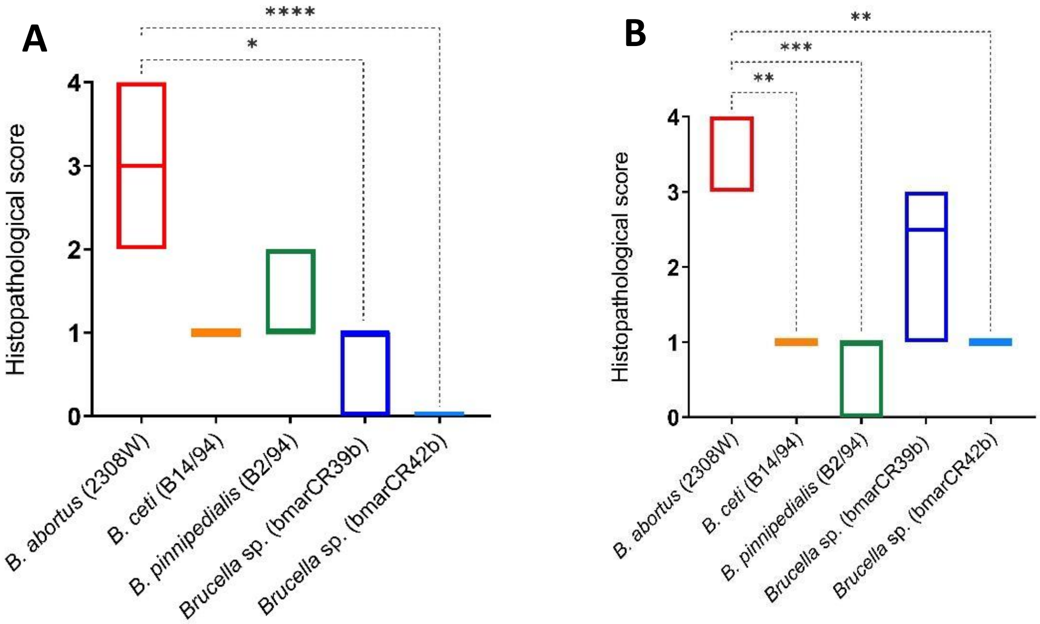

Figure 4. Granulomatous inflammation score. CD-1 mice were infected i.p. with 10^6 CFU of bacteria. Mice were then sacrificed at 8- and 30-days post-infection and sections of the spleen were analyzed by histopathological examination after hematoxylin-eosin staining. The histopathological score was determined based on the spleen lesions, where 0 is normal and 4 is severe. The results were expressed as the median ± minimum to maximum values of histopathological score. (A) Histopathological score at 8 days post-infection. (B) Histopathological score at 30 days post-infection. The p-values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), and ≤ 0.0001 (****) are indicated.

Infection in HeLa cells

Two types of cell infection patterns in HeLa cells were observed among *Brucella* sp. ST27 strains. Initially, both isolates demonstrated an intracellular multiplication of 4.2 log CFU (Fig. 5D). However, *Brucella* bmarCR39b strain reached a significant increase of 4.5 logs CFU after 48 hours p.i., while *Brucella* bmarCR42b strain exhibited a significant decrease in CFU by approximately 1 log after 48 hours p.i. Again, these distinctive infection patterns suggest potential *Brucella* sp. ST27 strain-specific differences in intracellular replication within epithelial cells. The *B. abortus* 2308W strain showed a typical strong intracellular replication pattern in HeLa cells after 48 hours post-infection (Fig. 5A). Less intensely, *B. ceti* B14/94 showed an increase in intracellular replication in HeLa cells after 48 hours p.i. (Fig. 5B), indicating this strain can also infect and replicate in these epithelial cells. In contrast, *B. pinnipedialis* B2/94 displayed limited intracellular replication (Fig. 5C), with an initial CFU log of 4.4, followed by a decrease of approximately one-half of log CFU after 48 hours p.i.

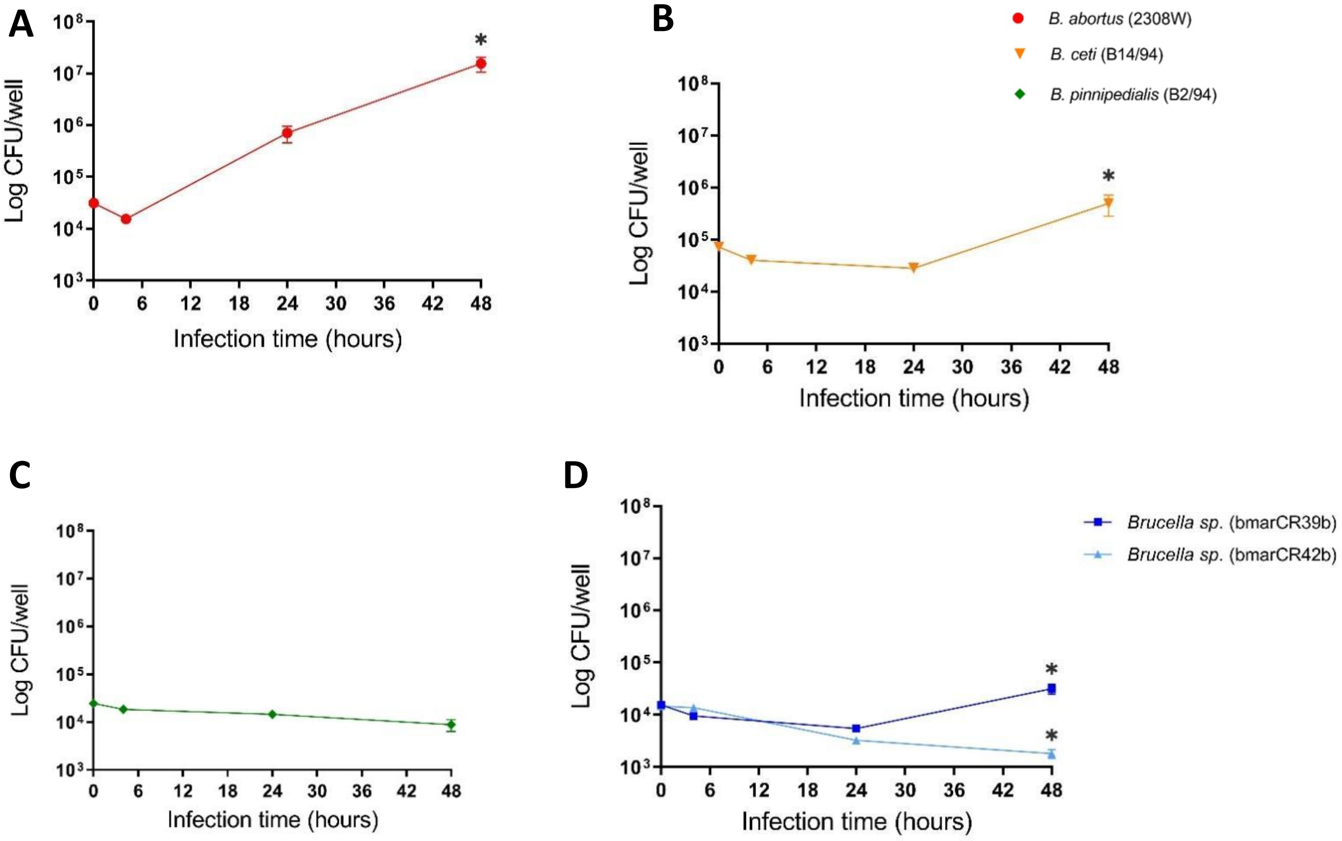

Figure 5. Infection of *Brucella* strains in HeLa cells. (A-D) HeLa epithelial cells were infected with bacteria at MOI of 500 in a gentamicin protection assay. The number of intracellular viable bacteria CFU was determined at different hours post-infection. Each bacterial infection pattern is represented with the average Log CFU/well ± standard deviation of an experiment performed in duplicate and the results representative of three independent experiments. Differences between the infection time points 0 and 48 are indicated as * ($p \leq 0.05$).

DISCUSSION

We previously reported two *Brucella* sp. ST27 strains isolated from a stranded *K. sima* closely related to phylogenetic relatives of *Brucella* sp. ST27 strain causing human disease [18]. The two *Brucella* sp. ST27 strains display pathogenic traits, such as (i) resistance to polymyxin B and complement, (ii) intracellular replication in cell culture, (iii) bacterial persistence in the mouse model and (iv) induction of granulomatous inflammation in the spleen. All these pathogenic characteristics are typically observed in the *Brucella* organisms [1,2]. However, similar to *B. ceti* and *B. pinnipedialis,* the *Brucella* sp. ST27 seemed more attenuated in mice and HeLa cells than *B. abortus* 2308W.

The resistance to polymyxin B and serum complement suggests that *Brucella* sp. ST27 possesses a similar cell envelope composition to *B. abortus* 2308W and marine strains *Brucella ceti* B14/94 and *Brucella pinnipedialis* B2/94, all smooth strains with A-type lipopolysaccharide (LPS) [18,29] and hydrophobic structure, which confers a resistant to cationic peptides and complement [30-32].

We observed that the *Brucella* sp. ST27 bmarCR39b differed in the replication kinetics from the *Brucella* sp. ST27 bmarCR42b in HeLa cells, an unexpected phenomenon since these bacteria strains isolated from the mother and fetus in the same stranding event do not show genomic differences, according to the phylogenetic analysis [18]. These finding, although striking, suggests different stages of physiological adaptation of the strains resulting from serendipitous events in their respective host.

Maquart and co-workers [33] showed that *Brucella* sp. ST27 was virulent in human macrophage cells to the same extent as the virulent *B. melitensis* 16M and *B. suis* 1330 reference strains. Likewise, *B. pinnipedialis* reference strain 12890 multiplied with a pattern like pathogenic *B. abortus* when infecting human macrophage cells, whereas another harbor seal isolate of *B.*

pinnipedialis M2533 was eliminated by 48 h p.i. In contrast, Larsen et al. [34] demonstrated that *B. pinnipedialis* 12890 could enter the epithelial cells like classical virulent strains but did not multiply or survive for extended periods intracellularly. This variability of the various species and strains to multiply and establish infections within host cells likely arises from multiple biological factors, some of which may include physiological adaptations genetically coded but others unrelated to genetic differences. To acquire a more comprehensive understanding of the intracellular behavior of these strains, it is also necessary to conduct infection using various cell types and to detect the intracellular expression of virulence factors such as BvrR/BvrS, VjbR, VirB, MucR, among others [22,35-40].

Despite these intracellular assays giving a closer approach to the pathogenic potential of these strains, it was necessary to evaluate if these intracellular differences impacted the persistence in the murine model *in vivo*. Our findings show, in general, that both *Brucella* sp. ST27 isolates displayed a degree of pathogenicity in mice similar to that of marine *Brucellae* reference strains *B. ceti* B14/94 and *B. pinnipedialis* B2/94 but reduced compared to *B. abortus* 2308W. In addition, the pathologic score induced in the spleen was less severe at both time points in all marine strains. These results agree with a previous study showing that *B. pinnipedialis* 12890 and *B. ceti* 12891 strains are attenuated in the spleen and liver by displaying low bacterial loads in a BALB/c mouse infection model [41].

It is also worth noting that, despite *Brucella* sp. ST27 bmarCR39b and bmarCR42b reached high levels of bacterial burden in the spleen during the early stage of infection, the hematological profiles and the spleen lesions of the infected mice remained less pronounced than reference strain *B. abortus* 2308W. The bacterial persistence in the mice spleen with limited signs of pathology has not been previously reported for the ST27 genotype. Although *Brucella* sp. ST27 bmarCR39b and bmarCR42b display an attenuated phenotype compared to *B. abortus* 2308W, they can induce significant inflammation and disease in their natural host [18]. Further investigations are required to draw more robust conclusions on this mouse model's pathology response and its relation to their zoonotic potential.

Altogether, using cell culture and mice models, our results describe the first extensive pathogenicity of two *Brucella* isolates from the ST27 genotype isolated from *K. sima*. Despite showing less virulent patterns in mice and cells, these strains display the traits expected for *Brucella* organisms. Moreover, the fact that *Brucella* sp. ST27 induced abortion and the death of the dwarf sperm whale *K. sima* [18] should prevent us from underestimating the virulent potential of this strain for cetaceans and maybe for other mammal hosts, including humans.

AUTHORSHIP CONTRIBUTION STATEMENT

Andrea Romero-Magaña: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing –review & editing. **Esteban Chaves-Olarte:** Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing –review & editing. **Carlos Chacón-Díaz:** Methodology, Resources, Supervision, Validation. **Edgardo Moreno:** Conceptualization, Methodology, Validation, Writing –review & editing. **Gabriela Hernández-Mora:** Resources. **Elías Barquero-Calvo**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no competing financial interests or personal relationships that could have inappropriately influenced or bias the content of the paper.

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CONCLUSIONES

- Los aislamientos de *Brucella* sp. ST27 mostraron un grado de patogenicidad en ratones similar al de las cepas marinas *B. ceti* B14/94 y *B. pinnipedialis* B2/94, pero reducido en comparación con *B. abortus* 2308W.

- La respuesta inflamatoria inducida por *Brucella* sp. ST27 en el bazo de los ratones no difiere significativamente del inducido por *B. ceti* y *B. pinnipedalis,* pero es considerablemente menor que el producido por *B. abortus* 2308W. Consecuentemente, los perfiles hematológicos de ratones infectados con *Brucella* sp. ST27 fueron menos severos que los infectados con *B. abortus* 2308W.

- La resistencia a la polimixina B y al complemento sugiere que *Brucella* sp. ST27 posee una composición de envoltura celular similar a *B. abortus* 2308W y a las cepas marinas *B. ceti* B14/94 y *B. pinnipedialis* B2/94.

- *Brucella* sp. ST27 es más atenuada en células HeLa que la cepa de referencia *B. abortus* 2308W.

- En conjunto, estos hallazgos mostraron que la cepa *Brucella* sp. ST27 asilada de la *Kogia sima* presentó características patogénicas comparables a las cepas marinas *B. ceti* B14/94 y *B. pinnipedialis* B2/94, tal como resistencia a la polimixina B y al complemento, replicación intracelular en cultivo celular, persistencia bacteriana en el modelo de ratón e inducción de inflamación. A pesar de mostrarse menos virulenta que la cepa de referencia *B. abortus* 2308W, *Brucella* sp. ST27 mostró los rasgos patogénicos esperados de los organismos *Brucella*.

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