UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

“BRUCELOSIS EN COSTA RICA: EPIDEMIOLOGÍA Y DESARROLLO DE ESTRATEGIA PARA SU CONTROL”

"BRUCELLOSIS IN COSTA RICA: EPIDEMIOLOGY AND DEVELOPMENT OF A CONTROL STRATEGY"

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Doctorado en Ciencias para optar al grado y título de Doctorado Académico en Ciencias

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DEDICATORIA

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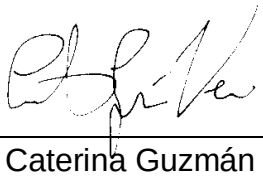

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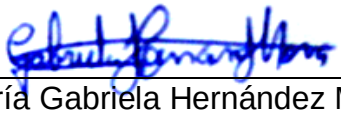

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TABLA DE CONTENIDO

RESUMEN

La brucelosis causada por *Brucella abortus* es una zoonosis de importancia mundial que afecta a animales domésticos, silvestres. La evolución histórica del problema en Costa Rica, desde su primer reporte en los inicios del siglo XX se describe en el Capítulo 1. Igualmente, se discuten las diversas estrategias de control aplicadas y las razones por la que esta infección no ha sido controlada ni erradicada en el país. Los datos más recientes (2014-2016) sobre la prevalencia por hato de la brucelosis bovina en Costa Rica (CR) muestran un rango de entre el 4.1% y el 10.5% dependiendo de la prueba diagnóstica usada. En el capítulo 2, se describe los muestreos realizados para conocer la seroprevalencia de esta enfermedad en cabras, ovejas, cerdos, búfalos y caballos, así como en 16 especies de cetáceos. En cabras, ovejas y cerdos se obtuvo una prevalencia nula y *B. melitensis* o *B. suis* no fueron detectadas en el territorio nacional en animales ni humanos. En el caso de caballos y búfalos se reportó una seroprevalencia colectiva de 6.5% y 21.7% e individual de 1.4% y 0.65% respectivamente. Se aislaron varias cepas de *B. abortus* en búfalos, pero no en caballos. En los cetáceos se tuvo una seroprevalencia individual de 46.9% y se confirmó la infección por *B. ceti* en el 70 % de los delfines rayados (*S. coeruleoalba*) a nivel de sistema nervioso central. En el capítulo 3 se describe los análisis del genoma completo (WGS) de éstas *B. ceti* que resultaron ser únicas a nivel mundial (P1-ST26) y su adaptación a estos hospederos marinos se debe a procesos de pseudogenización siendo esta una fuente de variación genética dentro del género. Adicionalmente, durante este período de estudio se logró aislar una *Brucella* marina (ST27) no clasificable, causado placentitis en un cachalote enano (*Kogia sima).* Esta cepa muestra un genoma semejante a las *Brucella* spp marinas aisladas de humanos. Por su parte, los análisis filogenéticos de las *B. abortus* aisladas, demostraron cinco grupos circulando en CR, el más antiguo introducido a mediados del siglo XIX mientras que los otros grupos se introdujeron más recientemente durante los siglos XX y XXI. Finalmente, en el capítulo 4 se propone una estrategia adaptada para el control la brucelosis bovina en Costa Rica. A pesar de que en América Latina existe tecnología suficiente para controlar y estudiar la enfermedad, incluidas las técnicas de aislamiento e identificación de nueva generación, pocos países han logrado describir sistemáticamente la realidad epidemiológica de la brucelosis para bovinos y otras especies de mamíferos que sirven de reservorio natural de la brucelosis. Lo anterior aunado con el desuso de herramientas diagnósticas y de profilaxis clásicas, baratas y comprobadas como efectivas en países que han logrado controlar la brucelosis bovina en el pasado, han limitado el avance y la sostenibilidad económica y de intervención en nuestros países donde los recursos son limitados. Por lo tanto, la información de este trabajo puede servir de ejemplo para los actores involucrados en la toma de decisiones para lograr un avance hacia el control y erradicación de la enfermedad en Latinoamérica.

ABSTRACT

Brucellosis caused by *Brucella abortus* is a worldwide zoonosis infecting domestic and wildlife animals. The history of the disease in Costa Rica, the strategies for its control and the reasons why this infection has not been eradicated in the country are discussed in Chapter 1. From 2014-2016, the herd seroprevalence of brucellosis in cattle in Costa Rica (CR) ranged between 4.1% and 10.5%, depending on the diagnostic tests used. Likewise, the seroprevalence of brucellosis in other mammals such as goats, sheep, pigs, water buffaloes, horses, and 16 species of cetaceans, are described in Chapter 2. *B. melitensis* or *B. suis* were not found in animals or humans of Costa Rica nor serology in goat, sheep, and pigs. However, horses and buffaloes showed a herd seroprevalence of 6.5%, 21.7%, and individual seroprevalence of 1.4% and 0.65%, respectively. Several strains of *B. abortus* were isolated in buffaloes, but not in horses. In stranded cetaceans, there was an individual seroprevalence of 46.9%, and central nervous system infections due to *B. ceti* were confirmed in 70% of striped dolphins (*S. coeruleoalba*). In chapter 3 the whole-genome sequence analyses (WGS) of these *B. ceti* showed that these strains are unique worldwide (P1-ST26) and did suffer host adaptation by pseudogenization, which result in a source of genetic variation within the genus *Brucella*. A new strain of marine *Brucella* named ST27 causing placentitis and abortion in a dwarf sperm whale (*Kogia sima*) was also isolated. This strain displayed similar genetic characteristics to those marine Brucellae causing zoonosis in humans. The genomic characterization and phylodynamic analysis of the *B. abortus* isolates, showed five clusters circulating in CR, been the oldest introduced during the mid of 19th century. The other four clusters were introduced more recently during the 20th and 21th century. Finally, in chapter 4, we propose a strategy to control brucellosis in Costa Rica.

Although there is sufficient technology in Latin America to control and study this disease, including identification and new generation techniques, few countries have been able to systematically describe the epidemiological reality of brucellosis for cattle and other species of mammals that serve as natural reservoir of brucellosis. All the above mentioned and combined with the abandonment of classic diagnostic and prophylaxis tools, cheap and proven effective in countries that achieve the control bovine brucellosis in the past, have limited the progress and sustainability of the interventions in our countries where resources are limited. Therefore, the information in this work can serve as an example for the actors involved in the decision-making process in order to achieve progress towards the control and eradication of the disease in Latin America.

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INTRODUCTION

Our goal has been the description of the epidemiology of brucellosis in Costa Rica and to report the presence of the vario species and strains *Brucella* organisms in domestic animals and marine mammals. In order to have a regional perspective, we also revise the historical and current situation of *Brucella abortus* and *B. melitensis* in the Americas and the different strategies used for the control of the disease for each country are included (Annex 1 and 2).

From 2012 to 2014, the Bacteriology Laboratory of the National Animal Health Service (SENASA), using commercial culture media, isolated the first three *B. abortus* strains by the Veterinary Services in Costa Rica. However, in 2015 a new culture media for *Brucella* genus previously developed by Agri-Food Research and Technology Center of the Government of Aragon, Spain (CITA), and currently recommended by the World Organization for Animal Health (OIE) was introduced into the laboratories. After that, greater efficacy was achieved in the isolation of *Brucellae* from clinical samples, secretions, and products of infected animals such as abortions, vaginal fluids, organs, milk, and even from tissues of dead animals in a moderate state of decomposition. From July 2015 to July 2019, 122 isolates of *B. abortus* from cattle (*Bos taurus*) and 36 from buffaloes (*Bubalus bubalis*), 30 *B. ceti* from striped dolphins (*Stenella coeruleoalba*), a strain from a common dolphin (*Delphinus delphis*), as well as a strain of *Brucella* spp. ST27, not classifiable with standard isolated techniques from a dwarf sperm whale (*Kogia sima*), was achieved. These last two isolates correspond to new hosts in the Eastern Tropical Pacific Ocean and not reported so far in the literature. Sera from bovine, goat, sheep, swine, equine, cetacean, and humans were collected to make the serological diagnosis and estimate the prevalence of brucellosis in the different species in Costa Rica.

These biological materials were the input for the epidemiological studies and the characterization and phylogenetic relationships of the *Brucellae* present in the country.

In Chapter 1, the history of bovine brucellosis in Costa Rica is described, as well as the control and eradication strategies used and the reasons why these

strategies have not been effective, and therefore the disease remains endemic in the country despite the efforts made. This information is included in the article “Epidemiology of bovine brucellosis in Costa Rica: lessons learned from failures in the control of the disease” PLoS ONE 12(8): e0182380. https://doi.org/10.1371/journal.pone.0182380.

Chapter 2 includes studies of brucellosis performed in Costa Rica in humans and other animal hosts other than cattle. The resulted data was published during 2017 in the article “Brucellosis in mammals of Costa Rica: an epidemiological survey” *PLoS ONE*, 12(8), e0182644. http://doi.org/10.1371/journal.pone.0182644

Chapter 3 presents the results of the phylogenetic analyses of marine and terrestrial strains obtained in Costa Rica. These analysis are presented in one published article and two articles submitted during 2020. The three articles are “Brucella genetic variability in wildlife marine mammals’ populations relates to host preference and ocean distribution. *Genome Biol Evol* 2017 evx137. https://doi.org/10.1093/gbe/evx137. The second article is “Dwarf sperm whale is a reservoir of *Brucella* sp. ST27, linked to human infections” Emerg Infect Dis (submitted), and the third article is “Persistence of *Brucella abortus* Lineages Revealed by Genomic Characterization and Phylo-temporal Analysis”. *PLOS Neglected Tropical Diseases* (submitted).

Finally, in Chapter 4, based on the results obtained in the previous chapters and the current prevalence of bovine brucellosis in the country, we propose a strategy for its control in the medium and long term.

METHODOLOGIAL STRATEGIES

The details of the materials methods that were used in this work are extensively described in chapters 1 to 3 and the corresponding attached publications. In this sense, here, we describe the methodological strategies and the experimental route that was followed for the realization of this thesis.

For Chapter 1, corresponding to “Bovine brucellosis in Costa Rica: lessons learned from failure in the control of the disease”, the total number of bovines in CR was close to 1.55 million, distributed in about 15000 farms and 50000 herds. For sampling purposes, the country was divided into six regions: Northern, Central, Brunca, Chorotega, Caribbean Huetar, and Central Pacific. Three different management systems are commonly carried out in the country: beef, dairy, and double purpose cattle. The seroprevalence of brucellosis in beef, dairy and double purpose animals was estimated in a non-random sample and, random sample, systematically taken in the different regions of CR. To assess both herd and animal prevalence by the management system in the later population, a random sample of 250 farms per strata, proportionally allocated by region, were sampled. This sample size was calculated using public access WinEpiscope 2.0 software (Thursfield *et al*., 2001). A farm was declared positive when at least one serum sample resulted positive. For sample size, the Cannon & Roe formula to demonstrate freedom from/absence of infection (Cannon & Roe, 1982). A total of 765 farms accounting for close to 13078 cows were sampled during 2012-2013: 250 dairy herds (3902 cows), 254 beef herds (4485 cows), and 261 dual- purpose herds (4691 cows). In addition, a non-random serum sample of 532199 cows comprising 7907 herds (~16% of CR herds), arriving during 2014-2016 to the laboratories of the Veterinary Services for routine diagnoses, were analyzed. For all purposes, repeated herds were considered. Relevant data concerning the geographical localization, area of the farm and management characteristics of the herd or individual animals were collected in addition to other relevant information. In both studies, the diagnostic strategy was first, screening all bovines by RBT, and then testing of the RBT positives (RBT+) by iELISA (OIE, 2018). Necropsies or sample collection were carried out as described elsewhere (OIE, 2018). Animal samples included blood, tissues, secretions, and

aborted fetuses Cultures were done using non-selective and selective media (OIE, 2018). The selected bacterial colonies were subjected to identification following regular bacteriological procedures (Alton *et al*., 1988). References from different *Brucella* species were used for comparative purposes during all the procedures (Le Flèche *et al.,* 2006). *Brucella* DNA from control and field isolates were extracted as described previously (Le Flèche *et al.,* 2006) with and the identification of *Brucella* species was performed by multiple-ocus variable-number tandem repeat analysis-16 (MLVA16) following standard procedures (Le Flèche *et al.,* 2006, CNRS, 2017). Control *Brucella* species were used for validation. In all procedures, we followed the ethical considerations established by the different organisms and ethical committees of Costa Rica (Procuraduría General de la República, 1994, 2006, 2012).

For Chapter 2, “Brucellosis in mammals of Costa Rica”, the national territory was divided into the same six regions, mentioned above. The estimated population of sheep and goats in CR was close to 12358 and 4626, distributed in about 164 and 271 herds, respectively. In the case of water buffaloes, the estimated population was 13000 animals within 100 herds, for pigs close to 435500 animals, for horses 67000 and 30 species of cetaceans. For sampling purposes, the herds of sheep and goat were divided into three sections. For sheep, the first section “A” included 6200 animals in 22 herds of broodstock farms with ≤150 individuals; section “B” were 3577 animals in 37 herds from farms with eventual broodstock activities, with populations ranging from 149-60 animals; and section “C” were 2691 in 105 herds for productive farms with population of ≤ 59 animals. For goats, we used the same criteria used for sheep. Section “A” included 1406 goats in 13 herds; section “B” were 1603 distributed in 14 herds; and section “C” were 1617 from 137 farms. Seventy-eight caprine and 139 ovine herds, corresponding to 2013 and 1668 animals, respectively, were selected. In the case of water buffaloes, a total of 2586 animals from 46 herds were sampled. A total of 2256 blood samples from pigs coming from eight herds, 160 blood from slaughterhouses, and 58 feral pigs from East side of Cocos Island National Park were taken. For horses, 1270 animals from 215 farms were sampled, and for stranded cetaceans 54 were analyzed. The sample sizes for sheep and goats were determined according to Cannon and Roe (Cannon & Roe, 1982), using

Win Episcope 2.0 software (Thursfield *et al*., 2001). This estimation included 500 sheep and 413 goats to be sampled, distributed in 10 and 13 herds respectively, selected by region as described above. Sheep and goat herds were chosen randomly from sections A and B, which are the broodstock herds, and largely reflected the sanitary conditions of section C. In the random sampling, a biased priority was given to females with a history of reproductive problems and low body condition, in order to increase the probability of positive results. Breeding rams in each farm were also examined for the detection of orchitis, epididymitis and reproductive problems. For feral pigs, the size of the sample was selected for an expected maximum population of 500 pigs distributed in the entire island. The rest of the animal species sampled corresponded to the surveillance performed as part of the National Brucellosis Control Program of the CR-NAHS and according to the OIE serological assays, as mentioned above (OIE,2018). The bacteriological and molecular analyses were performed, as mentioned for Chapter 1.

Protocols for the use of animal serum samples were revised and approved by the “Comite Institucional para el Cuido y Uso de los Animales de la Universidad de CR (CICUA 057-16366), and “Comite Institucional para el Cuido y Uso de los Animales of the National University, Heredia, CR (SIA 0545-15), and in agreement with the corresponding law “Ley de Bienestar de los Animales, CR” (Ley 7451 on Animal Welfare), and according to the “International Convention for the Protection of Animals” endorsed by Costa Rican Veterinary General Law on the CR-NAHS (Ley 8495).

For Chapter 3, corresponding to “Phylogenetic characterization of marine and terrestrial *Brucellae* isolated in Costa Rica”, 23 isolations of *Brucella ceti* from stranded striped dolphins with neurobrucellosis from the Eastern Tropical Pacific, as well as four from the Mediterranean Sea, nine from the North Atlantic Ocean, one from France, four *Brucella pinnipedialis* from the North Atlantic Ocean, and one *Brucella* sp. from California were used. Genotyping techniques such as multiplex PCR Bruceladder, MLST, PCR detection of ST27 or bcsp31, HRMRT-PCR and PCR targeting specific IS711 elements were performed either as previously described or in silico. The terrestrial strain included a total of 95 *B abortus* strains isolated from

bovines, water buffaloes, and humans in Costa Rica. Both marine and terrestrial strains were sequenced at the Wellcome Trust Sanger Institute on Illumina platforms according to in house protocols for wholegenome sequencing (WGS) (Quail *et al.,* 2008, 2012).

To construct a multiple sequence alignment for phylogenetic reconstruction, whole-genome sequence data from two *Ochrobactrum* species and the *Brucella* isolates from different hosts were aligned by bwa and mapped with SMALT v.0.5.8 against *B. abortus* 9-941, with an average coverage of 98.81%. Single Nucleotide Polymorphisms (SNPs) were called using samtools (Li *et al.,* 2009), and 311,780 variable sites were extracted using snp sites (Page *et al.,* 2016). The resulting alignment was used for maximum likelihood phylogenetic reconstruction with RAxML v7.0.4 (Stamatakis., 2006).

To detect pseudogenes in *B. ceti,* we selected five phylogenetically representative draft genomes from marine mammal *Brucellae* and automatically transferred the annotation of the manually curated draft genome working strain *B. abortus* 2308 Wisconsin (Suárez-Esquivel *et al.* 2016). Pseudogenes were defined, as any gene containing deletions or insertions that removed start or stop codons, or at least one in-frame stop codons and/or frame shifts compared with orthologs in *B. abortus* 2308 Wisconsin or reference genomes.To examine relevant phenotypic genes (virulence-related, outer membrane, lipopolysaccharide [LPS] and flagellar genes), regions of interest were examined, as mention before, through bwa alignment and SMALT mapping. The number of SNPs, insertions and deletions in each one of the genes was recorded.

For the study of *B. abortus* strains, a total of 167 isolates of *B. abortus* from bovines, 16 from humans, and 5 from water buffaloes were included in the study. From these isolates, 95 were analyzed by WGS, as previously described. The origin and dates of the introduction of circulating *B. abortus* strains in CR were explored by calibrating the *B. abortus* phylotemporal events and comparing the nodes and dates, according to the incursions of bovine species and breeds into the territory. A SNPs matrix, including 322266 sites from 228 *Brucella* spp. genomes and two *Ochrobactrum* spp. genomes as outgroup was aligned to the reference strain *B.*

abortus 9-941 to produce a maximum likelihood phylogenetic tree. To characterize each one of the CR lineages, we looked at known genomic traits associated with variability in *Brucella* (Ocampo-Sosa and García-Lobo, 2008b; Wattam *et al*., 2009; Mancilla *et al*., 2011; that could provide information to explain phenotypic or infective behavioral differences among the isolates. For that, we identified the SNPs position on specific coding sequences (CDS), checked for changes in genomics islands (GI) or anomalous regions, and assessed the number and positions of the insertion element IS711 within the lineages. Bayesian Evolutionary Analysis Sampling Trees (BEAST) were used, to determine the time of the introduction events of the different CR *B. abortus* lineages. All procedures involving live *Brucella* were carried out according to the "Reglamento de Bioseguridad de la CCSS 39975- 0", year 2012, after the "Decreto Ejecutivo #30965-S", year 2002 and research protocol NFEG06 approved by the National University, Costa Rica.

For Chapter 4, "Proposal for a suitable strategy to control brucellosis in Costa Rica" the information of brucellosis at national level mentioned in chapters 1 and 2 was used as well as, the information summarized in Annex 1 of the experience of all the countries in the Americas for the control of the disease and strategies used in the last ninety years, by each one of them. At national level, this information included the current epidemiological status of the disease, animals, and bacterial species involved in the natural cycle of brucellosis within the country, scarce or null economic support, or resources for control of the disease

Chapter 1: Bovine brucellosis in Costa Rica: lessons learned from failure in the control of the disease.

Brucellosis, caused by *Brucella abortus,* is a major disease of cattle and a zoonosis. Several studies for estimating the seroprevalence of bovine brucellosis in CR have been carried out. The last trial before this work was in 1982 (Vicente *et al*., 1983). Therefore, after more than three decades, we undertook a new investigation covering all regions of the country to estimate the prevalence and distribution of brucellosis in Costa Rica (CR) and describe the species and circulating strains of the genus *Brucella* in the country. The prevalence estimated by Rose Bengal test (RBT) ranged from 10.5%-11.4%; alternatively, the prevalence estimated by testing the RBT positives in iELISA ranged from 4.1%-6.0%, respectively.

However, cattle in CR are not vaccinated with *B. abortus* S19, but with RB51 (vaccination coverage close to 11%), and under these conditions, the RBT displays 99% specificity and 99% sensitivity. Therefore, the RBT herd depicted in the random analysis stands as a feasible assessment. Studies of three decades revealed that bovine brucellosis prevalence has increased in CR.

Biochemical and molecular studies identified *B. abortus* as the etiological agent of bovine brucellosis. Multiple locus variable-number tandem repeat analysis-16 revealed four *B. abortus* clusters. Cluster one and three are intertwined with isolates from other countries, while clusters two and four have only representatives from CR

Cluster one is widely distributed in all regions of the country and maybe the primary *B. abortus* source. The other clusters seem to be restricted to specific areas in CR. The implications of our findings, in relation to the control of the disease in CR, are critically discussed.

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RESEARCH ARTICLE

Epidemiology of bovine brucellosis in Costa Rica: Lessons learned from failures in the control of the disease

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Abstract

Brucellosis, caused by *Brucella abortus* is a major disease of cattle and a zoonosis. In order to estimate the bovine brucellosis prevalence in Costa Rica (CR), a total 765 herds (13078 bovines) from six regions of CR were randomly sampled during 2012–2013. A non-random sample of 7907 herds (532199 bovines) of the six regions, arriving for diagnoses during 2014–2016 to the Costa Rican Animal Health Service was also studied. The prevalence estimated by Rose Bengal test (RBT) ranged from 10.5%-11.4%; alternatively, the prevalence estimated by testing the RBT positives in iELISA, ranged from 4.1%-6.0%, respectively. However, cattle in CR are not vaccinated with *B. abortus* S19 but with RB51 (vaccination coverage close to 11%), and under these conditions the RBT displays 99% specificity and 99% sensitivity. Therefore, the RBT herd depicted in the random analysis stands as a feasible assessment and then, the recommended value in case of planning an eradication program in CR. Studies of three decades reveled that bovine brucellosis prevalence has increased in CR. *B. abortus* was identified by biochemical and molecular studies as the etiological agent of bovine brucellosis. Multiple locus variable-number tandem repeat analysis-16 revealed four *B. abortus* clusters. Cluster one and three are intertwined with isolates from other countries, while clusters two and four have only representatives from CR. Cluster one is widely distributed in all regions of the country and may be the primary *B. abortus* source. The other clusters seem to be restricted to specific areas in CR. The implications of our findings, in relation to the control of the disease in CR, are critically discussed.

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Introduction

As any other Latin American country, bovine brucellosis is a significant animal health problem and a relevant zoonosis in Costa Rica (CR). Consequently, the disease is of veterinary and of public health relevance. Bovine brucellosis (then recognized as "Bang´s disease") was clinically described in the Central Valley and in the volcanic highlands at the end of the XIX century, when different breeds of cattle were imported from United States and Europe. The introduction of zebu breeds to CR, mainly from Brazil, initiated at the start of XX century; thereafter, brucellosis was officially recognized as an endemic disease [1–7]. However, cattle exist in CR since 1560, after the introduction of European breeds by the Spanish conquerors from neighboring Nicaragua and Honduras countries. After this, recurrent abortions and reproductive problems of cattle due to brucellosis have been reported until the present time [6].

Although in 1900 the bovine population in CR was close to 350000 [6], brucellosis became just a notifiable disease in 1915, after the first *Brucella* sp. isolation from the blood of a human patient [7,8]. Intervention measures by the Costa Rican National Animal Health Service (CR-NAHS) aimed to the control of the disease in cattle started in 1950 [9]. At that time, reports of "epidemic" abortions, smooth *B*. *abortus* S19 vaccination and agglutination diagnostic tests were the only strategies followed. In 1958, the serological diagnosis of brucellosis in bovine herds was declared obligatory and a national campaign for the control and eradicated of the disease started under voluntary basis with *B*. *abortus* S19 calf vaccination and elimination of the positive reactor animals [10]. At that time the importation of S19 vaccine was under the supervision of the CR-NAHS.

From 1963 to 1965, CR suffered constant ash eruptions of the Irazú volcano, affecting areas of the Central Valley and the surrounding highlands. This natural disaster forced the authorities to abandon the brucellosis program and to allocate the economic resources and personnel in solving the emergency. This natural disaster favored the unrestricted traffic of animals from the affected areas to other regions. Nowadays, and despite the current legislation for traceability of bovine movements nationwide [11], the brucellosis status of the animals is seldom requested and, therefore, infected animals may still be mobilized from one region to another. However, this undisciplined movement of bovines was diminished during the recent ash eruptions of the Turrialba volcano in 2015–2016, when nearly 300 (90%) of the surrounding volcanic herds were tested for brucellosis and the positive animals slaughter before their transfer to safer areas [12,13].

In spite of the efforts, the first attempts for controlling brucellosis failed and in the seventies bovine brucellosis was already widespread in CR [9,10,14]. With a loan from the Inter-American Development Bank, additional actions to implement a brucellosis control program on an obligatory basis were undertaken [14]. Still, those were difficult times for Central America. Although CR did not have internal military conflicts, the critical growing political upheaval against authoritarian regimes in several neighboring Central American nations negatively impacted the country. In addition, during the early eighties CR suffered a severe economic recession. As consequence, the field activities devoted to the control of brucellosis, such as S19 vaccination, test and slaughter considerably diminished [10,15].

Although not implemented, the obligatory basis of the control program remained until 1999, period at which the legislation for the National Bovine Brucellosis Program was finally modified to a voluntary basis by the CR-NAHS in coordination with the livestock producers, the milk industry, other private enterprises and non-governmental organizations [16].

Following, the eradication of brucellosis in United States and Canada with *B*. *abortus* S19 and the corresponding banning of vaccination policies in these countries, rough *B*. *abortus* RB51 vaccine was implemented in CR in 1999 [17]. Although S19 vaccination is still allowed

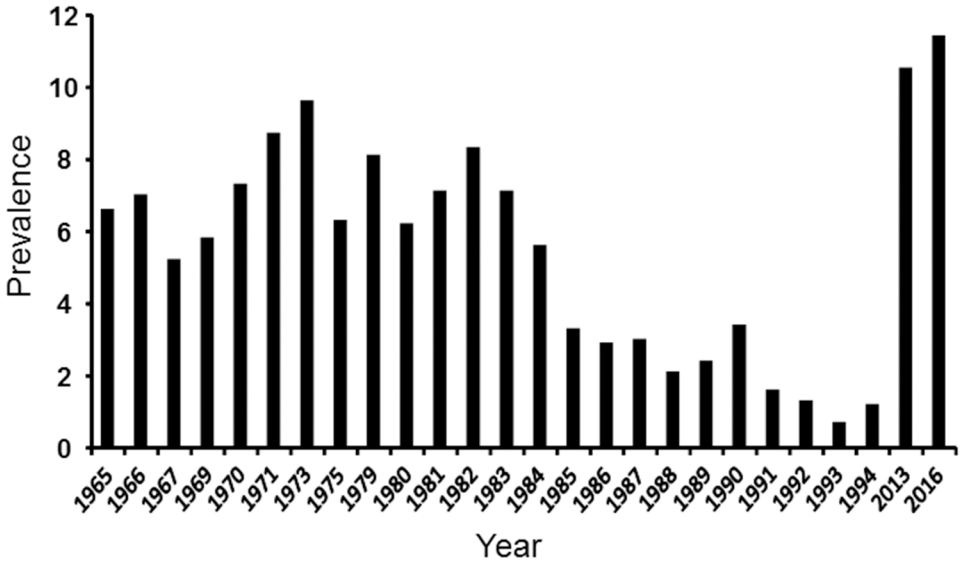

Fig 1. Prevalence of bovine brucellosis in CR during five decades estimated by agglutination tests. The prevalence from 1965–1969 was assessed by tube agglutination; the prevalence from 1970–1986 was assessed by card test in combination with 2- mercaptoethanol agglutination assay; the prevalence from 1987–1994 were estimated by RBT [10,14]. Prevalence values from 2012–2016 assessed by RBT are from this work.

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[18], the importation of this smooth vaccine strain was interrupted in 2000 by the CR-NAHS. For all practical purposes the vaccination with S19 was abandoned in the country and in the Central American region [17]. Since 1999, private enterprises, mainly the dairy companies, are devoted to immunize a low number of herds with RB51 vaccine [10,19].

Currently, vaccination and most of the serological testing of the bovines is on voluntary basis. However, CR-NAHS may request testing of the animals for epidemiological surveillance or upon suspicion of brucellosis. By law, all animals depicted as positive must be marked and thereafter slaughter with no further indemnity [19].

Several studies for estimating the prevalence of bovine brucellosis in CR have been carried out (Fig 1). The last trial before this work was made in 1982 [14]. Therefore, after more than three decades we undertook a new investigation covering all different regions of the country. In this work we describe the distribution of bovine brucellosis, the updated prevalence of the infection and the *B. abortus* strains circulating in CR during the lapse of 2012–2016. We also critically discuss the epidemiological implication of our findings in relation to the control programs and the vaccination strategies carried out in CR during the last decades. Distribution and prevalence of brucellosis in other susceptible hosts in CR such as sheep, goats, water buffaloes, pigs, horses, dolphins and humans are described in an accompanying paper [20].

Materials and methods

Geography of Costa Rica

CR is a country located in the middle of the Central American isthmus with a surface area of 51100 Km^2 with Pacific Ocean and Caribbean Sea coastlines of 1016 km and 212 km, respectively. To the North, CR borders with Nicaragua and to the Southwest with Panama. It has been estimated that CR has sixty volcanos, most of them extinct or dormant, but six of them are active. All the volcanos are aligned in a volcanic range were large part of the National parks

are located. The country is divided in seven provinces, with a human population close to five million, most of them living in the Central Valley, between the volcanic chain and the mountain range. Socioeconomically the country is divided in six regions: Northern, Central, Brunca, Chorotega, Caribbean Huetar and Central Pacific [21]. The total number of bovines in CR is close to 1.55 million, distributed in about 15000 farms and 50000 herds (S1 Table) [22]. Three different management systems are commonly carried out in the country: beef, dairy and double purpose cattle. Most dairy farms of European breeds (*Bos taurus*) are located in the highlands (from 1000–2500 m); while in the low lands (below 1000 m) are most of the zebu (*Bos indicus*) and mixed breeds (e.g. cebu-holstein cross), used for beef or double purpose production, respectively [22].

Study population and statistics

The seroprevalence of brucellosis in beef, dairy and double purpose animals were estimated in two bovine populations: i) a non-random sample from sera arriving to the CR-NAHS laboratories for regular diagnosis from herds with history of brucellosis, abortion, reproductive problems, commercial transactions, attendance to exhibitions, exportations and importation of cattle and bovines from herds declared "brucellosis free", and; ii) a random sample systematically taken in the different regions of CR. To assess both herd and animal prevalence by management system in the later population, a random sample of 250 farms per strata, proportionally allocated by region, were sampled. This sample size was calculated using public access WinEpiscope 2.0 software [23], fitting the following parameters: bovine herd prevalence of 10%, confidence level of 95% and accepted error of 4% for 235 farms; however, it was decided to sample a total of 250 farms per region. A farm was declared positive when at least one serum sample resulted positive. For sample size, the Cannon & Roe formula to demonstrate freedom from/absence of infection, the expected prevalence was adjusted to 15% and a confidence level of 95% [24]. The estimated herd prevalence was founded on the average herd prevalence obtained on pilot study performed in dairy herds in the highlands of the Central Valley of CR. This model does not strictly estimate the within-herd prevalence, but assess the presence of disease. In both studies, the diagnostic strategy was first, screening all bovines by RBT, and then testing of the RBT positives (RBT^+) by iELISA.

The univariate prevalence analysis at the global level and according to production system, were calculated by RBT and RBT^++iELISA. In addition, bivariate prevalence for production system by region was also estimated. The prevalence confidence intervals were calculated using beta distribution in the Program @risk [25]. Due to the 99% sensitivity and 99% specificity of the RBT in the absence of S19 vaccination [26,27], a perfection assay was assumed in the analyses.

Serum samples

For sampling purposes, the six socioeconomically divided regions of CR were tested (Fig 2). A total of 765 farms accounting for close to 13078 cows (2–6 years of age) were sampled ($\bar{X} = 18$ cows/farm) during 2012-2013-year period: 250 dairy herds (3902 cows), 254 beef herds (4485 cows) and 261 dual purpose herds (4691 cows). In addition, a non-random serum sample of 532199 cows (~35% of the CR bovines) of the six regions ($\bar{X} = 67$ cows/farm) comprising 7907 herds (~16% of CR herds), arriving during 2014–2016 to the laboratories of the CR-NAHS for routine diagnoses, were analyzed. For all purposes, repeated herds were taken into account. For epidemiological purposes, no distinction between breeds or bovine species was considered during the survey.

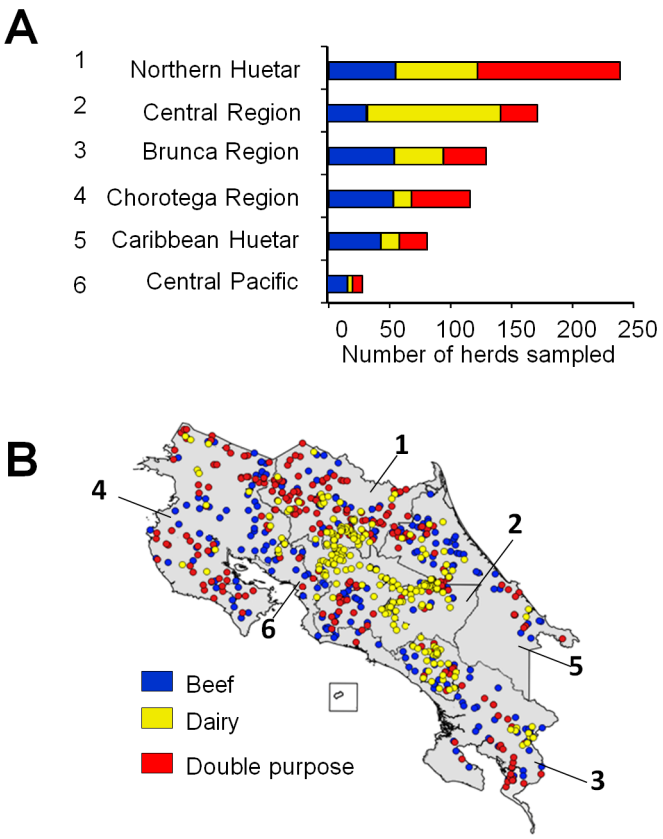

Fig 2. Sampling of cattle farms in the six regions of Costa Rica. (A) A total of 750 farms accounting for close to 18000 cows (2–6 year-old) were sampled during 2012–2013 year period: 250 dairy herds (3902 cows), 254 beef herds (4485 cows) and 261 dual purpose herds (4691cows). (B) Map of CR indicating the different sapling regions (depicted by numbers). Areas of low density of sampling correspond to national parks or protected areas devoid of cattle.

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Blood samples were collected with a syringe or a sterile vacutainer with Z serum clot activator (Vacutainer System, Greiner Bio-one), transported in refrigeration conditions and sera obtained by centrifugation. Each sampled received an individual consecutive number upon arrival to the laboratory. Analyses of the sera were performed within 24–72 hours after collection or arrival at the National Veterinary Laboratories of the CR-NAHS in Heredia, CR, or the Immunology Laboratory of Medicine Veterinary School of the National University, Heredia, CR.

Information collected for bovine sample

Relevant data concerning the geographical localization, area of the farm and management characteristics of the herd or individual animals, were collected. The information also included the presence of other domestic and wildlife species, veterinary services, reproductive parameters and history of abortion/stillbirth, replacement animals, and history of vaccination against brucellosis. Breed and individual identification was registered.

Serological tests

Rose Bengal test (RBT) (ID-VET, France) was used as general screening test [28]. Indirect protein A/G ELISA (iELISA) (ID-VET, France) and competitive ELISA (cELISA) (Svanovir,

SVANOVA, Sweden) were used as confirmatory tools as described elsewhere [28]. Standardizations of RBT, iELISA and cELISA were performed as described previously [27]. The cut-off values and the specificities and sensitivities of the iELISA and cELISA have been previously established [27,29]. All bovine sera samples were initially screened by RBT and the positives then tested by iELISA and cELISA.

Culture conditions and *Brucella* identification

The following strains obtained from PIET/CIET strain collections were used as controls for biochemical and molecular studies: *Brucella ceti* Atlantic dolphin type (B14/94), *B. ceti* Atlantic porpoise type (B1/94), *Brucella pinnipedialis* seal type (B2/94), *Brucella abortus* 2308W (biovar 1 virulent reference strain), *B. abortus* S19 (biovar 1 reference vaccine strain), *Brucella melitensis* Rev1 (biovar 1 reference vaccine strain), *Brucella suis* (S2 biovar 1), *Brucella canis* (CR206-10; CR isolate), *Brucella neotomae* 5K33 (reference strain), *Brucella ovis* PA (virulent reference strain) and *Brucella microti* (CCM4915, reference strain).

According to the National Brucellosis Control Program of the CR-NAHS of the Ministry of Agriculture and Livestock Management, all diagnosed seropositive cattle were selected for obligatory culling [19]. Necropsies or sample collection were carried out at the Pathology Department in the Veterinary School of Universidad Nacional, CR and official slaughterhouses. Animal samples included milk and other secretions such as vaginal swabs and semen, reproductive organs, lymph nodes, spleen, kidney and liver. In some cases, aborted fetuses were also collected and sampled. Cultures were done at the Bacteriology Laboratory of the Veterinary School and at the laboratories of SENASA, using non-selective and selective media including blood agar and Columbia agar, supplemented with 5% of dextrose and sheep blood as well as Modified *Brucella* Selective Supplement (Oxoid® (SR0209) and CITA medium [30]. Cultures were incubated in 10% CO_2 atmosphere at 37˚C for at least two weeks. The selected bacterial colonies were subjected to Gram staining, agglutination with acriflavine and acridine orange dyes and tested for urease and oxidase activity, citrate utilization, nitrate reduction, H_2S production, growth in the presence of thionin (20 μg/mL) and basic fuchsin (20 μg/mL) and uptake of crystal violet according to described procedures [31].

Brucella DNA samples from each isolate and control strains were extracted with DNeasy Blood & Tissue kit from QIAGEN ®, and stored at -80˚C until used. Identification of *Brucella* species was performed by multiple locus variable-number tandem repeat analysis-16 (MLVA16) following standard procedures [32]. *Brucella* control strains were used for validation. The profiles were entered in the database MLVA-NET for the corresponding analysis [33].

Ethical considerations

The sampling of bovines is part of the National Brucellosis Control Program of the CR-NAHS of the Ministry of Agriculture and Livestock Management [19] and the Law of Reportable Infectious Diseases of the Ministry of Health of CR [34]. Protocols for the use of bovine serum and tissue samples were revised and approved by the "Comité Institucional para el Cuido y Uso de los Animales de la Universidad de CR"(CICUA 057–16366), and "Comité Institucional para el Cuido y Uso de los Animales" of the Universidad Nacional, CR (SIA 0434–14 and SIA 0545–15), and in agreement with the corresponding law "Ley de Bienestar de los Animales", CR [35], and according to the "International Convention for the Protection of Animals" endorsed by Costa Rican Veterinary General Law on the CR-NAHS [36].

Results

In CR the CR-NAHS uses RBT as screening tests and iELISA and cELISA as confirmatory assays [37]. Following this, the results obtained in the analysis of non-random and random samples are presented in Table 1. The RBT herd prevalence levels obtained between the non-random and the random samples were 11.4 and 10.5, respectively. When positive RBT sera was tested by iELISA, the estimated herd prevalence values lowered to 6 and 4.1 respectively Comparable prevalence values observed by RBT^{+}+iELISA were obtained when RBT positives were tested by cELISA. The confidence limit 95% of the random sample was 3–6, in rounded numbers. Statistical significance comparisons were made among the different management systems, the random and non-random samples and among the various serological assays used. The only result that showed significant difference in RBT was the double purpose herds in the non-random sampling. When positive RBT samples were tested by iELISA, the results of dairy herds from the non-random sampling were significantly different from the other two management systems. Finally, when comparing both samplings procedures, there were significant differences in the results between beef and double purpose cattle (Table 1).

The higher brucellosis RBT prevalence levels in the non-random (Table 2) and random sampling (Table 3) were obtained with double purpose cattle from the Northern Huetar (17.2% and 17%, respectively) and the Caribbean Huetar (20.2% and 13%, respectively) been the latter one the poorer and less developed of CR. In the case of beef cattle, the regions with

Table 1. Herd and bovine brucellosis reactors according to management system and sampling procedures*.

		Management System	Number	RBT (%)	RBT^{+}+IELISA
Non-random sample from 2014-2016	**Herds**	Beef	806	90 (11.2) aα	56 (6.9) cδ
		Dairy	4479	431 (9.6) aβ	186 (4.2) dε
		Double purpose	2622	377 (14.4) bγ○	231 (8.8) cζ
		Total	**7907**	**898 (11.4)**	**473 (6.0)**
	Bovines	Beef	48129	414 (0.9)	320 (0.7)
		Dairy	346326	481 (0.1)	299 (0.1)
		Double purpose	137744	569 (0.4)	463 (0.3)
		Total	**532199**	**1464 (0.3)**	**1082 (0.2)**
Random sample from 2012–2013	**Herds**	Beef	254	24 (9.4) aα	8 (3.1) cη
		Dairy	250	22 (8.8) aβ	11 (4.4) cε
		Double purpose	261	34 (13.0) aγ	12 (4.6) cθ
		Total	**765**	**80 (10.5)**	**31 (4.1)**
	Bovines	Beef	4485	33 (0.7)	9 (0.2)
		Dairy	3902	37 (1.0)	15 (0.4)
		Double purpose	4691	90 (1.9)	50 (1.1)
		Total	**13078**	**160 (1.2)**	**74 (0.6)**

* Numbers in parenthesis indicate the seroprevalence. Latin alphabet letters (a-c) represent statistical differences of $p \leq 0.05$ values, among productive systems, within the sampling method. Greek alphabet letters (α-θ) represent statistical differences of $p \leq 0.05$ values among productive systems, sampling methods and according to type of serological test. Letters "a" and "c" within the same column indicate no significant statistical differences among the various management systems and among the non-random and random sampling. On the contrary, letters "b" and "d" indicate that there are significant statistical differences among the various management systems and among random and non-random sampling. Greek letters "α", "β" and "γ" indicate that there are not significant statistical differences among the RBT results between the non-random and the random sampling. Alternatively, Greek letters "δ", "ζ", "η", "θ" depict significant statistical differences among the results obtained in RBT^{+}+iELISA within the sampling method. On the contrary, the Greek letter "ε" indicates no significant statistical differences among the two sampling methods using RBT^{+}+iELISA. In the random sample, the confident limit 95% for beef cattle ranged from 1.6–6.1, for dairy cattle from 2.5–7.7, for double purpose cattle from 2.7–7.9 and for the total population of animals from 2.8–5.7. Bovine population in CR shown in S1 Table.

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Table 2. Herd prevalence in a non-random sample according to region and management system 2014–2016.

Region	Beef			Milk			Double purpose			Total		
	N° Herd	RBT	RBT⁺+ iELISA	N° Herd	RBT	RBT+ iELISA	N° Herd	RBT	RBT⁺+ iELISA	N° Herd	RBT	RBT⁺+ iELISA
1. Northern Huetar	73	15.1	8.2	1441	11.5	4.1	1048	17.2	11.0	2562	13.9	7.0
2. Central Region	74	5.4	2.7	2037	9.5	4.9	262	8.4	6.1	2373	9.3	4.9
3. Brunca Region	510	10.9	7.5	380	5.2	0.2	446	13.2	3.3	1336	10.1	4.0
4. Chorotega Region	82	6.1	2.4	431	6.0	3.2	365	7.9	5.7	878	6.8	4.2
5. Caribbean Huetar	46	23.9	19.6	114	15.8	11.4	396	20.2	15.4	556	19.6	14.9
6. Central Pacific	21	14.2	9.5	76	10.5	0.0	105	6.6	1.9	202	8.9	1.9
Total	**806**	**11.1**	**7.3**	**4479**	**9.6**	**4.1**	**2622**	**14.4**	**8.8**	**7907**	**11.3**	**6.0**

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the highest number of RBT positive herds in the random and non-random samples were also the Northern Huetar (15.1% and 9%, respectively) and Caribbean Huetar (23.9% and 23%, respectively); while the largest numbers of RTB positive dairy herds were detected in the Central (9.5% and 11.9%, respectively) and Caribbean Huetar (15.8% and 20%, respectively) regions. Due to the small number dairy herds in the Central Pacific region, fewer farms were sampled. In spite of this, positive herds were detected. As expected and regardless of the sample method, when positive RBT samples were tested by iELISA, the prevalence values were lower but commensurate to the RBT in the same regions (Tables 1 and 2). The RB51 animal vaccination coverage for five-year period was estimated in 11%, being more frequent in bovines from dairy farms. Although it was not possible to assess the actual numbers or RB51 revaccinated bovines, we confirmed that it was a common and a recommended practice in CR.

B. abortus has been isolated from dairy, meat and double purpose cattle in all the six regions of CR (Fig 3A). Consistent with previous findings [38,39], *B. abortus* biovar 1, 2 and 3 were isolated in different latitudes of CR. *B. abortus* MLVA16 clusters were estimated based on differences in three or less tandem repetitions. Following this, the MLVA16 analysis of 326 strains demonstrated that the CR *B. abortus* stains (S2 Table) clustered in four main groups (MLVA16 meta-data accessible at http://microbesgenotyping.i2bc.paris-saclay.fr/), suggesting at least four different *B. abortus* founders (Fig 3B). Bacteria in cluster one corresponds to the main group, harboring most of the CR isolates; while clusters two, three and four are represented by just a few isolates. Cluster one also includes clinical isolates from aborted fetuses which were identified as *B. abortus* RB51 vaccine by Bruce-ladder and supported by MLVA16 (baboCR58 and baboCR57). Clusters one and two are intertwined with *B. abortus* from different latitudes. For instance, within cluster one there are isolates from central Europe, USA,

Table 3. Herd prevalence in a random sample according to region and management system 2012–2013.

Region	Beef			Milk			Double purpose			Total		
	N° Herd	RBT	RBT⁺+ iELISA	N° Herd	RBT	RBT⁺+ iELISA	N° Herd	RBT	RBT⁺+ iELISA	N° Herd	RBT	RBT⁺+ iELISA
1. Northern Huetar	55	9.0	3.6	67	4.4	3.0	117	17.0	8.5	239	11.7	5.9
2. Central Region	32	12.5	0.0	109	11.9	4.6	30	6.7	0.0	171	11.1	2.9
3. Brunca Region	54	1.9	1.9	40	10.0	0.0	35	1.9	0.0	129	4.7	0.8
4. Chorotega Region	53	9.4	1.9	15	6.6	0.0	48	12.5	2.1	116	10.3	1.7
5. Caribbean Huetar	43	23.2	9.3	15	20.0	20.0	23	13.0	4.3	81	16.0	9.9
6. Central Pacific	17	11.7	0.0	4	25.0	25.0	8	0.0	0.0	29	10.3	3.4
Total	**254**	**10.6**	**3.1**	**250**	**10.0**	**4.4**	**261**	**12.3**	**4.6**	**765**	**10.5**	**4.1**

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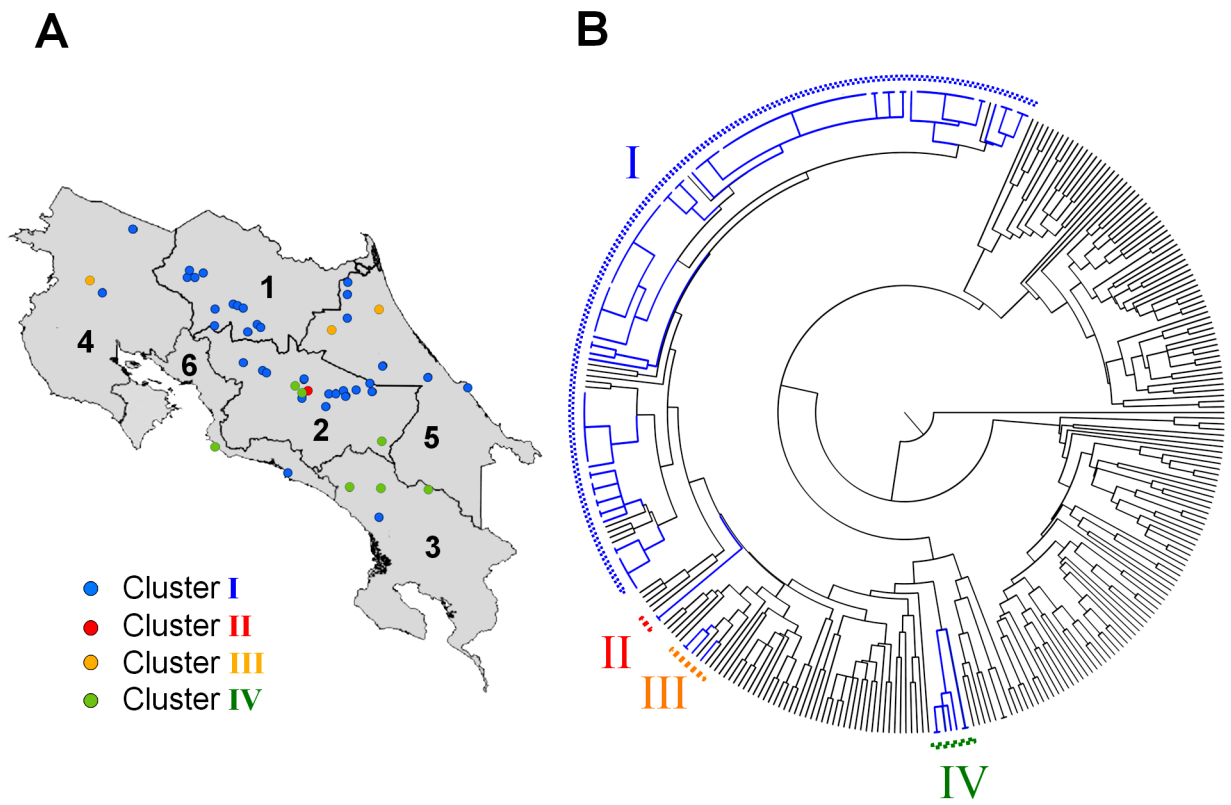

Fig 3. MLVA16 dendogram of *B. abortus* isolates from different regions of Costa Rica. (A) Map of CR indicating the different regions from which *B. abortus* were isolated (circles). The color of the circles corresponds to the I-IV clusters, respectively. (B) MLVA16 dendogram constructed from the analysis of 107 *B. abortus* isolates (depicted in blue lines) are compared with MLVA16 of 219 *B. abortus* representative isolates from other latitudes (indicted in black lines). Clusters I to IV are indicted in the figure. S2 MLVA16 genetic profiles for the CR *B. abortus* isolates are presented in S2 Table.

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India and Brazil. Likewise, cluster three is intertwined with isolates from central Europe, India and Brazil. In contrast, cluster two and four seem to have only representatives from CR. While cluster one is found in all the six regions of CR, cluster three seems confined to the northern areas of the Caribbean Huetar and Chorotega regions and cluster four mainly to the Central and southern areas of the Brunca region. Cluster two is represented just by two isolates confined to the Central region.

Discussion

We have analyzed the brucellosis herd prevalence in CR by random and non-random methods. The rational of these two schemes is different: while the random sampling is based on a probability theory in which each herd in the population is identified, and has an equal chance of being in the sample; the non-random sampling takes advantage of the samples routinely available for diagnoses. This last non-probability sample is useful for quick and inexpensive studies and for developing hypotheses. When non-random schemes include a large number of individuals and herds within a given population -as it is our case- the values rendered by the analysis may complement the random analysis, and therefore, useful to enforce or deny the hypothesis.

Depending on the strategy employed, brucellosis prevalence varies. For instance, if the RBT results are used as sole parameters, then the prevalence ranges from 10.5% to 11.4%.

Alternatively, if the criterion used is the screening of the RBT positives by iELISA, then the prevalence span from 4.1% to 6%. The confidence limit 95% for the random analysis was 3–6%. However, these data deserve careful interpretation. First, detection of RBT false positives due to residual antibodies after vaccination is ruled out in CR. Indeed, the only vaccine used is rough RB51 devoid of O-chain lipopolysaccharide and the vaccine animal coverage in CR is rather low (11%). Under these conditions and in our hands, with a collection of sera from negative and culture positive animals [27], the RBT performs with 99% specificity and 99% sensitivity, values that are commensurate with the findings of other investigators [26]. In spite of this, the RBT may still detect cross reacting antibodies against other bacteria (e.g., *Yersinia enterocolitica* O:9) sharing antigenic determinants with *Brucella*, and then render some false positive reactions [40,41]. Nevertheless, under high brucellosis prevalence, the RBT false positives may have little impact. Moreover, the iELISA and cELISA may also detect cross reacting antibodies [40,41]. Second, the specificity (~98%) and sensitivity (~97%) of the so called "confirmatory assays", such as iELISA and cELISA [28], depend on the cut off values established [26,27;41]. The current iELISA and cELISA cut off values used in CR and in other Latin American countries were adjusted under S19 vaccination [29], and then intended for detecting antibodies in the infected but not in the S19-vaccinated animals. Finally, the RBT and the iELISA or cELISA may detect different subsets of positive animals [27,41]. This is relevant, taking into account that not all animals were tested by iELISA or cELISA, but just the RBT positives.

Regarding the model used here, there are some drawbacks that deserve attention. Accordingly, a herd was declared positive when at least one serum sample was positive in the RBT following the Cannon & Roe strategy [24]. Sticking to this, it seemed that the average number of 18 animals/herd sampled, became somewhat short. Since the test is not perfect (99% specificity) the probability that 18 bovines in a negative herd, all tested negative, was close to 83%. Then, it follows that the probability that at least one bovine was false-positive –and in consequence the whole herd–, was close to 17%. Likewise, the probability of obtaining a false-positive in given herd decreased with the increased number of positive-diagnosed animals within the group. Testing the RBT positives by iELISA (RBT^{+}+iELISA) ensured higher specificity, and the lowest possible prevalence, but not the highest prevalence, which was given by the RBT. It is worth mentioning that while the RBT does not depend on quantitative measures; the iELISA and cELISA depend upon cut-off values, which may vary depending on the epidemiological conditions.

In spite of the limitations of the model and the possibility of cross reactions by the RBT, this test stands as the most reliable assay in the absence of S19 vaccination and low RB51 vaccination coverage [41]. Considering this, it is likely that the RBT herd prevalence depicted in the random analysis is closer to the reality of the country and then, the standing prevalence in case of planning an eradication program in CR. Although the rational of the non-random scheme is different from that of the random sampling, the data in the former somewhat supports the values obtained in the latter.

At least four different *B. abortus* MLVA16 clusters are circulating in CR, indicating that the bacterium was introduced more than once in the territory. Cluster one and three are intertwined with isolates from other countries, while clusters two and four have only CR representatives. Since cluster one is widely distributed in all different regions of the country, it seems to be the dominant and the primary source. The relationship of the local strains with *B. abortus* from North America, Brazil and Central Europe is not surprising, taking into account that CR cattle came from those lands. The other *B. abortus* clusters may be of more recent introduction. It seems to be some association between the MLVA16 clusters and the distribution of the

CR isolates. However, in order to unambiguously determine this association and the origin of the clusters, more isolates from different regions are required.

Through the years, efforts have been carried out by the CR animal health authorities to control bovine brucellosis. Unfortunately, these efforts -mainly based in control programs from other latitudes- have been erratic and constantly interrupted [10,14,42,43]. For instance, it is evident that the brucellosis prevalence (estimated by agglutination tests) has increased in relation to that observed in the second half of the eighties and first half of the nineties (Fig 1). During the period of 1978–1985 -after a loan from the Inter-American Development Bank-, a brucellosis control program, known as National Program of Animal Health (PRONASA), was undertaken. PRONASA was intended for ten years and it was coordinated by the CR-NAHS of the Ministry of Agriculture and Livestock Management [10,44,45]. The plan included obligatory *B. abortus* S19 vaccination of young replacements, monitoring of abortions, compulsory diagnoses by RBT, 2-mercaptoetanol, rivanol and milk-ring agglutination tests, culling of the serological positive animals with no compensation, and control of animal displacements at specific regional checkpoints [10,14]. During the early years of PRONASA the national vaccine coverage reached close to 43% of bovines and the surveillance was actively taken [14].

Unfortunately, the strong economic recession initiated in 1982 undermined the brucellosis control program. In addition, new political endeavors endorsed the end of PRONASA which was then substituted by PROGASA [44]. In time, this caused the dismantled of the majority of the veterinary field services devoted to the program and, in practical terms, the end of the brucellosis surveillance campaign [10]. By 1984, S19 vaccination reached only one third of the expected coverage [14]. By 1990 the vaccination coverage was less than 15% and finally by the end of the decade, S19 vaccination was interrupted with the subsequent advent of rough *B. abortus* RB51 vaccine handled by private hands, mainly by the dairy industry [10,14,19,46]. As stated, the current RB51 vaccination coverage at five year lapse at the animal level is not more that 11%, as estimated in this study and by the annual importation of RB51 vaccine doses to CR [47]. However, this value does not take into consideration revaccination protocols, which are common practices in Costa Rica, and which may interfere with the diagnosis.

Considering the PRONASA 1978–1985 brucellosis control campaign, some errors were made [14,42]. Regardless of the type of vaccine employed, the vaccination coverage in CR has never reached the required levels for adequate herd immunity (at least 70% of coverage). Moreover, the serological testing necessary to detect the brucellosis positive herds reached during the campaigns, was always lower than expected. In addition, the removal of the positive animals was not systematically applied and the economy and political situation of the country did not allow compensation for culling of the reactors. This favored hiding of the positive animals, clandestine sales and transfer of infected cattle to other areas. Moreover, the sole vaccination of young replacements with S19 seemed not enough. Indeed, the logic behind calf S19 vaccination implies extensive survey and constant identification and removal of the positive bovines. However, since testing was not extensively applied, then a significant number of susceptible and infected adult bovines were not identified. All these aspects favored the permanence and spreading of the infection in the country.

One key factor that worsened the problem and deserves attention, concerns to the vaccination policy during the last two decades. In order to "avoid diagnosis confusion" in the detection of *Brucella* infected animals, the regular use of *B. abortus* S19 was banned in countries free of bovine brucellosis (e.g. United States and Canada). The Animal Health authorities replaced S19 with RB51 in 2000, before achieving any control of the disease. In addition, the vaccination platform was transferred into private hands mainly to dairy and pharmaceutical companies [18]. We were unable to find documents justifying the rational for these "technical" decisions carried out in CR. This caused the practical obliteration of *B. abortus* S19 from the program

and the introduction of RB51 as the canonical vaccine [17]. This is not trivial since S19 is the only vaccine that has demonstrated to be successful in eradicating bovine brucellosis [48]. All these events have caused additional problems. Two of them relate to the frequent revaccination, practice known to induce diagnostic problems and increase costs [17, 49–51]. In addition, the unrestricted use of RB51 may promote a "false sense of security", relaxing the surveillance protocols in the vaccinated herds [52].

Experiences of the various brucellosis eradication programs have demonstrated that the first campaigns were mostly unsuccessful [48]. In countries such as United States, Canada, Australia, New Zeeland or those from Western Europe, eradication of brucellosis was achieved only after the development of joint efforts among the livestock producers, authorities and industry who finally understood the scientific and epidemiological data. They embraced the eradication of brucellosis as their own problem and perceived it as an opportunity to reduce the losses, increase the value of their products and ending with human suffering caused this zoonotic disease [48]. Among the most successful strategies followed by these countries were [48]: i) widespread *B. abortus* S19 vaccination coverage of female bovine at risk; ii) single dose immunization of female bovine with complete or reduced S19 vaccine; iii) extensive diagnoses of bovines and herds by sensitive and specific serological assays; iv) obligatory culling of the serological positive animals with compensation actions, and; v) restriction in the traffic of animals from infected areas to free areas.

Although these experiences are relevant, it is unlikely that eradication of bovine brucellosis in CR would be achieved by just applying fixed strategies from other latitudes. Indeed, the eradication of bovine brucellosis is far more complex than just vaccination, testing and slaughtering of the reactors. It is mandatory to consider the idiosyncrasy of each country at the time of initiating campaigns towards the elimination of the disease. For instance, due to the high brucellosis prevalence in CR, immediate slaughtering of all the rectors and confining the herds seem unpractical and not economically feasible. First, it would be necessary to lower the prevalence by limiting the rate of infection and reducing the number of abortions. These may be achieved by extensive and unrestricted vaccination of all female bovines (young and adults) by the conjunctival route with reduced dose S19 vaccine; this, without previous diagnoses and without testing of the animals for two years. Such a strategy—which seems unorthodox–, is known to practically eliminate the clinical disease and to diminish the degree of cattle infection at risk [53]. After few years (e.g. two years), this approach would reduce the prevalence and density of the bacteria in the bovine population to numbers where "a clean" vaccination program of young replacements with S19 (e.g. reduce dose by the conjunctiva route) would be feasible. Then, a serological identification and slaughter of the positive animals might be initiated under more favorable herd infection conditions, allowing some compensation for culling the reactors.

Since the first surveillances performed eighty years ago [7], it is clear that brucellosis remains as a relevant disease of cattle in CR. The steady increase in the brucellosis detection and the consistent isolation of the bacterium in all regions supports the high prevalence and validate the notion that in CR *B. abortus* is a source of important economic losses and human health suffering [17,20]. Within this perspective, it seems that the brucellosis conditions prevailing in CR are not unique, and other regions in Latin America display similar vaccination strategies and epidemiological profiles [54–56]. Therefore, our findings are relevant within a broadest context.

Why does after one hundred years of the first isolation of *Brucella* in CR, this small country has not been capable to lower the prevalence and eradicate brucellosis? Certainly, countries about the same size as CR have eradicated brucellosis. Moreover, CR has been able to resolve very complex problems [57–59]. For instance, since 1949 the army was abolished, and since

1970 the natural protected areas of the country cover 26% of the territory of CR. Likewise, the Costa Rican public healthcare system is ranked among the highest in the American Continent. Literacy is also comparatively high for a middle range income country. Regarding the cattle industry, a large part of the milk and meat producers are well organized in cooperatives and associations. Above 97% of the farms are electrified, communicated by roads and the veterinary services attending the farms are well trained [6,36]. It seems, therefore, that in order to achieve brucellosis eradication in CR, joint efforts are necessary among scientists, producers, cattle industry and the government. Without cooperation among these parties, even good intentions and first-class strategies are condemned to failure.

Conclusions

1. Bovine brucellosis due to *B. abortus* is widespread in CR and the prevalence of the disease has increased in relation to the last three decades.
2. In the absence of S19 vaccination, the RBT herd prevalence depicted in the random analysis tends to lay close to the reality and then, the suggested value in case of planning an eradication program in CR.
3. In the absence of S19 vaccination, the iELISA and cELISA used as "confirmatory tests" need to be adjusted to the required levels of sensitivity and specificity to fulfill the brucellosis epidemiological conditions of CR.
4. The vaccination campaigns in CR have never been adequately adopted to increase the herd immunity required to decrease the number of susceptible animals below a desired threshold, for control programs.
5. The vaccination coverage in CR is rather low and revaccination with RB51 is a common practice in CR.
6. At least four different *B. abortus* MLVA16 clusters are circulating in CR, indicating that the bacterium has been introduced more than once in the territory. Cluster one -widely distributed in all different regions of the country- seems to be the dominant and the primary *B. abortus* source in CR.
7. The brucellosis campaigns have been interrupted due to economic problems, deficient animal health services, absence of personnel and weak political support to technical and scientific concerns.
8. The availability of reliable epidemiological data on bovine brucellosis in all regions of CR establishes a background level to envision strategies for the control of bovine brucellosis in the country.

Supporting information

S1 Table. Estimated number of bovines by geographical region and by management system in Costa Rica (2011–2014).
(DOCX)

S2 Table. MLVA16 genetic profiles for the CR B. abortus isolates.
(XLSX)

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References

1. Campos E, Vicente G, Ramírez JA, Moreno E. Evaluación seroepidemiológica de la brucelosis humana en poblaciones de riesgo ocupacional. Acta Med Cost (Costa Rica). 1984; 47:8–9.
2. Ministerio de Economía y Hacienda. Anuario Estadístico de Costa Rica. Dirección General de Estadísticas y Censos. San José, Costa Rica. 1953. http://biblioteca.ccp.ucr.ac.cr/handle/123456789/1303.
3. Szyfres B, Blood BD, Moya V. Estado actual de la brucelosis en la América Latina. Bol Oficina Sanit Panam. 1959, 46:48–64.

4. Ministerio de Economía y Hacienda. Anuario Estadístico de Costa Rica. Dirección General de Estadísticas y Censos. San José, Costa Rica. 1956. Avaliable from: http://biblioteca.ccp.ucr.ac.cr/handle/123456789/1299.
5. Pérez-Román C, Nema-Vidaurre J, Fonseca-Araya E. Brucelosis en personal del matadero de Nicoya, Guanacaste. Acta Méd Cost (Costa Rica) 1984, 27: 41–44.
6. Quirós-Quirós E. Historia de la ganadería bovina en Costa Rica. Corporación Ganadera (CORFAGA). Archivos Institucionales. 2006. http://corfoga.org/carne-de-costa-rica/historia-de-la-ganaderia%20/
7. Zeledón-Alvarado J. Primera historia clínica de brucelosis humana en Costa Rica. Rev. Med. 1940; 72:153–167.
8. Quirós D. El primer caso de fiebre de Bruce en Costa Rica. Gaceta Médica de Costa Rica. 1915; 11:241–244.
9. Ministerio de Agricultura y Ganadería. Dirección de Salud Animal. Proyecto Salud Animal MAG-BID. San José, Costa Rica. 1978.
10. Programa de información agropecuaria (PIAGRO). Brucelosis y tuberculosis. Taller de evaluación y perspectivas de los programas oficiales de brucelosis, tuberculosis y leptospitosis. Ministerio de Agricultura y Ganadería, San José, Costa Rica. 1996. http://www.mag.go.cr/bibliotecavirtual/L73-2398.pdf.
11. Procuraduría General de la República. Control de ganado bovino, prevención y sanción de su robo, hurto y receptación. Sistema costarricense de información jurídica. Ley N˚ 8799. La Gaceta N˚ 88, San José, Costa Rica. 2010. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=67888&nValor3=80618&strTipM=TC.
12. Jiménez E. Ceniza del Turrialba afecta a 8252 animales y 261 hectáreas de cultivos. La Nación (26-05-2016) 2016. http://www.nacion.com/sucesos/desastres/volcan_Turrialba-erupcion-afectacion-cultivos-animales_0_1563043796.html.
13. Recio P. SENASA confirma brote de brucelosis en fincas cercanas al volcán Turrialba. La Nación (16-03-2015) 2015. http://www.nacion.com/nacional/salud-publica/Senasa-confirma-brucelosis-cercanas-Turrialba_0_1475652544.html.
14. Vicente GS, Kirschbaum A, Ureña M. Consideraciones sobre la vacunación antibrucelosa en Costa Rica 1978–1982. Ciencias Veterinarias (Costa Rica). 1983;123–127.
15. Ministerio de Agricultura y Ganadería. Informe sobre brucelosis. Archivos de informes semestrales y anuales. Dirección de Salud animal. San José, Costa Rica. 1990.
16. Ministerio de Agricultura y Ganadería. Reglamento sobre el control de la brucelosis en los animales. Decreto N˚ 28514-MAG. La Gaceta N˚ 53, San José, Costa Rica. 2000. http://www.ecolex.org/fr/details/legislation/decreto-no-27946mag-reglamento-sobre-el-control-de-la-brucelosis-en-los-animales-lex-faoc017344/
17. Moreno E. Brucellosis in Central America. Vet Microbiol. 2002; 90:31–38. PMID: 12414131
18. MediVet-SENASA. Vacuna antibrucélica. Dirección de Medicamentos Veterinarios. 2016. http://www.senasa.go.cr/medivet/WebForm2.aspx?id=AR-48-64-51-5680.
19. Ministerio de Agricultura y Ganadería. Reglamento para la intervención de la brucelosis bovina. Decreto 34858-MAG-2008. La Gaceta N˚ 230, San José, Costa Rica. 2008. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=64425&nValor3=74734&strTipM=TC.
20. Hernández-Mora G, Bonilla-Montoya R, Barrantes-Granados O, Esquivel-Suárez A, Montero-Caballero D, González-Barrientos R, et al. Brucellosis in mammals of Costa Rica: an epidemiological survey. 2017; Plos One (pending decision).
21. Vargas-Ulate T. Geografía de Costa Rica. Editorial Tecnológica, Cartago, Costa Rica. 2004;124.
22. Ministerio de Agricultura y Ganadería. Sistema integrado de registro de establecimientos Agropecuario-SIREA. Servicio Nacional de Salud Animal-SENASA. 2017. http://registrosenasa2.addax.cc/.
23. Thrusfield M, Ortega C, de Blas I, Noordhuizen JP, Frankena K. WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet Rec. 2001; 148:567–572. PMID: 11370882
24. Cannon R.M., Roe R.T., Livestock Disease Surveys. A field manual for veterinarians. Canberra. 1982;35.
25. Palisade Corporation. Risk analysis using Monte Carlo simulation, 2017. http://www.palisade.com/risk/.
26. Omer MK, Skjerve E, MacMillan AP, Woldehiwet Z. Comparison of three serological tests in the diagnosis of *Brucella* infection in unvaccinated cattle in Eritrea. Prev Vet Med. 2001; 48:215–222. PMID: 11182464
27. Moreno E, Rojas N, Nielsen K, Gall D. Comparison of different serological assays for the differential diagnosis of brucellosis. In International Atomic Energy, Diagnosis and epidemiology of animal diseases in Latin America IAEA-TECDOC 1055. Viena; 1998;153–161.

28. OIE-World Organization for Animal Health. Bovine brucellosis: In manual of diagnostic tests and vaccines for terrestrial animals. 12 rue de Prony 75017. Paris; 2009. http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.

29. Gall D, Colling A, Marino O, Moreno E, Nielsen K, Pérez B, et al. Enzyme immunoassays for serological diagnosis of bovine brucellosis: A trial in Latin America. Clin Diagn Lab Immunol. 1998; 5:654–661. PMID: 9729532

30. De-Miguel MJ, Marín CM, Muñoz PM, Dieste L, Grilló MJ, Blasco JM. Development of a selective culture medium for primary isolation of the main *Brucella* species. J Clin Microbiol. 2011; 49:1458–1463. https://doi.org/10.1128/JCM.02301-10 PMID: 21270216

31. Alton GG, Jones LM, Angus RD,Verger JM. Techniques for brucellosis laboratory. INRA, France.1988.

32. Le Flèche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoeud F, et al. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. 2006; 6:9. https://doi.org/10.1186/1471-2180-6-9 PMID: 16469109

33. CNRS-Centre National de la Recherche Scientifique. MLVA-Net for Brucella. 2017. http://mlva.u-psud.fr/brucella/.

34. Procuraduría General de la República. Reglamento de vigilancia de la salud. Sistema costarricense de información jurídica. Norma 37306-S. La Gaceta N˚ 203, San José, Costa Rica. 2012. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&param2=18&nValor1=1&nValor2=73471&nValor3=90176&strTipM=TC&lResultado=180&nValor4=1&strSelect=sel.

35. Procuraduría General de la República. Ley de Bienestar de los Animales. Sistema costarricense de información jurídica. Ley N˚ 7451. La Gaceta N˚ 236, San José, Costa Rica. 1994. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=24319&nValor3=25739&param2=1&strTipM=TC&lResultado=2&strSim=simp.

36. Procuraduría General de la República. Ley General del servicio nacional de salud animal. Sistema costarricense de información jurídica. Ley N˚ 8495. La Gaceta N˚ 93, San José, Costa Rica. 2006. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=57137&nValor3=80913&strTipM=TC.

37. Jiménez-Arias JC. Programa Nacional de brucelosis bovina. Servicio Nacional de Salud Animal (SENASA), Ministerio de Agricultura y Ganadería, Heredia, Costa Rica. 2009; http://www.senasa.go.cr/senasa/sitio/files/151211052133.pdf.

38. Sequeira A, Campos E, Mendoza L, San-Román MA, Moreno E. Identificación de especies y biotipos de Brucella aisladas en Costa Rica. Turrialb. 1984; 34:525–526.

39. Villalobos-Zúñiga MA, Barrantes-Valverde E, Monge-Ortega P. Endocarditis por *Brucella abortus*: reporte del primer caso en C. R. Acta Méd Cost (Costa Rica). 2011; 53:154–157.

40. Muñoz PM, Marín CM, Monreal D, González D, Garin-Bastuji B, Díaz R, et al. Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. Clin Diagn Lab Immunol. 2005; 12:141–151. https://doi.org/10.1128/CDLI.12.1.141-151.2005 PMID: 15642999

41. Ducrotoy MJ, Conde-Álvarez R, Blasco JM, Moriyón I. A review of the basis of the immunological diagnosis of ruminant brucellosis. Vet Immunol Immunopathol. 2027; 171:81–102.

42. Ministerio de Agricultura y Ganadería. Evaluación del Programa de Salud Animal. Programa Nacional de Salud Animal MAG/BID, San José, Costa Rica. 1979. http://www.mag.go.cr/bibliotecavirtual/L70-5002.pdf.

43. Programa de Desarrollo Ganadero y Salud Animal -PROGASA. Dirección de Salud y Producción Pecuaria de Costa Rica, San José Costa Rica. 1983; http://www.mag.go.cr/bibliotecavirtual/Memoria-1983.pdf.

44. Programa de Desarrollo Ganadero y Salud Animal -PROGASA. Contrato Préstamo N˚ 439/SF-CR suscrito entre el gobierno de Costa Rica y el Banco Interamericano de Desarrollo para un programa ganadero y de sanidad animal. Ley N˚ 7060. La Gaceta N˚65, San José, Costa Rica. 1987. http://www.senasa.go.cr/sitioanterior/Documentos/legislacion/Ley%207060.pdf

45. Ministerio de Agricultura y Ganadería. Préstamo del Banco Interamericano de Desarrollo para un programa de salud animal. Ley N˚ 6240. La Gaceta N˚198, San José, Costa Rica. 1978. http://www.asamblea.go.cr/Centro_de_informacion/Consultas_SIL/Pginas/Leyes.aspx.

46. León EA, De-García A. Evaluación de Servicios Veterinarios de Costa Rica mediante herramienta PVS. World Organization for Animal Health- OIE. 2007. http://www.senasa.go.cr/senasa/sitio/files/060711095741.pdf.

47. OIE-WAHIS interface. Selected disease. Brucellosis (Brucella abortus) Costa Rica 2006–2015. World Animal Health Information Database Version 1. 2017. https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Vaccination.

48. Crawford RP, Hidalgo RJ. Bovine Brucellosis: An international Symosium. Texas A&M University Press. 1rst ed. Texas, USA. 1977.

49. Barrio MB, Grilló MJ, Muñoz PM, Jaques I, González D, De Miguel MJ et al. Rough mutants defective in core and O-polysaccharide synthesis and export induce antibodies reacting in an indirect ELISA with smooth lipopolysaccharide and are less effective than Rev1 vaccine against *Brucella melitensis* infection in sheep. Vaccine. 2009; 27:1741–1749. https://doi.org/10.1016/j.vaccine.2009.01.025 PMID: 19186196

50. Nielsen K, Smith P, Yu W, Nicoletti P, Elzer P, Robles C, et al. Towards single screening tests for brucellosis. Rev Sci Tech Off Int Epiz. 2005; 24:1027–1037.

51. Moriyón I, Grilló MJ, Monreal D, González D, Marín C, López-Goñi I, Mainar-Jaime RC, Moreno E, Blasco JM. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res. 2004, 35:1–38. https://doi.org/10.1051/vetres:2003037 PMID: 15099501

52. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Frontiers Microbiol. 2014;1–18. https://doi.org/10.3389/fmicb.2014.00213 PMID: 24860561

53. Nicoletti P. The effects of adult cattle vaccination with Strain 19 on the incidence of brucellosis in dairy herds in Florida and Puerto Rico. Proc Annu Meet US Anum Health Assoc. 1979; 83:75–80.

54. Calderón-Range A, Angulo-Maza LA, Tique-Salleg VP, Rodríguez-Rodríguez V.C, Ensuncho-Hoyos CF. Seroprevalencia de brucelosis bovina en dos localidades del caribe colombiano. Orinoquia (Colombia). 2015, 19:203–209.

55. Borba MR, Stevenson MA, Gonçalves VS, Neto JS, Ferreira F, Amaku M, Telles EO, Santana SS, Ferreira JC, Lôbo JR, Figueiredo VC, Dias RA. Prevalence and risk-mapping of bovine brucellosis in Maranhão State, Brazil. Prev Vet Med. 2013, 110:169–176. https://doi.org/10.1016/j.prevetmed.2012.11.013 PMID: 23218657

56. Poulsen Keith P., Hutchins Frank T., McNulty Chase M., Tremblay Marlène, Zabala Carmen, Barragan Veronica, Lopez Luis, Trueba Gabriel, Bethel Jeffrey W. Brucellosis in dairy cattle and goats in northern Ecuador. Am J Trop Med Hyg. 2014, 90: 712–715. https://doi.org/10.4269/ajtmh.13-0362 PMID: 24591429

57. United Nation Development Program- UNDP. Human development report. 2010. The real wealth of nations: Pathways to Human Development. Consolidated Graphics. Washington DC, USA. http://hdr.undp.org/en/content/human-development-report-2010.

58. Aguilar Bulgarelli O. La Constitución de 1949. Antecedentes y proyecciones. San José, Costa Rica: Editorial Costa Rica. 1974;188.

59. Sáenz M, Bermúdez JL, Acosta M. Universal coverage in a middle income country: Costa Rica. WHO World Health Report. 2010. http://www.who.int/healthsystems/topics/financing/healthreport/CostaRicaNo11.pdf.

Chapter 2: Brucellosis in mammals of Costa Rica

Brucellosis has been an endemic disease of cattle and humans in Costa Rica since the beginning of the 20th century. However, brucellosis in sheep, goats, pigs, water buffaloes, horses,and cetaceans, has not been reported in the country. In this work, published during 2017 (Hernández-Mora *et al*., 2017b), we have performed a brucellosis survey in these host mammal species from 1999-2016.

The individual brucellosis seroprevalence in goat and sheep flocks was 0.98% and 0.7%, respectively, with no *Brucella* isolation using commercial *Brucella* medium as well as CITA and Farrell culture media. Antibodies against *Brucella* .were not detected in feral or domestic pigs. This data suggest the absence of *B. melitensis, B.suis,* and *B. ovis* in these animal species in Costa Rica. This information is also supported by the lack of isolations from humans infected with this *Brucella* species. In horses, the individual seroprevalence of brucellosis and water buffaloes were estimated at 6.5% and 21.7%, respectively, with no *Brucella* isolation.

Six cetacean species including striped dolphin (*S. coeruleoalba*), bottlenose dolphin (*Tursiops truncatus*), spotted dolphin (*S. attenuata*), common dolphin (*Delphinus delphis*), rough-toothed dolphin (*Steno bredanensis*), and Cuvier beaked whale (*Ziphius cavirostris*), showed positive reactions against *Brucella* antigens in RBT, cELISA, iELISA and inmuchromatographic rapid test (B-*Brucella* Rapid Test). *B. ceti* was isolated in 70% (n= 29) of striped dolphins (*S. coeruleoalba*) using culture mentioned above. A steady increase in the diagnosis of human brucellosis cases was observed. Considering the prevalence of brucellosis in the various host mammals of Costa Rica, different measures are recommended.

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RESEARCH ARTICLE

Brucellosis in mammals of Costa Rica: An epidemiological survey

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Abstract

Brucellosis has been an endemic disease of cattle and humans in Costa Rica since the beginning of XX century. However, brucellosis in sheep, goats, pigs, water buffaloes, horses and cetaceans, has not been reported in the country. We have performed a brucellosis survey in these host mammal species, from 1999–2016. In addition, we have documented the number of human brucellosis reported cases, from 2003–2016. The brucellosis seroprevalence in goat and sheep herds was 0.98% and 0.7% respectively, with no *Brucella* isolation. Antibodies against *Brucella* were not detected in feral or domestic pigs. Likewise, brucellosis seroprevalence in horse and water buffalo farms was estimated in 6.5% and 21.7%, respectively, with no *Brucella* isolation. Six cetacean species showed positive reactions against *Brucella* antigens, and *B. ceti* was isolated in 70% (n = 29) of striped dolphins (*Stenella coeruleoalba*). A steady increase in the diagnosis of human brucellosis cases was observed. Taking into account the prevalence of brucellosis in the various host mammals of Costa Rica, different measures are recommended.

Introduction

Costa Rica (CR) is a Central American country with a surface area of 51100 Km^2 and a human population close to five million. Most of the inhabitants are located in the Central Valley, flanked by the volcanic chain and the mountain range. The country is divided in six administrative areas: Chorotega, Central Pacific, Brunca, Central, Northern Huetar and Caribbean Huetar. CR has two ocean fronts: the Pacific Ocean and the Caribbean Sea. In addition, there is the Cocos Island located in the Pacific Ocean [1].

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Bovine brucellosis is a significant problem in CR [2] and human brucellosis has been endemic since the beginning of last century [3,4]. However, the presence of *Brucella* organisms in sheep, goats, pigs, water buffaloes, horses and cetaceans and the impact that brucellosis has in these animals has been barely explored in CR [5]. Moreover, very little information in the number of human cases arriving to the CR health centers has been recorded.

Up to now, five species of *Brucella* have been isolated in CR: *Brucella abortus* (biotypes 1, 2 and 3) in cattle and humans, *Brucella suis* (biotype 1) in domestic swine, *Brucella canis* in dogs, *Brucella neotomae* in humans and *Brucella ceti* (dolphin type) in dolphins [2,5–7]. *B. melitensis* and *B. ovis* have not been reported in CR.

In this work we describe the distribution and the prevalence of brucellosis in different mammal species and the cumulative number of human brucellosis cases in CR. We discuss our findings in concordance to the conditions and measures carried out in the country and the zoonotic potential. Brucellosis in cattle is not reported here, since it has been thoroughly described in the accompanying manuscript [2].

Materials and methods

Serum samples

Sheep and goats. The total number of sheep and goats in CR is close to 12358 and 4626, distributed in about 164 and 271 herds, respectively (Table 1). For sampling purposes CR was divided in six administrative areas by the Costa Rican National Animal Health Service (CR-NAHS) of the Ministry of Agriculture and Livestock Management: Chorotega, Central Pacific, Brunca, Central, Northern Huetar and Caribbean Huetar. Herds from each species were divided in three sections. For sheep the first section "A" included 6200 animals in 22 herds of broodstock farms with $\geq$150 individuals; section "B" were 3577 animals in 37 herds from farms with eventual broodstock activities, with populations ranging from 149–60 animals; and section "C" were 2691 in 105 herds for productive farms with population $\leq$59 animals. For goats, we used the same criteria used for sheep. Section "A" included 1406 goats in 13 herds; section "B" were 1603 distributed in 14 herds; and section "C" were 1617 from 137 farms. Seventy-eight caprine and 139 ovine herds, corresponding to 2013 and 1668 animals respectively, were sampled nationwide as part of the surveillance program, during 2014–2016.

Water buffalos. The estimated water buffalo population in the country corresponds to 13000 animals, distributed in about 100 herds. About 70% of the water buffalo farms are devoted to mozzarella cheese production. The rest, are dedicated to meat production, leather industry or as wild fauna in zoological parks [8,9]. A total of 2586 animal blood samples, corresponding to 46 herds located in the six administrative areas were taken during 2014–2016.

Pigs. The estimated number of domestic swine in continental CR is close to 435500, most of them under intensive management farms, located in the Northern Huetar and Central Pacific regions [10]. A total of 2256 pigs from eight herds were sampled from 2014–2016. In addition, 160 blood samples collected at the slaughter house in the Central region were also studied. As part of the control of Wildlife Service of National Parks of CR, 58 feral pigs were sampled in the East side of Cocos Island National Park (23.85 km^2) located in the West Pacific Ocean (5˚31′08″N 87˚04′18″O), during 1998–2000. This region included close to half of the area. The sampling spots were chosen randomly and their location estimated on the basis of recognized pathways and reference points already established in maps used by the National Park rangers. Ages were estimated on the basis of size, weight, secondary sexual organ development, hair distribution, hoof size and dentition. Samples were analyzed at the CR-NAHS Laboratory or at the Veterinary Medicine School, National University, Heredia, CR.

Table 1. Numbers of ovine and caprine herds and numbers of animals by geographical region in Costa Rica (2015).

Region	Ovine		Caprine	
	Herd	Animals	Herd	Animals
1. Northern Huetar	36	2440	39	2077
2. Central	59	4295	117	1973
3. Brunca	21	1246	41	128
4. Chorotega	28	2792	22	312
5. Caribbean Huetar	9	637	28	79
6. Central Pacific	11	948	24	57
Total	**164**	**12358**	**271**	**4626**

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Horses. The estimated population of horses in CR is close to 67000 in about 20000 farms [10]. In CR there is little tradition for eating horse meat. Therefore, most of the equines are devoted to sports, recreation and work. A total of 1270 horse blood samples from 215 farms located in the six administrative areas were taken during 2014–2016.

Cetaceans. Thirty cetacean species have been documented in Costa Rican waters, representing about 36% of the 83 species known worldwide [11]. From 2004–2016, 115 individuals from sixteen species were reported stranded in the Costa Rican shorelines (Table 2). Cetacean blood samples were taken at the stranding sites. After death, the animals were transported to the Veterinary School of the National University of CR, for necropsy and bacteriological studies.

Humans. Brucellosis in humans has been documented in CR since 1915 [3,4]. A survey for human brucellosis from 2003–2016 was carried out at the laboratories of Public Health Services (CCSS) of CR. In addition, a total of 250 abattoir workers were monitored for antibodies against *Brucella* antigens, from 2015–2016. All human case reports and bacteriology were received at the National Reference Bacteriology Laboratory at the Costa Rican Institute for Research and Training in Nutrition and Health (INCIENSA), for confirmation.

Table 2. Number of cetaceans stranded in Costa Rica from January 2004 to September 2016.

Common name	Specie	Number of animals
Striped dolphin	*Stenella coeruleoalba*	51
Bottlenose dolphin	*Tursiops truncatus*	10
Spotted dolphin	*Stenella attenuata*	8
Humpback whale	*Megaptera novaengliae*	8
False killer whale	*Pseudorca crassidens*	6
Spinner dolphin	*Stenella longirostris*	4
Rough tooth dolphin	*Steno bredanensis*	4
Dwarf sperm whale	*Kogia sima*	4
Cuvier beaked whale	*Ziphius cavirostris*	3
Risso's dolphin	*Grampus griseus*	2
Pilot whale	*Globicephala macrorhynchus*	2
Sperm whale	*Physeter machrocephalus*	2
Common dolphin	*Delphinus delphis*	1
Beaked whale	*Mesoplodon* spp.	1
Beaked whale	*Mesoplodon* spp.	1
Sei Whale	*Balaenoptera borealis*	1
Unknown species*	Unknown	7
Total		**115**

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Information collected and blood animal samples

Relevant data concerning geographical localization, size of the farm, management and characteristics of the herds or individual animals were collected. The information also included veterinary services, reproductive parameters, history of abortion/stillbirth and the presence of other domestic and wildlife species in the farms. Breeds and identifications were registered.

Blood samples were collected with syringes or a sterile vacutainers with Z serum clot activator (Vacutainer System, Greiner Bio-one), transported under refrigeration, and sera obtained by centrifugation. Each sample received a consecutive number. Analyses of the sera were performed within 24–72 hours after collection at the CR-NAHS Brucellosis Serology Laboratory or at the Immunology Laboratory at the School of Veterinary Medicine, National University, Heredia, CR. Humans blood samples were sent to the National Reference Bacteriology Laboratory (INCIENSA) for confirmation.

Serological tests

Rose Bengal test (RBT) (ID-VET, France), indirect protein A/G ELISA (iELISA) (ID-VET, France) and competitive ELISA (cELISA) (Svanovir, SVANOVA, Sweden) and fluorescent polarization assay (FPA) (Sentry 100 instrument, Diachemix, United States) were used as diagnostic tools, as described elsewhere [12–14]. For the standardization of small ruminant brucellosis diagnostic tests, positive and negative sera from sheep and goats were obtained from Spain and Mexico respectively. Twenty sera from *B. melitensis* biotype 1 culture positive sheep, twenty sera from *B. melitensis* biotype 1 culture positive goats, twenty- one sera from non-vaccinated negative sheep and twenty-one sera from non-vaccinated negative goats were obtained and used for validation as previously described [14,15]. In Costa Rica sheep and goats are not vaccinated. Therefore, the specificity of RBT in the absence of vaccination has been estimated to be ~100%; likewise, under these conditions the sensitivity has also been estimated in ~100% [14]. The cut off values for iELISA, cELISA and FPA in sheep and goats were 120% S/P, 30% positivity and 20 milipolarization units, respectively. Since standardized diagnostic tests for water buffalo brucellosis are not available, RBT, iELISA and cELISA, including the cut-off values, were used as reported for cattle [16]. Dolphin sera were collected and tested in RBT, iELISA and cELISA as described before [17]. For swine, modified RBT, iELISA and cELISA was used as described elsewhere [18]. Likewise, for horses, background levels for the same tests were estimated with sera from 20 healthy horses with no signs of brucellosis and with no contact with cattle or small ruminants. All animal sera samples were initially screened by RBT and then by iELISA, cELISA and FPA, following the procedures described elsewhere [13,15,17]. For humans, RBT and microagglutination in 96/well round bottom plates were used for screening, as described before [19].

Culture conditions and *Brucella* identification

Bacteriological cultures and identification of *Brucella* isolates were performed as described in the accompanying paper [2]. Briefly, various reference *Brucella* species were used as positive controls for genetic and bacteriological identification of samples [2]. According to the National Brucellosis Control Program of the CR-NAHS, seropositive sheep, goats, buffalos or pigs are selected for obligatory culling and pathological examination [20]. Necropsies were carried out at the Pathology Department in the Veterinary School of the National University, CR. Animal samples, included milk and other secretions such as vaginal swabs, semen and cerebrospinal fluid. Tissues samples included reproductive organs lymph nodes, spleen, kidney, liver and brain. In some cases aborted fetuses were also collected and sampled. Cultures were performed at the CR-NAHS or at the Bacteriology Laboratory of the Veterinary School. Non-selective

and selective media, including blood agar and Columbia agar, supplemented with 5% of dextrose and sheep blood as well as Modified *Brucella* Selective Supplement Oxoid® (SR0209) and CITA medium, under 10% CO2 atmosphere, were used [21]. The selected bacterial colonies were subjected to Gram staining, agglutination with acriflavine and acridine orange dyes, tested for urease and oxidase activity, citrate utilization, nitrate reduction, H_2S production, growth in the presence of CO_2, thionin (20 μg/mL) and basic fuchsin (20 μg/mL) and uptake of crystal violet, according to described procedures [12].

Brucella DNA samples from each isolate and control strains were extracted with DNeasy Blood & Tissue kit from QIAGEN, and stored at -80˚C until used. Identification of *Brucella* species was performed by bruce-ladder, single-nucleotide polymorphisms and MLVA16 analysis following standard procedures [22–25]. *Brucella* control strains were used for validation. The profiles were analyzed following standardized procedures (http://mlva.u-psud.fr/brucella/) and thereafter entered in the database MLVA-NET (http://microbesgenotyping.i2bc.paris-saclay.fr/).

Ethical considerations

Sampling of domestic and wildlife animals is part of the National Brucellosis Control Program of the CR-NAHS [20] and the Law of Reportable Infectious Diseases of the Ministry of Health of CR [26]. Dolphin serum samples were taken from stranded dolphins following the procedures described before [27]. Protocols for the use of animal serum samples were revised and approved by the "Comité Institucional para el Cuido y Uso de los Animales de la Universidad de CR" (CICUA 057–16366), and "Comité Institucional para el Cuido y Uso de los Animales" of the National University, Heredia, CR (SIA 0545–15), and in agreement with the corresponding law "Ley de Bienestar de los Animales", CR (Ley 7451 on Animal Welfare), and according to the "International Convention for the Protection of Animals" endorsed by Costa Rican Veterinary General Law on the CR-NAHS (Ley 8495).

Human samples were handled by the authorities of the Public Health Service of CR (Social Security Services CCSS and Ministry of Health) and then submitted to National Reference Bacteriology Laboratory at INCIENSA for diagnostic confirmation. In this institution the samples were handled according to the INCIENSA ethical committee specifications and the agreement between INCIENSA and SENASA (Oficio 16-06-2013). Upon registration to the Medical Health Centre, all patients were informed regarding the purpose of the work and provided the corresponding written consents according to the respective Law (Ley 9234, La Gaceta 79). All samples were taken following the procedures dictated by the Costa Rican National Health system (Ley 9234, La Gaceta 79), and the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, General Assembly, Seoul, October 2008), regarding blood samples.

Statistics

For sheep and goats, the sample sizes were determined according to Cannon and Roe [28] using Win Episcope 2.0 software [29], with an expected brucellosis prevalence of 0.6% for sheep and 0.7% for goats, with a confidence level of 95%. This estimation included 500 sheep and 413 goats to be sampled, distributed in 10 and 13 herds respectively, sorted by region as described above. Herd selection was chosen assuming that the management and biosecurity actions, regarding these two ruminants, are similar in CR. Herds were chosen randomly from sections "A" and "B", which are the broodstock herds, and largely reflected the sanitary conditions of section "C". From each herd selected, a proportional sample population was calculated based on the clinical signs compatible with brucellosis, with a confident level of 95% and an

expected prevalence of 5%, according with Cannon and Roe [28]. In addition to the random sampling, and in order to increase the probability of positive results, a biased priority was given to females with a history of abortions, weak or stillborn births, placenta retention, or with conditions that rendered individuals more susceptible to any infection, such as low body condition and pale mucous membranes. If the total number of animals defined for the herd was not covered with these specifications, random adult females were selected. Breeding rams in each farm were also examined for the detection of orchitis, epididymitis and reproductive problems. For feral pigs, the size of the sample was selected for an expected maximum population of 500 pigs distributed in the entire island, with a 95% confidence level and a tentative prevalence of 5%. The rest of the animal species sampled corresponded to the surveillance performed as part of the National Brucellosis Control Program of the CR-NAHS and according to the OIE specifications [13].

Results

Sheep and goats

Most of the ovine and caprine herds are located in the lowlands of CR (below 1000 m) and are mainly devoted to dairy (caprine) and meat (ovine) production (Table 1). The sampling procedure was carried out at the indicated regions, from 2015–2016 (Fig 1). From a total of 510 sheep sampled, corresponding to 10 herds, eleven animals (five herds) were RBT positive and five cELISA positive. None of the RBT positive animals were positive in iELISA, cELISA or FPA. Likewise, from a total of 424 goats, covering close to 10% of the Costa Rican population, only five animals demonstrated positive reactions in RBT. However, none of these RBT positive samples resulted positive in iELISA, cELISA or FPA. According to these results, the estimated brucellosis RBT prevalence values for goat and sheep herds were 0.98% and 0.7%, respectively. The RBT positive animals were culled and tested for the presence of *Brucella* spp. in lymph nodes, spleen, liver, placenta, mammary gland, milk and fetus organs. All cultured samples tested negative for *Brucella* spp. Epidemiological and clinical surveys of the sheep and goat populations and the corresponding farms did not demonstrate clinical brucellosis.

From the 3681 ovine and caprine routinely sampled at the CR-NAHS laboratories for regular diagnosis, only one caprine was classified as positive in RBT and iELISA. The animal was slaughter and their various organs tested for the presence of *Brucella*, with negative results. Clinical disease compatible with *B. ovis* infection was not detected in rams. Likewise, this bacterium was not isolated from semen samples. Taken together these data, the "positive" RBT reactions were estimated as unspecific and the presence of brucellosis in ovine and caprine herds ruled out.

Water buffalos

Most water buffalos are located in the low lands, since they require fresh water habitats for subsistence. From a total of 2586 samples distributed in 46 herds, collected from 2014–2016 (Fig 1), 17 animals tested positive in RBT, 38 in cELISA and 77 in the iELISA. The total number of herds positive in these three assays was ten. All RBT positive samples were also positive in iELISA and cELISA; and all samples positive for cELISA were also positive in iELISA. FPA was not performed. In spite of the efforts, *Brucella* organisms were not isolated from vaginal swabs, dairy products, placental tissues, fetuses, testes, lymph nodes, mammary gland, blood, spleen or liver of the culled seropositive animals. However, due to the reported clinical characteristics and the testimonies of persistent abortions and positive serological reactions, *Brucella* infections were suspected. Moreover, it is likely that *B. abortus* constitutes an infection source for

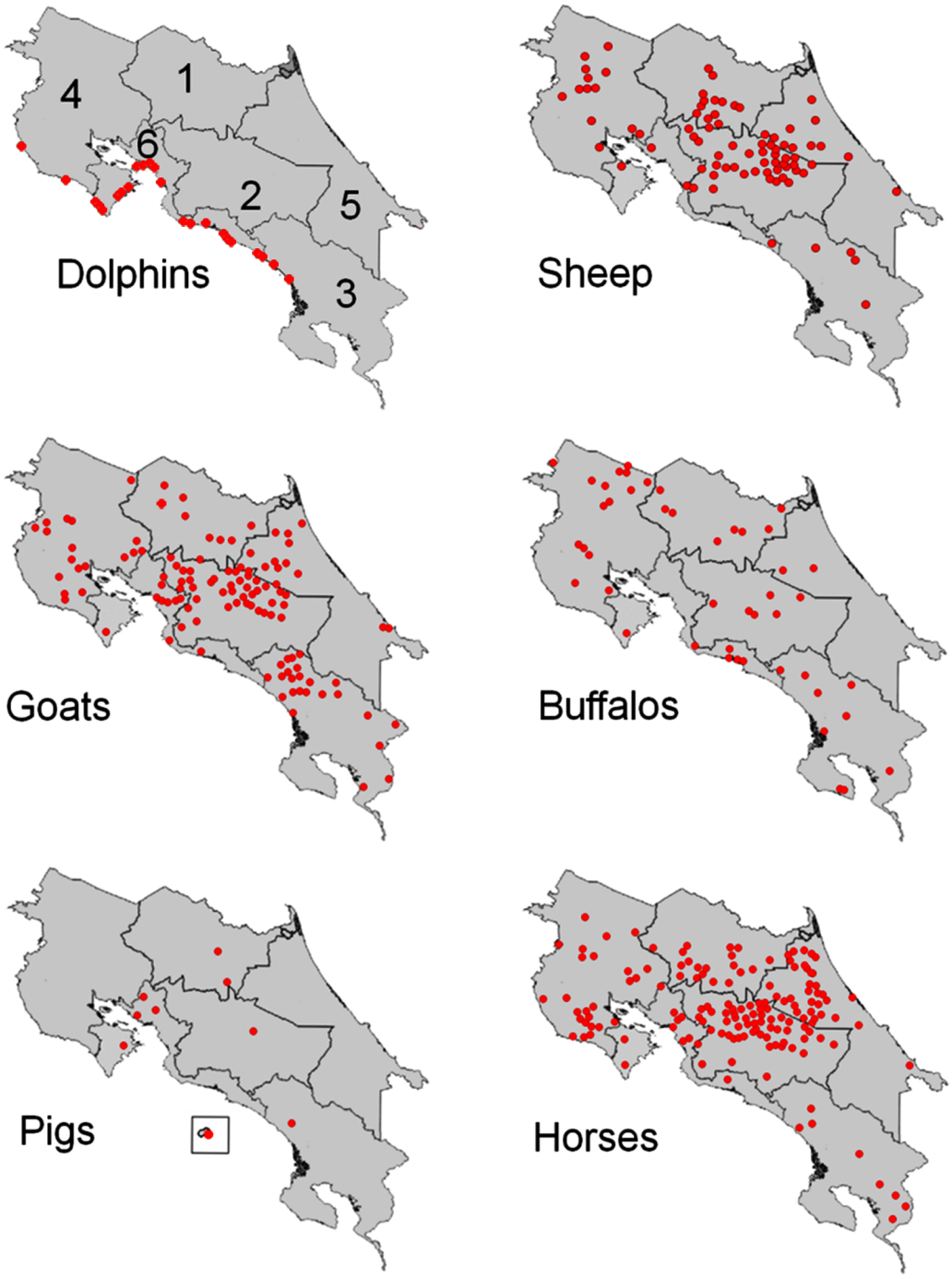

Fig 1. Sampling of animal stocks, in the six regions of CR. The epidemiological regions are as follow: 1, Northern Huetar; 2, Central; 3, Brunca; 4, Chorotega; 5, Caribbean Huetar; 6, Central Pacific. Each red dot represents an animal stock facility.

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water buffaloes, since bovine brucellosis caused by this *Brucella* specie is highly prevalent in CR [2].

Pigs

From the number of herds studied and the samples obtained at the slaughter house (Fig 1), only two pigs of one herd were RBT positive. From these, only one pig was also positive in iELISA and cELISA. The FPA assay was not performed. Positive animals were culled and different tissues were cultured for the presence of *Brucella*, with negative results. In addition, tissues of aborted fetuses in some farms were also tested for the presence of *Brucella*, all with negative results. Likewise, positive serological reactions were not detected in the feral pig population in the Cocos Island. Histopathological examination of the liver in the feral swine sample showed chronic inflammation in 84% of the cases, while 20% had multifocal granulomatous inflammation with eosinophilic infiltration, probably related to the presence of parasite nematode *Stephanurus dentatus*, but not *Brucella*. Taken together these data, the positive serological reactions were estimated as non-specific.

Horses

Most horses are located in North Huetar, Chorotega and the northern part of the Caribbean Huetar regions of CR. Therefore, most of the samples are from these areas (Fig 1). From the total number of farms studied 14 (6.5%) had seropositive animals, including 18 horses positive in RBT; from these, only four were also positive in both iELISA and cELISA. In spite of the efforts, *Brucella* was not isolated from horses. However, it is likely that *B. abortus* is a source of infection in horses, since many of these animals are in close contact with infected bovines in CR. In addition, some clinical features such as fistulous withers and nonspecific lameness due to joint infection, have occasionally been observed in horses.

Cetaceans

Cetacean brucellosis in Costa Rican was investigated from 2004–2016. RBT and iELISA, designed for cetacean diagnosis, were positive in 54 (46.9%) individuals from six different species. They included 38 striped dolphins (*Stenella coeruleoalba*), one bottlenose dolphins (*Tursiops truncatus*), one spotted dolphins (*S. attenuata*), one common dolphin (*Delphinus delphis*), one rough toothed dolphin (*Steno bredanensis*), and one Cuvier beaked whale (*Ziphius cavirostris*). However, striped dolphin (*S. coeruleoalba)* remains as the only cetacean specie from which *B. ceti* has been isolated from different organs in CR.

Strong positive RBT and iELISA reactions were obtained in sera from 37 out of 38 striped dolphins stranded at the Pacific coast of CR (Fig 1). Thirty-seven out of 38 striped dolphins, stranded alive. At the time of stranding, all live animals presented neurological symptoms such as tremors, buoyancy difficulties, weakness, seizures and locomotion problems. With exception of two dolphins (one seropositive and one seronegative), all other *S. coeruleoalba* dolphins displayed neurobrucellosis, following previous diagnosis [27]. All of them died at the stranding site within hours after the event. Necropsy was performed in all cases and *B. ceti* was isolated from the cerebrospinal fluid of 29 individuals (70%). In addition, *B. ceti* was also present in placenta, umbilical cord, amniotic and allantoic fluids, multiple fetal organs, milk, cardiac valve, atlanto occipital joint fluid, lung and lung nematodes (*Halocercus spp*.) [6,27,30,31]. All *B. ceti* isolates belonged to the MLVA16 type P [32], corresponding to the Pacific Ocean (data accessible at: http://microbesgenotyping.i2bc.paris-saclay.fr/ [and the following entries: public databases, Brucella v4_1, bmarCR+number, years 2006–2014]).

Humans

According with the Costa Rican National Reference Bacteriology Laboratory (INCIENSA), the number of human cases reported by the health centers over 12 year (2003–2015) period

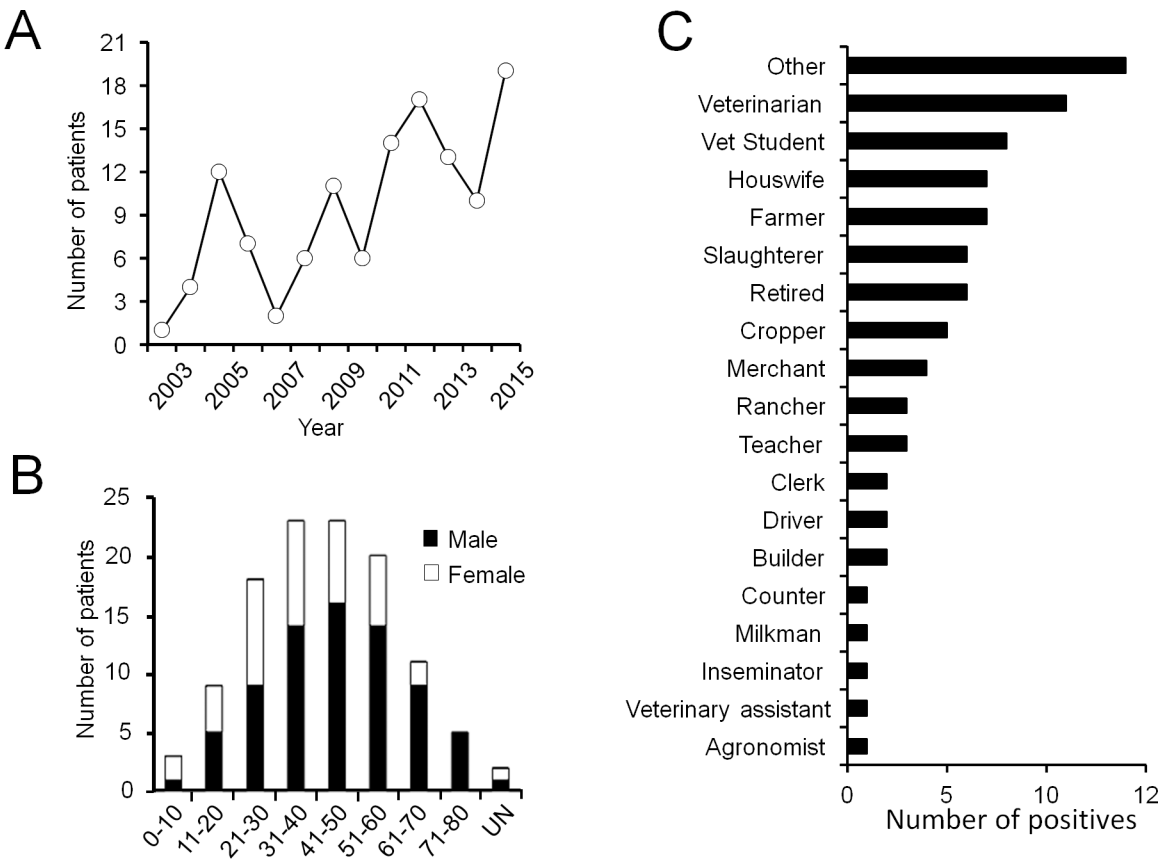

Fig 2. Occurrence human brucellosis cases in CR from 2003–2015. (A) Number of human brucellosis cases diagnosed per year in CR for the period. All cases recorded were due to *B. abortus*. (B) Distribution per age and proportion of male and female brucellosis cases in CR, diagnosed for the period. (C) Proportion of 250 seropositive abattoir workers from 2015 to 2016.

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corresponded to 124 patients (Fig 2A): fifty one were from the Central region 37 from the Caribbean Huetar region and 36 cases from all other regions. Male and female patients represented 79 and 41 cases (Fig 2B), respectively, with ages ranging between 8–76 year-old, with a large proportion of veterinarians, farmers and slaughter plant workers (Fig 2C). From a total of the 250 abattoir workers only three presented high antibody titers (>1/160) compatible with an active brucellosis. With the exception of two *B. neotomae* isolates [7], all other human brucellosis cases corresponded to *B. abortus*.

Discussion

For most of the history of CR, sheep and goats have been raised in very low numbers and the dairy products and meat of these animals barely consumed [33]. Until 1975 the number of goats and sheep in the country were close to 1000 animals, all together [33]. However, in the nineties the population of these small ruminants started to increase. Already, in the first decade of the XXI century, the numbers of goats and sheep were close to 5000 and 3000, respectively [34]. With the enhanced acceptance of ovine and caprine dairy and meat products, the emergent industries for small ruminants have increased. Indeed, the numbers of goats and sheep have augmented almost three fold (12852) and twelve fold (35800) [10], respectively.

An epidemiological survey for caprine and ovine brucellosis was performed from 2015–2016. Although we detected a minor number of RBT positive reactions in small ruminants, they were regarded as false positives. In spite of the high specificity and sensitivity displayed by the RBT under controlled conditions with a limited number of known sera, this assay is not perfect and some non-specific reactions are expected to occur under field conditions. Therefore, exhaustive clinical, pathological and epidemiological investigations in the serologically positive sheep and goats were carried out, all rendering negative results for the presence of *Brucella* infections. Bacteria displaying similar antigenic determinants as smooth brucellae may be the source of false positive reactions [35]. In addition, positive serological reactions due to *B. abortus* infections cannot be ruled out, since this bacterium is highly prevalent in CR [2]. However, we did not isolate *B, abortus* or any other brucellae from the tissues of goats and sheep. Although *B. melitensis* may be present in some Central American countries [36], this bacterium has never been isolated in animals or humans in CR [5, 36]. Following this, it is important to keep these small ruminants free of brucellosis, restricting the importation of animals and semen from *B. melitensis* free countries.

Similar to goats and sheep, the number of water buffalos has steadily increased in CR during the last ten years. In 2006 the number of water buffalos in CR was close to 615 animals [37]; in ten years the population has increased twenty fold, most of them devoted to the production of dairy products. Taking into account the persistent positive serological reactions, their close association of water buffalo with *B. abortus* infected cattle and the reported cases of abortions compatible with clinical disease; we believe that some water buffalo populations are infected with *Brucella* in CR. Moreover, a significant number of the CR water buffalo population originates from Trinidad-Tobago, country endemic for water buffalo brucellosis [8, 38]. The fact that we did not isolate *Brucella* from water buffalos may be related to the natural resistance of these animals to brucellosis in relation to other bovines [39].

B. suis was isolated from a domestic pig in the Central region of CR in 1984 [5]. Since then, the bacterium has not been isolated from boars, in spite of the efforts. In CR pigs seldom roam freely around the houses and most animals are confined to intensive management facilities, under good health conditions. Moreover, with the exception of Cocos Island, no feral pigs are present in the CR territory. Since no clinical or epidemiological surveys indicate swine brucellosis, it is unlikely that *B. suis* is currently infecting pigs in the country.

Horses are not primary *Brucella* hosts and commonly they do not have the ability to transmit the bacterium to other animals or humans. Therefore, horses are not of epidemiological relevance in keeping the bacterium life cycle; however, these animals are sentinels for the presence of *Brucella* in other animals, mainly in cattle. Like humans, they become infected by contact with abortions or with infected cattle, and display a wide range of clinical manifestations including articular swelling and general weakness [40]. The fact that close to 18 horses displayed recurrent positive reactions against *Brucella*, may be an indication of the high seroprevalence of *Brucella* infections in cattle [2], including water buffalo.

B. ceti infections in dolphins stranded in the CR Pacific coast were detected for the first time in 2004 [6]. A total of 115 stranding events from at least 16 different species of cetaceans have been recorded in CR seashores from 2004–2016 (Table 2). From these, six species displayed positive serological reactions. However, *B. ceti* active infections have been only documented in striped dolphins from the Pacific Ocean of CR. All *B. ceti* isolates belong to the same MLVA16 type P. This bacterial group corresponds to a particular cluster distinct from other *B. ceti* strains isolated in various oceanic latitudes, and it is a hallmark for *S. coeruleoalba* infections in the Eastern Tropical Pacific [32]. Moreover, all the 29 dolphin cases in which *B. ceti* organisms were isolated suffered from neurobrucellosis [27]. It seems, therefore, that this dolphin specie is highly susceptible to *B. ceti* and that many of the stranding events were due to

brain infections, as recorded in other latitudes [41]. The surveillance of cetacean brucellosis in Central American littorals requires attention. This is mandatory to understand the impact that brucellosis has in the Eastern Tropical Pacific marine mammal populations and to ensure prevention measures for potential human and animal infections [42].

In a previous study in the Central region (Cartago, CR), in which 71% of the human population consumed unpasteurized dairy products; an overall seroprevalence of 0.87% was detected [19]. However, no statistically significant association was found between unpasteurized milk consumption and the presence of antibodies against *Brucella* organisms. Here, we reported a steady increase in the number of human brucellosis cases during a lapse of 12 years. Whether the steady increase of human brucellosis reports corresponded to improved diagnosis or to intensification in the number of cases, is not known. The number of human brucellosis cases due to *B*. *abortus* is consistent with the high prevalence of bovine brucellosis in CR, and the absence of *B*. *melitensis* in sheep and goats, and *B*. *suis* in pigs, two *Brucella* species that display a higher zoonotic potential than former bacteria [43]. In CR there are other zoonotic brucellae such as *B*. *neotomae* [7] and *B*. *canis* [44], which were not considered in this study. Nevertheless, a careful identification of strains is required, even with those *Brucella* species that are considered of low zoonotic risk.

From the epidemiological perspective, it seems that the population of sheep, goats and pigs in CR are free of *B*. *melitensis* infections. This seems to be also the case for *B*. *ovis* in rams and *B*. *suis* for pigs. Consequently, humans are also free of these bacterial species. However, with the increasing number of small ruminant species in the country the risk of *Brucella* infections arriving from other latitudes requires permanent surveillance, improved management and sensitive and specific diagnostic tools.

Conclusions

1. Domestic ovine, caprine and swine herds are free of brucellosis in CR.
2. The presence of *Brucella* infections in water buffaloes is highly suspected in CR.
3. The presence of *B*. *abortus* infections in horses is highly suspected in CR.
4. Striped dolphins from the Pacific Ocean of CR are the main host of *B*. *ceti* cluster type P.
5. The main clinical symptom found in striped dolphins corresponded to neurobrucellosis.
6. Detection of human infections, due to *B*. *abortus*, has steadily increased since 2005 in CR.
7. Estimating the presence of *Brucella* infections in different hosts inhabiting CR is relevant for understanding the impact that brucellosis has in the country and for prevention measures.

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References

1. Vargas-Ulate T. Geografía de Costa Rica. Editorial Tecnológica, Cartago, Costa Rica. 2004; 124.
2. Hernández-Mora G, Ruiz-Villalobos N, Bonilla-Montoya R, Romero-Zúñiga JJ, Jiménez-Arias J, Barquero-Calvo E, et al. Epidemiology of bovine brucellosis in Costa Rica: lessons learned from failures in the control of the disease. Submitted to Plos One. 2017 (accompanying manuscript).
3. Quirós D. El primer caso de fiebre de Bruce en Costa Rica. Gaceta Médica de Costa Rica. 1915; 11:241–244.
4. Zeledón-Alvarado J. Primera historia clínica de brucelosis humana en Costa Rica. Rev. Med. 1940; 72:153–167.
5. Sequeira A, Campos E, Mendoza L, San-Román MA, Moreno E. Identificación de especies y biotipos de *Brucella* aisladas en Costa Rica. Turrialb. 1984; 34:525–526.
6. Hernández-Mora G, González-Barrientos R, Morales JA, Chaves-Olarte E, Guzmán-Verri C, Baquero-Calvo E, et al. Neurobrucellosis in stranded dolphins, Costa Rica. Emerg Infect Dis. 2008; 14:1429–1433.

7. Suárez-Esquivel M, Ruiz-Villalobos N, Jiménez-Rojas C, Barquero-Calvo E, Chacón-Díaz C, Víquez-Ruiz E, et al. Human neurobrucellosis caused by *Brucella neotomae*. Emerg. Infect. Dis. 2017; 23:997–1000.
8. Rosales-Rodríguez R. Situación del búfalo de agua en Costa Rica. Tecnología en Marcha (Costa Rica). 2011; 24:19–24.
9. Rosales-Rodríguez R. Costa Rica. In: Crudeli GA, Konrad JL, Patiño EM, editors. Reproducción en búfalas. 1st ed. Moglia.2016;251.
10. Instituto Nacional de Estadísticas- INEC. VI Censo Nacional Agropecuario. San José, Costa Rica. 2014;72–74.
11. May Collado L. Marine Mammals. In: Wehrtmann IS, Cortes J, editors. Marine biodiversity of Costa Rica, Central America. Monographiae Biologicae. 1st ed. Springer. 2009;479–496.
12. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for brucellosis laboratory. INRA: Paris;1988.
13. OIE-World Organization for Animal Health. Bovine brucellosis: In manual of diagnostic tests and vaccines for terrestrial animals. 12 rue de Prony 75017. Paris; 2009.
14. Marín CM, Moreno E, Moriyón I, Díaz R, Blasco JM. Performance of competitive and indirect enzyme-linked immunosorbent assays, gel immunoprecipitation with native hapten polysaccharide, and standard serological tests in diagnosis of sheep brucellosis. Clin Diagn Lab Immunol. 1999; 6:269–272. PMID: 10066666
15. OIE-World Organization for Animal Health. Caprine and ovine brucellosis: In Manual of Diagnostic Tests and Vaccines. 12 rue de Prony 75017. Paris; 2009.
16. Moreno E, Rojas N, Nielsen K, Gall D. Comparison of different serological assays for the differential diagnosis of brucellosis. In International Atomic Energy, Diagnosis and Epidemiology of Animal Diseases in Latin America IAEA-TECDOC 1055. Viena; 1998;153–161.
17. Hernández- Mora G, Manire CA, González-Barrientos R, Barquero-Calvo E, Guzmán-Verri C, Staggs L, et al. Serological diagnosis of *Brucella* infections in odontocetes. Clin Vaccine Immunol. 2009; 16:906–915. https://doi.org/10.1128/CVI.00413-08 PMID: 19386800
18. Muñoz PM, Blasco JM, Engel B, De Miguel MJ, Marín CM, Dieste L, et al. Assessment of performance of selected serological tests for diagnosing brucellosis in pigs. Vet Immunol Immunopathol. 2012; 146:150–158. https://doi.org/10.1016/j.vetimm.2012.02.012 PMID: 22445082
19. Chanto G, Rojas N, Ching A, Zúñiga R, Castro E, Chaverri S, et al. Prevalencia de anticuerpos séricos contra la bacteria *Brucella* sp. en una población humana tropical. Rev Biol Trop 2007; 55:385–391. PMID: 19069753
20. Reglamento para la intervención de la brucelosis bovina. Decreto 34858-MAG-2008. Gaceta, 230, (7-11- 2008) 2008; Available from: http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=64425&nValor3=74734&strTipM=TC
21. De Miguel MJ, Marín CM, Muñoz PM, Dieste L, Grilló MJ, Blasco JM. Development of a selective culture medium for primary isolation of the main *Brucella* species. J Clin Microbiol. 2011; 49:1458–63. https://doi.org/10.1128/JCM.02301-10 PMID: 21270216
22. Le Flèche P, Jacques I, Grayon M, Al Dahouk S, Bouchon P, Denoeud F, et al. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. 2006; 6:9. https://doi.org/10.1186/1471-2180-6-9 PMID: 16469109
23. García-Yoldi D, Marín CM, de Miguel MJ, Muñoz PM, Vizmanos JL, López-Goñi I. Multiplex PCR Assay for the identification and differentiation of all *Brucella* Species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin Chem. 2006; 52:779–781. https://doi.org/10.1373/clinchem.2005.062596 PMID: 16595839
24. López-Goñi I, García-Yoldi D, Marín CM, de Miguel MJ, Barquero-Calvo E, Guzmán-Verri C, et al. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Vet Microbiol. 2011; 154:152–155. https://doi.org/10.1016/j.vetmic.2011.06.035 PMID: 21782356
25. Scott JC, Koylass MS, Stubberfield MR, Whatmore AM. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. Appl Environ Microbiol. 2007; 73:7331–7337. https://doi.org/10.1128/AEM.00976-07 PMID: 17890328
26. Reglamento de Vigilancia de la Salud. Decreto 37306-S-2012. Gaceta (Costa Rica), No 203, (27-8-2012). 2012; Available from: http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&param2=18&nValor1=1&nValor2=73471&nValor3=90176&strTipM=TC&lResultado=180&nValor4=1&strSelect=sel.
27. González-Barrientos R, Morales J-A, Hernández-Mora G, Barquero-Calvo E, Guzmán-Verri C, Chaves-Olarte E, et al. Pathology of striped dolphins (*Stenella coeruleoalba*) infected with *Brucella ceti*. J Comp Pathol. 2010; 142:347–352. https://doi.org/10.1016/j.jcpa.2009.10.017 PMID: 19954790

28. Cannon RM, Roe RT. Livestock disease surveys: a field manual for veterinarians. Australian Government, Australia.1982.
29. Thrusfield M, Ortega C, de Blas I, Noordhuizen JP, Frankena K. WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet Rec. 2001; 148:567–572. PMID: 11370882
30. Guzmán-Verri C, González-Barrientos R, Hernández-Mora G, Morales J- A, Baquero-Calvo E, Chaves-Olarte E, et al. *Brucella ceti* and brucellosis in cetaceans. Front Cell Infect Microbiol. 2012; 2:3. https://doi.org/10.3389/fcimb.2012.00003 PMID: 22919595
31. Oliveira JB, Morales JA, González-Barrientos RC, Hernández-Gamboa J, Hernández-Mora G. Parasites of cetaceans stranded on the Pacific coast of Costa Rica. Vet Parasitol. 2011; 182:319–328. https://doi.org/10.1016/j.vetpar.2011.05.014 PMID: 21665367
32. Suárez-Esquivel M, Baker KS, Ruíz-Villalobos N, Hernández-Mora G, Barquero-Calvo E, González-Barrientos R, et al. *Brucella* genetic variability in wildlife marine mammals populations relates to host preference and ocean distribution. Genome Biol Evol. 2017; (in press).
33. CATIE-Centro Agronómico Tropical para la Investigación y Enseñanza. La situación caprina en Costa Rica: Situación de la producción caprina en centroamérica y República Dominicana. 1987. Turrialba, Costa Rica. 1987; 114:120.
34. FAOSTAT-Organización de las Naciones Unidas para la Alimentación y la Agricultura. Dirección de Estadística.2016: Available from: http://faostat3.fao.org/download/Q/QA/S.
35. Ducrotoy MJ, Conde-Álvarez R, Blasco JM, Moriyón I. A review of the basis of the immunological diagnosis of ruminant brucellosis. Vet Immunol Immunopathol. 2017; 171:81–102.
36. Moreno E. Brucellosis in Central America. Vet Microbiol. 2002; 90:31–8. PMID: 12414131
37. Rosales RR, Wingching JR. Sistemas de producción bufalina en Costa Rica. Cuantificación de la población y caracterización de los sistemas. Rev Agron Costarricense (Costa Rica) 2007; 31:65–69.
38. Fosgate GT, Diptee MD, Ramnanan A, Adesiyun AA. Brucellosis in domestic water buffalo (*Bubalus bubalis*) of Trinidad and Tobago with comparative epidemiology to cattle. Trop Anim Health Prod. 2011; 43:1479–1486. https://doi.org/10.1007/s11250-011-9846-9 PMID: 21479842
39. Borriello G, Capparelli R, Bianco M, Fenizia D, Alfano F, Capuano F, et al. Genetic resistance to *Brucella abortus* in the water buffalo (*Bubalus bubalis*). Infect Immun. 2006; 74:2115–2120. https://doi.org/10.1128/IAI.74.4.2115-2120.2006 PMID: 16552040
40. Denny HR. A review of brucellosis in the horse. Equine Vet J. 1973; 5:121–125. PMID: 4208703
41. Isidoro-Ayza M, Ruiz-Villalobos N, Pérez L, Guzmán-Verri C, Muñoz PM, Alegre F, et al. *Brucella ceti* infection in dolphins from the Western Mediterranean sea. BMC Vet Res. 2014; 10:206. https://doi.org/10.1186/s12917-014-0206-7 PMID: 25224818
42. Whatmore AM, Dawson CE, Groussaud P, Koylass MS, King AC, Shankster SJ, et al. Marine mammal Brucella genotype associated with zoonotic infection. Emerg Infect Dis. 2008; 14:517–518. https://doi.org/10.3201/eid1403.070829 PMID: 18325282
43. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Frontiers Microbiol. 2014;1–18. https://doi.org/10.3389/fmicb.2014.00213 PMID: 24860561
44. Chacón-Díaz C, Altamirano-Silva P, González-Espinoza G, Medina M- C, Alfaro-Alarcón A, Bouza-Mora L, et al. *Brucella canis* is an intracellular pathogen inducing a lower proinflammatory response than smooth zoonotic counterparts. Infect Immun. 2015; 83:4861–4870. https://doi.org/10.1128/IAI.00995-15 PMID: 26438796

Chapter 3. Phylogenetic characterization of marine and terrestrial *Brucellae* isolated in Costa Rica

Costa Rica started the investigation of marine brucellosis in 2004 (Hernández-Mora *et al*., 2008). Since then, out of the nineteen species reported as stranded in Costa Rica coastal shores, 45% are striped dolphins (*Stenella coeruleoalba*). These animals stranded with tremors, swimming difficulties, buoyancy problems, lack of coordination and they died within a few hours after the event (Hernández-Mora *et al*., 2008). *B. ceti* was isolated from the cerebrospinal fluid in 70% of these dolphins, associated with the presence of meningoencephalomyelitis (97%),that caused the death of these dolphins (González Barrientos *et al*., 2010; May Collado *et al.,* 2017). This bacterium was also present in the vaginal and uterine fluids, placenta, umbilical cord, allantoidal and amniotic fluids, fetal organs, milk, cardiac valve, atlanto-occipital joint fluid, and in lung nematodes (*Halocerchus* spp) (May Collado *et al.,* 2017).

Using whole-genome sequencing (WGS) on these *B. ceti* isolations, we described in the paper Suárez-Esquivel (2017a), the elements of genetic variation in *B. ceti* isolated from wild dolphins inhabiting the Pacific Ocean, the Atlantic Ocean, and the Mediterranean Sea. The *B. ceti* strains showed distinctive traits according to oceanic distribution and preferred host. *B. ceti* isolates displayed genetic variability, represented by an important number of IS711 elements as well as specific IS711 and SNPs genomic distribution clustering patterns. Extensive pseudogenization was found among isolates from cetaceans as compared with terrestrial ones, causing degradation in pathways related to energy, transport of metabolites, and regulation/transcription.

Costa Rican dolphin *B. ceti* isolates, showed further degradation of metabolite transport pathways as well as pathways related to cell wall/membrane/envelope biogenesis and motility. Thus, gene loss through pseudogenization is a source of genetic variation in *Brucella*, which in turn, related to adaptation to different hosts. This is relevant to the understanding of the natural history of bacterial diseases, their zoonotic potential, and the impact of human interventions such as domestication.

Characterization by MLST *in silico* of the *B. ceti* isolates has also been performed. As a result, sequence type ST26 has been described in all the bacteria from the stranded striped dolphins regardless of the origin of the strains, including CSF, lung nematodes, placenta, and milk. In 2018, a *Brucella* ST27 was identified in a stranded dwarf sperm whale (*Kogia sima*) in Playa Herradura, Puntarenas (Figure 1). The *Brucella* ST27 isolate was also obtained from the fetus, the placenta, and other organs of the two dwarf sperm whales, causing reproductive problems.The above mentioned confirmed a new *Brucella* ST27 host in the Eastern Tropical Pacific. Previously, *Brucella* ST27 was isolated in humans with neurobrucellosis and spinal osteomyelitis in Peru and New Zealand in 2003 and 2006, respectively. However, the source of infection in these patients remains unknown.

Likewise, as part of the clinical presentation of the dolphin cases with neurobrucellosis in Costa Rica, we used computerized axial tomography before performing the necropsy (virtopsy), which represents a pioneering advance in imaging diagnosis in the country. The information obtained has allowed comparisons between human and dolphin neurobrucellosis (Figure 2).

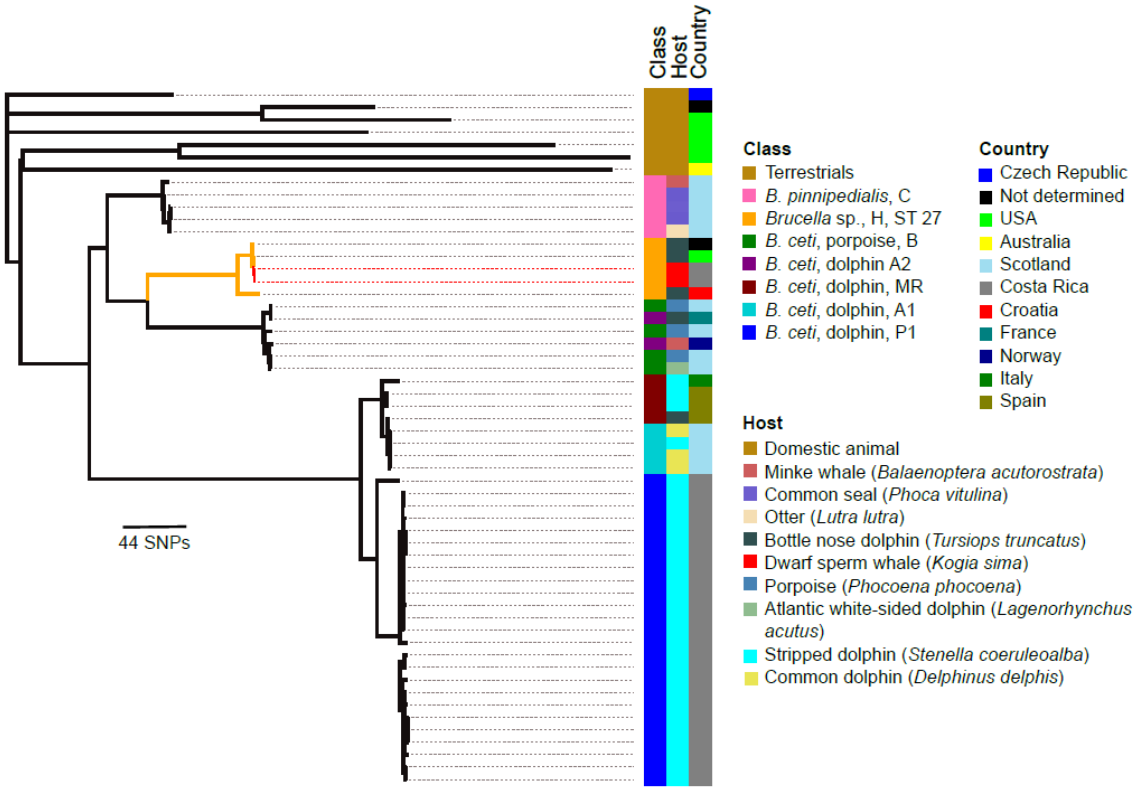

Figure 1. WGS phylogenetic reconstruction of *B. ceti* isolates. The tree is based on 27,365 SNPs of different *Brucella* WGS. The isolates related to marine mammals are classified into seven categories, corresponding to clusters revealed by MLVA-16 analysis and previously described according to geographic origin or host association. Cluster H (human-associated isolates– ST27), is highlighted in orange. This cluster includes *Brucella* sp. F5-99, *B. ceti* strain Cudo, *B. ceti* strain CR0350 and the *K. sima* isolates. WGS from *K. sima* are shown with red lines. *Ochrobactrum* sp., used as the original root for the tree, was trimmed from the figure to increase tree resolution. Each cluster defining branch showed a 100-bootstrap value. Color codes for MLVA-16 classification, host, and country of origin are specified next to the tree. For increased resolution, visit https://microreact.org/project/xaQYIdp96.

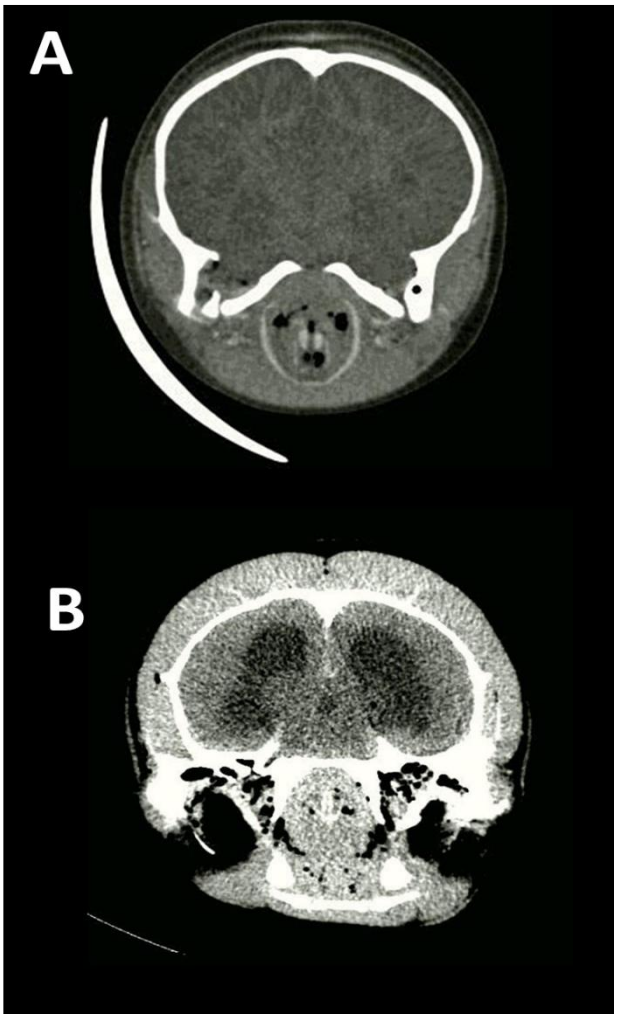

Figure 2. Axial tomography Scan on stranded cetacean in Costa Rica. A) Brain of a spinner dolphin (*S. longirostris*) with no visible lesions. Third and fourth ventricles are barely perceptible, negative to brucellosis by serology and culture. The young male stranded in Bajamar beach in Puntarenas Costa Rica in November 2017. **B**) Brain of a striped dolphin (*S. coeruleoalba*) with ventriculomegaly and secondary hydrocephalus, positive to brucellosis by serology and culture of *B. ceti.* The adult female, stranded in Parque Marino Ballena in Puntarenas Costa Rica in January 2018.

For *B. abortus* isolated in Costa Rica from bovines, humans, and water buffaloes, we analyzed by whole-genome sequencing (WGS) and performed the phylo-temporal analysis of the incursion in Costa. For this purpose, a total number of 95 *B. abortus* isolated in Costa Rica showed five *B. abortus* lineages, phylogenetically related to isolates from the United States (US), United Kingdom (UK) and South America (SA). We demonstrated that the predominant CR lineages of *B. abortus*, displaying modest diversity and introduced more than 100 years ago, have circulated and spread in the territory in spite of new introductions that seemed to be less dispersed. Our findings are relevant from the epidemiological perspective. Following the brucellosis prevalence and the idiosyncratic settings of several middle- and low- income countries, similar scenarios could be found in other latitudes.

Chapter 3 includes the following papers:

- Suárez-Esquivel, M., Baker, K.S., Ruiz- Villalobos, N., Hernández Mora, G.,Barquero-Calvo, E., González-Barrientos, R., Castillo-Zeledón, A., Jiménez-Rojas, C., Chacón-Díaz, C., Cloeckaert, A., Chaves Olarte E., Thomson N., Moreno, E. (2017). *Brucella* genetic variability in wildlife marine mammals' populations relates to host preference and ocean distribution. *Genome Biol Evol* 2017 evx137. https://doi.org/10.1093/gbe/evx137
- Hernández-Mora G., González-Barrientos, R., Palacios Alfaro, J.D., Suárez-Esquivel M., Ruiz-Villalobos N., Barquero-Calvo E., Cordero X.M., Bettoni, G., Roca, K., Guzmán-Verri C., Moreno E. *Brucella spp* ST27 in dwarf sperm whale (*Kogia sima*), Costa Rica. Emerg Infect Dis (submitted)
- Suárez-Esquivel, M., Hernández-Mora G., Ruiz-Villalobos, N., Rojas-Campos N., Barquero-Calvo E., Oviedo-Sánchez G., Foster J.T., Ladner J.T., Chacón-Díaz C., Chaves-Olarte E., Baker K.S., Thomson N.R., Moreno E., Guzmán-Verri C. (2019). Phylo-temporal Analysis of *Brucella abortus* Incursions in Costa Rica. Sometido a *PLOS Neglected Tropical Diseases* (submitted)

Brucella Genetic Variability in Wildlife Marine Mammals Populations Relates to Host Preference and Ocean Distribution

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Data deposition: This project has been deposited at the European Nucleotide Archive (ENA) under the accessions ERR418023-ERR418025, ERR471310-ERR471313, ERR471316-ERR471328, ERR471330-ERR471333, ERR473728-ERR473729, ERR485943-ERR485951, ERR554829 and ERR775250-ERR775251 (details in Supplementary data set S1).

Abstract

Intracellular bacterial pathogens probably arose when their ancestor adapted from a free-living environment to an intracellular one, leading to clonal bacteria with smaller genomes and less sources of genetic plasticity. Still, this plasticity is needed to respond to the challenges posed by the host. Members of the *Brucella* genus are facultative-extracellular intracellular bacteria responsible for causing brucellosis in a variety of mammals. The various species keep different host preferences, virulence, and zoonotic potential despite having 97–99% similarity at genome level. Here, we describe elements of genetic variation in *Brucella ceti* isolated from wildlife dolphins inhabiting the Pacific Ocean and the Mediterranean Sea. Comparison with isolates obtained from marine mammals from the Atlantic Ocean and the broader *Brucella* genus showed distinctive traits according to oceanic distribution and preferred host. Marine mammal isolates display genetic variability, represented by an important number of IS*711* elements as well as specific IS*711* and SNPs genomic distribution clustering patterns. Extensive pseudogenization was found among isolates from marine mammals as compared with terrestrial ones, causing degradation in pathways related to energy, transport of metabolites, and regulation/transcription. *Brucella ceti* isolates infecting particularly dolphin hosts, showed further degradation of metabolite transport pathways as well as pathways related to cell wall/membrane/envelope biogenesis and motility. Thus, gene loss through pseudogenization is a source of genetic variation in *Brucella*, which in turn, relates to adaptation to different hosts. This is relevant to understand the natural history of bacterial diseases, their zoonotic potential, and the impact of human interventions such as domestication.

Key words: *Brucella*, marine mammals, genome degradation.

Introduction

Bacteria living in isolation or stable habitats, such as the intracellular milieu, tend to have clonal populations with smaller and degraded genomes than free-living ancestors, which keep larger and more versatile genomes (Moreno 1998; Toft and Andersson 2010). Still, some versatility must be preserved in order to confront environmental challenges.

Most of the emergent human pathogens have a zoonotic origin where transgression of host barriers is critical (Greger 2007; Jones et al. 2008). Understanding how microorganisms are able to surpass such barriers, particularly host range adaptation is relevant to comprehend the emergence of pathogens.

It has been proposed that genetic drift and speciation in extant clonal bacteria will depend exclusively on mutation and internal genetic rearrangements (Moreno 1997). Several mechanisms had been described in mammal bacterial pathogens with small genomes to keep genetic variability (Bolotin and Hershberg 2015). However, it is possible that these mechanisms are underrepresented when studying bacterial pathogens of domesticated animals. In this sense, domestication may represent a microbial population bottleneck for diversity: By selecting animals genetically suited for human benefit, there is probably selection of their microorganisms. Within this context, to study bacteria infecting wildlife populations, closely related to bacteria isolated from domesticated animals, may bring light to pathways followed by these selection processes.

Members of the *Brucella* genus are facultative extracellular intracellular α 2-Proteobacteria responsible for causing brucellosis in a variety of mammals. This chronic disease results in abortion and infertility in livestock causing economic losses mainly in middle and low income countries (Moreno and Moriyón 2006). Humans are infected through contaminated animal-derived food products or infected animals. It is considered by the WHO as a "forgotten neglected zoonosis", estimating that for every reported human case, there are 25–50 unreported cases (World Health Organization 2014).

Brucella species share 97–99% identity at genome level. In spite of this close genetic relatedness and genomes with no lysogenic phages or detected plasmids, there is a strong correlation between genotypes, virulence, and host preference (Moreno and Moriyón 2006). These traits make *Brucella* an appropriate model for understanding bacterial host adaptation. Interestingly, pseudogene accumulation in prokaryotes has been demonstrated as a hallmark of recent host adaptation. It is also inversely related to host-range, that is, narrow host-range pathogens tend to have a higher number of pseudogenes, and similar phenomena had been studied in *Brucella* (Chain et al. 2005; Tsolis et al. 2009; Wattam et al. 2009; Goodhead and Darby 2015).

Here we used *Brucella* isolates from free-living marine mammals in three of the world's major oceanic basins to look for elements of genetic variation and their relation to host specialization of this zoonotic pathogen. We characterized *Brucella ceti* isolates from dolphins from the Pacific Ocean and the Mediterranean Sea, and compared them with isolates obtained from marine mammals (dolphins, porpoises, and seals) from the Atlantic Ocean. The distinctive traits observed among the isolates showed signatures of host preference, speciation, and oceanic distribution. Expanding that comparison to *Brucella* sp. isolates, revealed genetic variability elements among isolates from wildlife marine mammals as compared with those from terrestrial domesticated animals. This variability is demonstrated through a SNPs and IS*711* specific clustering pattern across genomes and a higher number of IS*711* elements. There is also an important number of pseudogenes affecting specific metabolic pathways and inducing gene loss according to host preference. Therefore, gene loss should be considered a source of genetic variation in *Brucella*, which in turn, relates to adaptation to different niches and host preference.

Materials and Methods

Bacterial Strains

The list of isolates used in this study is presented in supplementary data set S1, Supplementary Material online and includes 23 *B. ceti* isolates from stranded striped dolphins from the Eastern Tropical Pacific of Costa Rica as well as several previously described isolates: Four from the Mediterranean Sea, nine from the North Atlantic Ocean, one from France, four *Brucella pinnipedialis* from the North Atlantic Ocean, and one *Brucella* sp. from California. These were analyzed alongside with reference strains from other *Brucella* species (*Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella microti*, *Brucella neotomae*, *Brucella ovis* and *Brucella suis*).

Brucella Phenotypic Characterization

All procedures involving live *Brucella* were carried out according to the "Reglamento de Bioseguridad de la CCSS 39975-0", year 2012, after the "Decreto Ejecutivo #30965-S", year 2002 and research protocol NFEG06 approved by the National University, Costa Rica. Phenotypic analysis of *Brucella* isolates was carried out as described (Hernández-Mora et al. 2008). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) studies of *Brucella* protein extracts and gas chromatographic analysis of fatty acid methyl esters were performed as previously described (Isidoro-Ayza et al. 2014). A dendogram derived from the analysis of concatenated data based on the retention time of the fatty acid methyl esters, and on the protein masses detected, was constructed using an Agglomerative Hierarchical Clustering (AHC) algorithm, using Microsoft Excel 2000/XLSTAT-Pro (Version 4.07, 2013, Addinsoft, Inc., Brooklyn, NY). Proximities were calculated using Squared Euclidean Distance, and aggregation was calculated using the unweighted pair-group average method. Raw data are in supplementary data set S2, Supplementary Material online.

DNA Molecular Studies

DNA was extracted with DNeasy Blood & Tissue kit from QIAGEN or Promega Wizard Genomic DNA Purification kit, and stored at −70 °C until used.

Multiple loci variable number of tandem repeats (MLVA-16) analysis and the corresponding cladograms were generated according to described protocols (Le Flèche et al. 2006; Al Dahouk et al. 2007; Maquart et al. 2009; Isidoro-Ayza et al. 2014) using the MLVA-NET database (http://microbesgenotyping.i2bc.paris-saclay.fr/ (last accessed July 24, 2017); Grissa et al. 2008). Values obtained for each MLVA marker are in supplementary data set S2, Supplementary Material online. DNA polymorphism at the *omp2* locus was performed as described (Cloeckaert et al. 2001).

Other genotyping techniques such as multiplex PCR Bruceladder, MLST, PCR detection of ST27 or *bcsp31*, HRM RT-PCR and PCR targeting specific IS*711* elements, were performed either as previously described (references in data set S1, supplementary Material online) or in silico (supplementary data set S3, Supplementary Material online).

Whole genome sequencing (WGS) was performed at the Wellcome Trust Sanger Institute on Illumina platforms according to in house protocols (Quail et al. 2009, 2012). For WGS assembly and alignment sequencing reads were de novo assembled using Velvet Optimiser (Zerbino and Birney 2008) and contigs were ordered using abacas (Assefa et al. 2009) against *B. abortus* 9-941 under accession numbers NC_006932 and NC_006933 at the National Center for Biotechnology Information (NCBI). To detect miss-assemblies, raw data were mapped back against the genome assemblies using SMALT v.0.5.8 (http://www.sanger.ac.uk/science/tools/smalt-0; last accessed July 24, 2017). All sequencing data have been deposited at the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena/; last accessed July 24, 2017) under the accession codes listed in Supplementary data set S1, Supplementary Material online. Other WGS sequences from various *Brucella* strains used for comparative purposes were obtained from GenBank (supplementary data set S1, Supplementary Material online). Incomplete genomes, or low N50 scaffolds from databases were not included in the analysis.

Phylogenetic Reconstruction

To construct a multiple sequence alignment for phylogenetic reconstruction, whole-genome sequence data from two *Ochrobactrum* species and the *Brucella* isolates from different hosts (Supplementary data set S1, Supplementary Material online) were aligned by bwa and mapped with SMALT v.0.5.8 against *B. abortus* 9-941, with an average coverage of 98.81%. Single Nucleotide Polymorphisms (SNPs) were called using samtools (Li et al. 2009), and 311,780 variable sites were extracted using snp sites (Page et al. 2016). The resulting alignment was used for maximum likelihood phylogenetic reconstruction with RAxML v7.0.4 (Stamatakis 2006). The phylogenetic tree was rooted using *Ochrobactrum anthropi* ATCC49188 and *Ochrobactrum intermedium* strain type LMG3301. Within this data set the *B. ovis* lineage shared the most recent common ancestor with *Ochrobactrum*, therefore it was subsequently used to root phylogenies constructed using only *Brucella*.

All analyses relevant to reference annotation (e.g., dN/dScalculation and SNP ascription to coding sequences—CDS) were relative to *B. abortus* 9-941 (accession numbers NC_006932 and NC_006933). The alignment and the tree files were used to generate a tab file containing coordinates of SNPs position relative to the root; all three files were used to produce a visual reconstruction of the SNPs distribution along the genome per branch (as seen in supplementary fig. S5, Supplementary Material online).

Comparative Genomics from Whole Genome Sequences

Comparative genomics was facilitated by annotation of *B. ceti* draft genome assemblies by Prokka (Seemann 2014) and by annotation transfer from *B. abortus* 2308 Wisconsin (Suárez-Esquivel et al. 2016). The annotation of genes absent in *B. abortus* 2308 Wisconsin was completed through manual comparison against reference genomes (Supplementary data set S1, Supplementary Material online): *B. suis* 1330, *B. ovis* ATCC 25840, *B. melitensis* 16M and *B. pinnipedialis* B2/94. We identified orthologous protein groups and the number of new, conserved and total genes added by each genome included in the analysis (discovery rate) by using Roary (Page et al. 2015).Visualizations were done with Artemis and comparisons with the Artemis Comparison Tool (ACT; Carver et al. 2005). The presence of recombination events was analyzed by Genealogies Unbiased By recomBinations In Nucleotide Sequences (Gubbins) (Croucher et al. 2014).

Pseudogene Analysis

To detect pseudogenes in *B. ceti*, we selected five phylogenetically representative draft genomes from marine mammal *brucellae* (*B. ceti* bmarCR17 -P1 cluster-, *B. ceti* bmarMR26 -MR cluster-, *B. ceti* M644/93/1 -A1 cluster-, *B. ceti* M187/00/1 -A2/B cluster-, and *B. pinnipedialis* M2466/93/4 -C2 cluster-) and automatically transferred the annotation of the manually curated draft genome working strain *B. abortus* 2308 Wisconsin (Suárez-Esquivel et al. 2016).

Pseudogenes were defined as any gene containing deletions or insertions that removed start or stop codons, or at least one in-frame stop codons and/or frame shifts compared with orthologs in *B. abortus* 2308 Wisconsin or reference genomes as described above. Pseudogenes were detected manually using Artemis and ACT. Pseudogenes from marine mammal brucellae with no homologs in terrestrial *Brucella* were compared against the NCBI nonredundant protein database using BlastX. The putative cellular localization was predicted by PSORT and the function was classified based on: The product description in the references annotation; BLAST comparison with several *Brucella* species and other genus;

metabolic assigned pathway according to KEGG (Kanehisa et al. 2016). In depth metabolic pathway analysis of pseudogenes from particular phylogenetic branching points was carried out using BioCyc (Caspi et al. 2014).

Specific Search for Regions of Interest

In order to examine relevant phenotypic genes (virulence related, outer membrane, lipopolysaccharide [LPS] and flagellar genes), regions of interest were examined through bwa alignment and SMALT mapping. The number of SNPs, insertions and deletions in each one of the genes was recorded.

The number and position of the insertion sequence IS*711* were searched in the analyzed genomes by mapping the reads to the 842 bp IS*711* of *B. ovis* (accession number M94960). Those reads that showed 99% mapping, were then mapped against the reference WGS *B. ovis* ATCC 25480 in order to judge where IS*711* might be inserted. The reads that mapped >90% to the WGS were filtered to 50× coverage and used to produce a visual representation displaying the identified sites per genome and approximate location according to *B. ovis* sequence coordinates.

The presence, orientation, and distribution of 24 previously reported genomic islands (GIs) or anomalous regions (regions apparently acquired by horizontal gene transfer; Mancilla 2012; Rajashekara et al. 2004; Wattam et al. 2009) were examined across the four phylogenetically representative *B. ceti* genomes (see above). For this, a "genomic-island pseudomolecule" was formed by concatenation of 23 genomic regions obtained from nonmarine *Brucella* reference sequences (supplementary data set S1, Supplementary Material online). Islands were concatenated and ordered as follows: GI-4, GI-3, SAR 1-2, wbk, SAR 1-5, GI-2, GI-1, SAR 1-17, 4 kb, 13 kb, GI-9, GI-8, 26.5 kb, IncP, 12 kb, GI-7, GI-6, GIBs2, GIBs3, SAR 2-10, GI-5, mtgC, and virB.

A BLAST comparison between the representative *B. ceti* genomes and the pseudo-molecule was performed and visualized using ACT. The described orientation of the islands was checked in several reference genomes (*B. suis* 1330, *B. microti* CCM 4915, *B. abortus* 9-941, and *B. ovis* ATCC 25840) to confirm the presence of inversions. The 24th GI, a 67 kb sequence found mainly in isolates from marine mammals (Audic et al. 2011; Maquart et al. 2008; Bourg et al. 2007) was similarly analyzed independently.

Results and Discussion

Brucella ceti Clusters According to Geographical Region and Host Type

To study host preference in nondomesticated *Brucella* hosts, we performed genotypic analysis of *B. ceti* isolated from dolphins from the Pacific Ocean and the Mediterranean Sea (table 1), and compared the results with those of isolates obtained from marine mammals (dolphins, porpoises, and seals) from the Atlantic Ocean. These findings were then related to host and geographical origin.

MLVA-16 results were analyzed in the context of a worldwide *Brucella* databank and indicated that isolates from marine mammals showed dispersion and clustering according to the host from which they were isolated (fig. 1). Five *B. ceti* clusters were observed; two correspond to isolates mainly from different dolphin species (clusters A1 and A2) inhabiting the North Atlantic Sea. A third one is represented mostly by isolates from porpoises (cluster B) from the same sea (Maquart et al. 2009). Two additional *B. ceti* clusters affecting dolphins from the Pacific Ocean and the Mediterranean Sea were evident (Guzmán-Verri et al. 2012; Garofolo et al. 2014; Isidoro-Ayza et al. 2014). These clusters are herein referred as P1 and MR, respectively. The new isolates described in this study from the Eastern Tropical Pacific of Costa Rica belong to the P1 cluster affecting striped dolphins (*Stenella coeruleoalba*) (table 1, supplementary fig. S1, Supplementary Material online).

Brucella pinnipedialis isolated from the North Atlantic Sea was divided in three different MLVA-16 clusters that also related to host preference: Two were represented by isolates mainly from harbor seals (*Phoca vitulina*) (clusters C1 and C2) and one was represented by isolates from hooded seals (*Cystophora cristata*, cluster C3; Maquart et al. 2009). In addition, a human *Brucella* sp. isolate from New Zealand (*Brucella* sp. 02611), with no zoonotic link, an isolate from an aborted dolphin (*Brucella* sp. F5/99), and isolates from a stranded bottlenose dolphin from the Adriatic Sea (Cvetnić et al. 2016) define a distinct cluster (Maquart et al. 2009) herein named cluster H.

To determine if the dispersion and clustering observed by MLVA-16 could be reproduced by using higher resolution methods and establish possible explanations for this, we performed WGS of *Brucella* isolates from marine mammals from the North Atlantic, Eastern Tropical Pacific and Mediterranean Sea and analyzed them together with publically-available high quality *Brucella* genomes (table 1, supplementary data set S1, Supplementary Material online). The number of studied genomes ($n = 50$) was adequate to describe basic genomic characteristics of the genus, because the pan and core genome reached a plateau value within the data set (supplementary fig. S2, Supplementary Material online).

The overall genetic structure of *Brucella* from marine mammals is in tune with the classical pathogenic *Brucella* from land mammals. Some conserved traits are: Presence of two chromosomes, absence of plasmids, no major recent recombination events, similar GIs/anomalous regions, and conservation of genes encoding virulence factors (fig. 2, supplementary figs. S3, S4, and supplementary data set S3, Supplementary Material online). Phylogenetic analysis using *O. anthropi* ATCC49188 and *O. intermedium* LMG3301 as an outgroup showed that *B. ovis* shared the most recent common ancestor within this data set with *Ochrobactrum*, so it was subsequently used to root phylogenies constructed using only

Table 1
Marine Mammal *Brucella* Isolates Used for WGS Analysis (detailed information in supplementary data set S1, Supplementary Material online)

Species/Host	N. of Isolates	Location[a]
B. ceti		
Balaenoptera acutorostrata (minke whale)	1	NA
Delphinus delphis (common dolphin)	2	NA
Lagenorhynchus acutus (Atlantic white-sided dolphin)	1	NA
Phoca vitulina (common seal)	1	NA
Phocoena phocoena (porpoise)	3	NA
Stenella coeruleoalba (striped dolphin)	27	ETP, MS, NA
Tursiops truncatus (bottle nose dolphin)	2	MS, France
B. pinnipedialis		
Balaenoptera acutorostrata (minke whale)	1	NA
Lutra lutra (otter)	1	NA
Phoca vitulina (common seal)	2	NA
***Brucella* sp.**		
Tursiops truncatus (bottle nose dolphin)	1	USA

[a]NA, North Atlantic. ETP, Eastern Tropical Pacific. MS, Mediterranean Sea.

Brucella isolates. When *Ochrobactrum* was excluded from the alignment, a total of 24,340 SNPs were found among the *Brucella* genomes. Of these, 19,081 SNPs were located in coding regions with a dN/dS ratio of 1.61.

The general topology of the SNPs based phylogenetic tree was consistent with those of similar studies using mainly terrestrial isolates, showing a clonal genus (Wattam et al. 2014, 2009) (fig. 2) or when *B. suis* 1330 was used as reference genome. It is also similar to a dendogram obtained by concatenation of results of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with gas liquid chromatography analysis of the fatty acid methyl esters (GLC) of *Brucella* cell extracts (supplementary fig. S5*A*, Supplementary Material online). When compared with the MLVA-16 study (fig. 1 and supplementary fig. S1, Supplementary Material online), the WGS analysis showed at least four *B. ceti* clusters, corresponding to MLVA-16 clusters P1, MR, and A1. The MLVA-16 clusters A2 and B are grouped in a single cluster that we refer as the A2/B genotype. The H cluster was represented by *Brucella* sp. F5/99, had its most recent common ancestor with *B. pinnipedialis* C cluster, and was also closely related to the *B. ceti* A2/B cluster.

When SNPs positions across each genome are visualized relative to the tree root, a barcode-like pattern due to different SNPs density regions within the genomes was observed. Some SNPs clusters could be identified, specific for a group of *Brucella* genotypes from marine mammals, or a single genotype (supplementary fig. S6, Supplementary Material online).

All together, these results expand the panorama observed in previous genotypic studies (Audic et al. 2011; Garofolo et al. 2014; Wattam et al. 2014; Maquart et al. 2009) and indicate a correlation between the evolutionary traits of *Brucella* isolated from marine mammals, its geographical origin and preferred host.

In order to benchmark other molecular techniques described for identification or typing of *Brucella*, we compared results generated using ten different techniques to the WGS classifications of the marine mammals *Brucella* isolates. Of these, multiplex PCR Bruce-ladder, adopted by the OIE for identification of *Brucella* species (OIE 2009) was able to classify but not discriminate all marine isolates (Supplementary data set S2, Supplementary Material online). Phylogenetic analysis based on DNA polymorphism at the *omp2* locus essentially replicated the genomic and phenotypic analysis results (supplementary fig. S5*B*, Supplementary Material online).

Multiple Sources and Consequences of *B. ceti* Genome Variation

To establish if there were genetic traits that could be related to *Brucella* host preference and virulence using isolates from wild animals, a detailed analysis of the genome structure of *B. ceti* clusters as compared with other *Brucella* genomes was performed.

Analysis of amount of SNPs found in genes encoding virulence traits such as the type IV secretion system *virB*, some of its effectors (see below), LPS, membrane lipids, BvrR/BvrS two component system regulatory network and flagella did not show significant variations among the isolates (Supplementary data set S2, Supplementary Material online).

Genome alteration through the active transposon insertion sequence IS*711*, used as a *Brucella* genus fingerprint (Ocampo-Sosa and García-Lobo 2008), was examined. An increased number of this element was detected in *brucellae* from marine mammals as compared with those from terrestrial strains (fig. 3), consistent with previous reports (Bricker et al. 2000; Dawson et al. 2008; Bourg et al. 2007; Audic et al. 2011). This indicates that marine isolates show greater genome variability than terrestrial ones. Interestingly, several IS*711* insertion patterns along the genome assemblies were observed and related to phylogenetic position. Some variation among isolates within phylogenetic clusters was also observed (e.g., Cluster P1, fig. 3).

To study the *en bloc* gain or loss of syntenic genes across the *Brucella* isolates from marine mammals, further detailed comparative genomics of representative from each of the four genome clusters P1, MR, A1, and A2B was performed. Presence of previously reported 24 GIs, important as evidence of gene horizontal transfer and gain of virulence traits within the genus (Mancilla 2012) was investigated (supplementary fig. S4, Supplementary Material online). Inversion of a GI as compared with reference sequences was a frequent event found in all four genomes, particularly those found in

Color	Cluster	Species	Host	Geographical location
	A1	*B. ceti*	Dolphin	North Atlantic
	A2	*B. ceti*	Dolphin	North Atlantic
	B	*B. ceti*	Porpoise	North Atlantic
	C1	*B. pinnipedialis*	Seal	North Atlantic
	C2	*B. pinnipedialis*	Seal	North Atlantic
	C3	*B. pinnipedialis*	Hooded seal	North Atlantic
	H	*Brucella* sp.	Human / Dolphin	Eastern Indo Pacific / Adriatic Sea
	MR	*B. ceti*	Dolphin	Mediterranean
	P1	*B. ceti*	Striped dolphin	Eastern Tropical Pacific

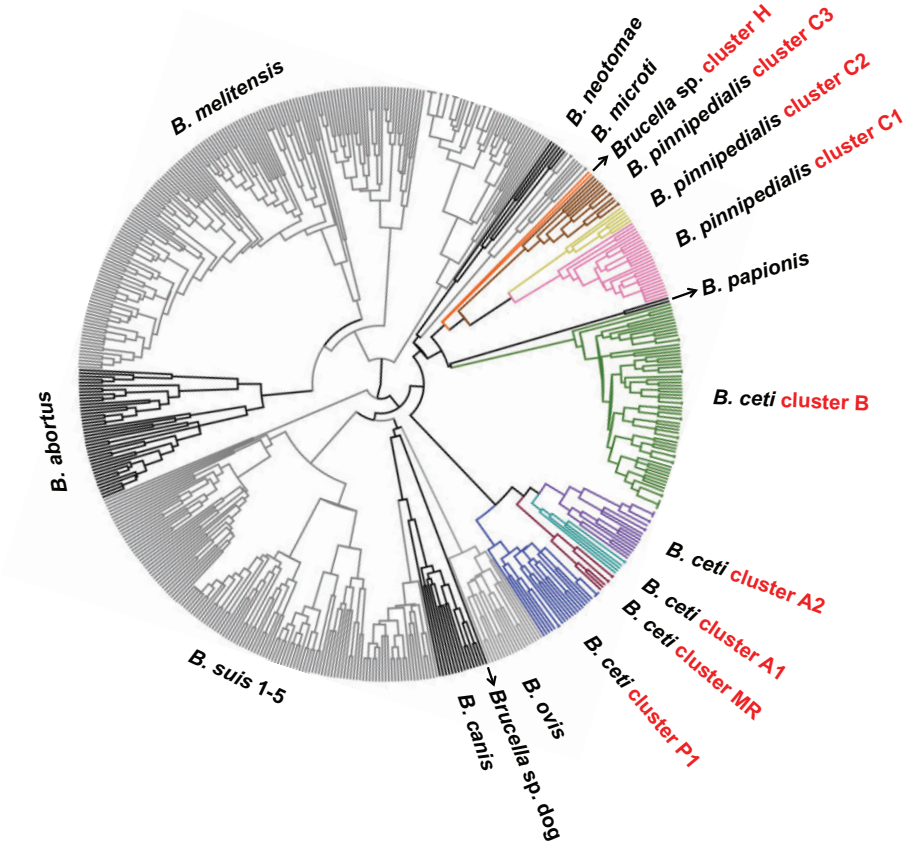

Fig. 1.—MLVA-16 analysis dendogram of *Brucella* related to geographic location and host. Analysis was performed according to: http://microbesgenotyping.i2bc.paris-saclay.fr/ (last accessed July 24, 2017). Increased resolution of marine isolates shown in supplementary fig. S1, Supplementary Material online.

chromosome II. The 12 kb and the 26.5 kb GIs were absent in all four genomes. GI-1 was absent in the P1 cluster and as previously reported, GI-3 was absent in the A2/B cluster representative (Wattam et al. 2014). The *wbk* GI, related to LPS synthesis, a virulence factor, has a particular rearrangement in the P1 cluster representative, caused by transposon and IS derived elements. However, they do not affect codifying genes as compared with the *B. melitensis* 16M *wbk* GI. The 67 kb GI related to *B. pinnipedialis* and to cluster H (Bourg et al. 2007; Audic et al. 2011) was found in the *B. pinnipedialis* isolates included in this study and in *B. ceti* bmarMR24. GI IncP was absent in *B. pinnipedialis* B2/94.

Comparative analysis of draft genome contiguous sequences ordered against *B. abortus* 2308W revealed a deletion due to repetitive sequences in the P1, MR, and A1 isolates representatives relative to the A2B cluster, including nine genes encoding mainly sugar transporters (BAW_20470-BAW_20476 and BAW_20479-BAW_20480) and four adjacent pseudogenes.

Pseudogenization Is a Source of Genetic Variability That Relates to Host Preference

To study correlations among pseudogene accumulation and host adaptation, we performed manual pseudogene

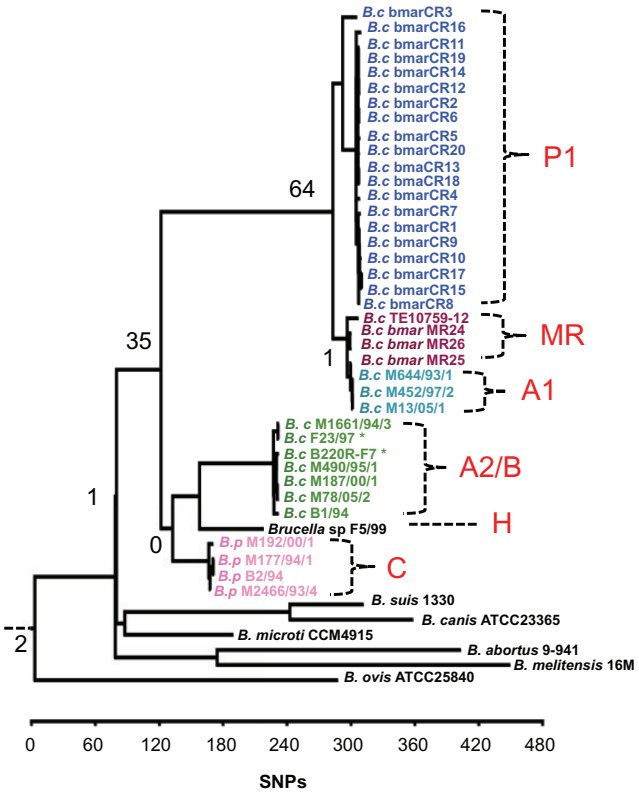

Fig. 2.—Whole genome sequence analysis of marine mammal *Brucella* shows phylogenetic correlation to host and geographic location. Phylogenetic tree based on 24,340 SNPs of different *Brucella* WGS. The isolates related to marine mammals showed six clusters, corresponding to those revealed by MLVA-16 analysis: P1, MR, A1, A2/B (which includes isolates from MLVA-16 A2—marked with asterisk—and B clusters), H and C. *Ochrobactrum* sp., used as the original root for the tree, was trimmed from the figure to increase tree resolution. Each cluster defining branch showed a 100 bootstrap value. The number of pseudogenes found is indicated in each defining node. Core genome analysis displayed similar tree topology.

annotation in the four *B. ceti* representative genomes, one representative *B. pinnipedialis*, *B. abortus*, *B. ovis*, and *B. suis* genomes and compared pseudogene traits according to genome (fig. 4*A*). In all genomes, the proportion of pseudogenes was higher in chromosome II than in the larger chromosome I (supplementary data set S4, Supplementary Material online). A total of 706 pseudogenes were found among these genomes and only two were shared among them. The mutation site within each gene was often conserved, suggesting that they occurred once in a common ancestor. The main cause of pseudogenization, was frame shift (410/706, 58%), followed by deletions (90/706, 13%) (fig. 4*A* and supplementary data set S4, Supplementary Material online). Their distribution according to former gene product, subcellular location and function is in Supplementary data set S4, Supplementary Material online.

Putative primary events targeting specific metabolic pathways that have become fixed in this population can be identified by looking at the extent of gene degradation at nodes of the phylogenetic tree (fig. 2). At the branching point between the marine mammal isolates and the *B. suis*/*B. canis*/*B. microti* clade only one shared pseudogene was found. Likewise, no shared pseudogenes were found at the branching point between the *B. ceti* A2B genotype and *B. pinnipedialis*, and only one pseudogene was shared among the *B. ceti* MR and A1 genotypes, suggesting that little gene degradation occurred when they diverged.

However, extensive pseudogenization was found among the isolates from marine mammals diverging from the *B. suis* clade (fig. 4*A*). Most of the 35 found pseudogenes, related to energy metabolism (8/35, 23%), amino acid transport and metabolism (5/35, 14%), gene regulation/transcription (4/35, 11%), or unknown function (5/35, 14%) (fig. 4*B*). Frame shift (22/35, 63%) was the main cause of pseudogenization followed by insertions (6/35, 17%) (fig. 4*C*). Functional analysis of the cognate wild type genes indicated that several pseudogenes were related to relevant metabolic pathways (Supplementary data set S4, Supplementary Material online). Notably, multiple pseudogenizations had occurred in pathways that alter fatty acid metabolism. Specifically, an acetyl-CoA acyltransferase and an acetyl-CoA C acetyltransferase very likely lost function in the marine mammal isolates. Lack of these enzymes is expected to influence fatty acids synthesis and beta-oxidation. In line with this finding, AceB, a malate synthase, catalyzing the conversion of glyoxylate to malate during the TCA, glyoxylate cycle (Zúñiga-Ripa et al. 2014) has probably lost its function. A functional glyoxylate shunt provides succinate and malate from acetyl-CoA and isocitrate for the TCA cycle and it is responsible for the bacteria ability to grow on fatty acids as carbon source (Barbier et al. 2011).

Synthesis of betaine glycine an osmoprotectant and source of carbon and nitrogen, important for *B. abortus* virulence (Lee et al. 2014) is probably affected, because two related genes lost function: Choline dehydrogenase and a glycine betaine/L-proline ABC transporter. Two more genes related to *Brucella* virulence probably lost function in the analyzed marine mammal isolates: One of the four predicted autotransporters in *Brucella* encoded by *btaE*, required for full virulence and defining a specific adhesive pole in *B. suis* (Ruiz-Ranwez et al. 2013) and the predicted sugar porin encoded by BR0833, required in *B. suis* for late stages of macrophage infection (Kohler et al. 2002).

There are 64 genes commonly pseudogenized in *B. ceti* genotypes P1, MR, and A1 representatives infecting dolphins, relative to the remaining marine mammal *brucellae* clusters (figs. 2, 4*A* and supplementary data set S4, Supplementary Material online), most of them related to amino acid transport and metabolism (11/64, 17%), carbohydrate transport and metabolism (10/64, 16%) or unknown function (12/64, 19%; fig. 4*B*). Although frame shift was still the most important mechanism of pseudogenization in this group (25/64,

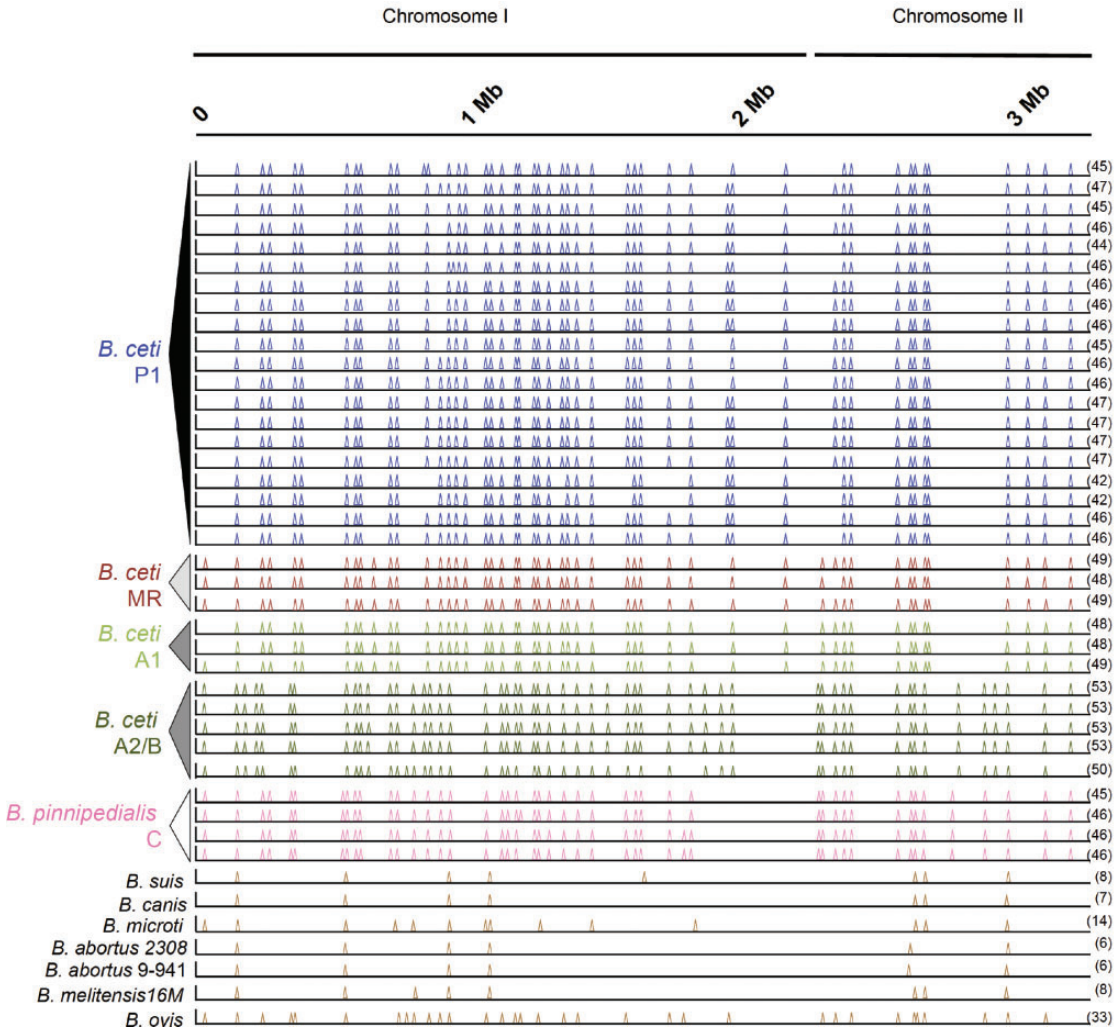

Fig. 3.—IS*711* insertion signatures for *Brucella* sp. Each peak represents the location of 50× coverage IS*711* insertion. The position in the first and second chromosomes (shown as a concatenated molecule) is indicated by the scale bar (in Mb) above. The number of IS*711* insertions is shown in parentheses at the end of each genome.

39%), deletion and premature stop codons were found in higher proportions (28 and 27%, respectively) as compared with the group of all marine isolates (18 and 17%, respectively). Insertions on the other hand were not as common (2%) as in the second group (17%; fig. 4*C*).

The P1, MR, and A1 genotypes show a higher proportion of gene degradation in functions related to carbohydrate transport and metabolism as well as those encoding transporters and cell envelope biogenesis functions as compared with the shared pseudogenes in the marine mammal representatives (fig. 4*B*). Several pseudogenes were tracked to specific pathways. Neither degradation of amino acids such as cysteine, glutamine, arginine, histidine, alanine, and aspartate nor pyruvate fermentation seem essential for survival in their dolphin host. The highly conserved sigma-54 factor *rpoN*, related to control of nitrogen metabolism, shows a frame shift mutation that very likely impairs its function (Ronneau et al. 2014).

Some genes related to virulence showed mutations. Degradation of outer membrane protein encoding genes as well as the flagellum operon, was also observed in the P1MRA1 *B. ceti* as in terrestrial *Brucella* (Martín-Martín et al. 2009; Moreno and Moriyón 2006). One of the type IV secretion system VirB effectors encoding gene, *vceC* contains an internal in frame deletion, resulting in loss of 10 amino acid residues as compared with *B. abortus* 2308 VceC. This mutation was present in all 30 *B. ceti* P1MRA1 genomes studied (Supplementary data set S3, Supplementary Material online) and is different from a previous reported one in terrestrial isolates (de Jong et al. 2008). This indicates that either that particular deletion does not affect protein function or that VceC is not needed for survival in the dolphin host.

Gene *galE-1* encoding an UDP-galactose 4-epimerase related to smooth LPS biosynthesis and attenuation (Rajashekara

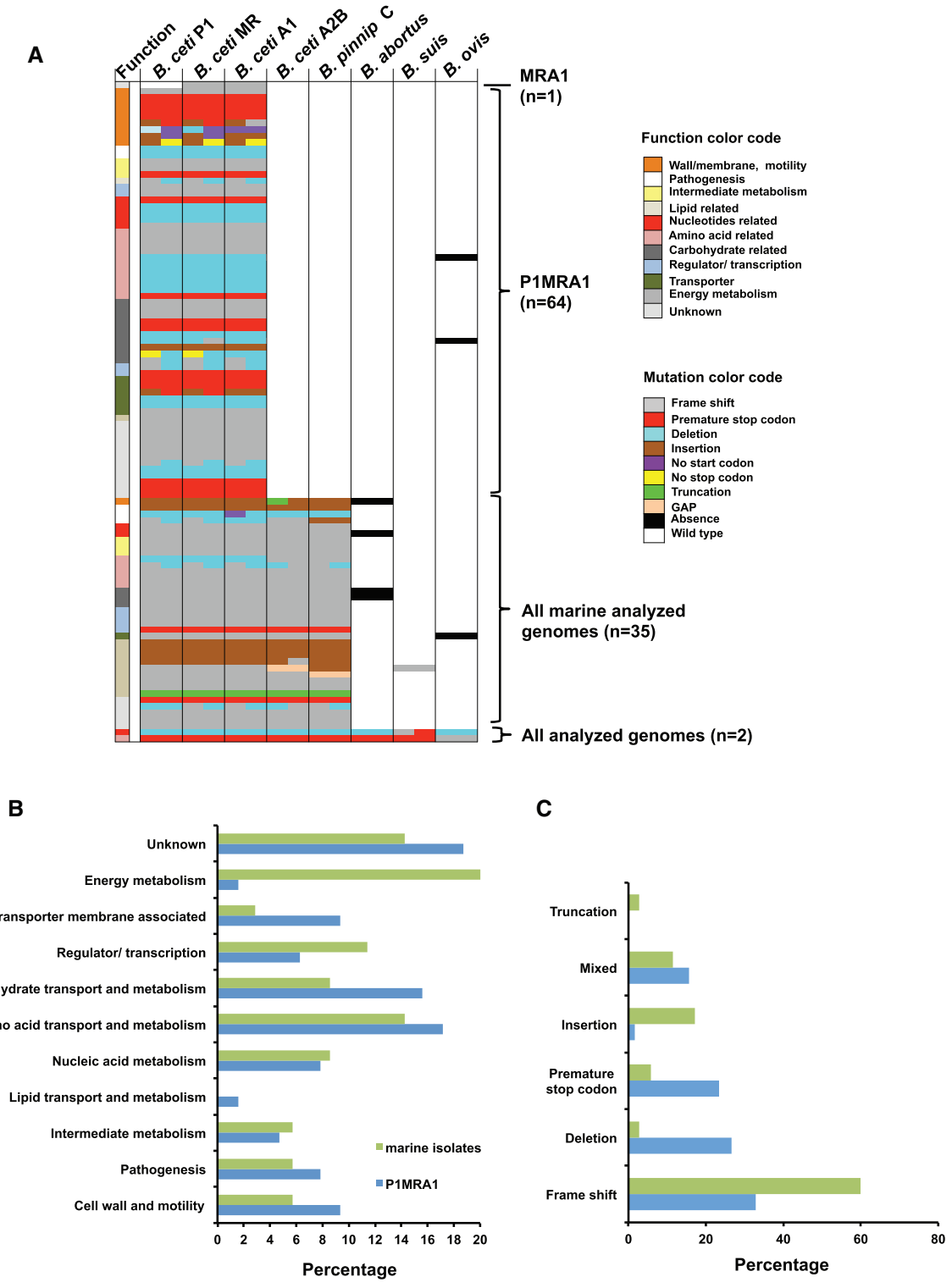

Fig. 4.—Classification of *Brucella* pseudogenes in relevant tree branching points found in representative genomes. (*A*) The left bar graph indicates function of each pseudogene according to color code and distributed according to four branches (MRA1, PMRA1, all marine analyzed genomes and all analyzed genomes). Every other bar represents the pseudogenes in each genome and colors correspond to a specific pseudogene type. "No stop codon" mutation refers to longer genes as compared with other *Brucella* reference genes. The number of pseudogenes for each branch is indicated in parenthesis. Details in Supplementary data set S4, Supplementary Material online, spreadsheet "at branch pseudo" (*B*, *C*). Proportional distribution of pseudogenes classified by their function (*B*) and by mutation type (*C*), according to two branching points (marine isolates and P1MRA1) in the phylogenetic tree.

et al. 2006) has an internal stop codon that probably renders inactive its product and could be related to the fact that some *B. ceti* isolates may appear as a "rough" phenotype (Guzmán-Verri et al. 2012). The premature stop codon was consistently found in all 30 P1MRA1 analyzed genomes.

It seems then, that when *Brucella* infects marine mammals, several important pathways related to energy, transport of metabolites and regulation/transcription are being degraded mainly via frame shift mutations. Marine isolates infecting particularly dolphin hosts showed further degradation of metabolites transport pathways as well as pathways related to cell wall/membrane/envelope biogenesis and motility, via not only frame shift mutations but also by premature stop codons and even gene absence. Altogether these findings indicate that degradation of metabolic pathways in *Brucella* is related to host preference with pseudogenization being a source of genetic variability. This is important for the establishment of host–bacterial interactions among the different *Brucella* species and their preferred hosts.

At least three barriers to successful bacterial replication and transmission exist for an intracellular pathogen in a given host population. The first is the immune system that will select for variants capable of withstanding host defenses. The second one is the intracellular milieu, which imposes conditions such as requirements for lysosome evasion, intracellular trafficking, and metabolic requirements. The third one relates to the mechanisms for transmission to other hosts, which may vary among different animal species. In the case of *Brucella* organisms from terrestrial domesticated mammals, at least two additional anthropogenic conditions may play a relevant role in biasing *brucellae* recovered from these populations: Domestication of a finite genetic line of the host species and population management controls such as vaccination and slaughter strategies (Moreno 2014). It is feasible that selection towards increased virulence, transmissibility, replication and zoonotic potential observed in *B. abortus*, *B. melitensis*, and *B. suis* (biotype 1 and 3) from domesticated animals, has taken place through successive infections in confined domesticated hosts, as proposed for the evolution of other infectious diseases (Ewald 2004).

Conclusion

Genetic variation is evident in *Brucella* from marine mammals and manifests in a variety of ways: 1) specific IS*711* insertion patterns across the genome, 2) higher numbers of IS*711* elements compared with *Brucella* from terrestrial mammals, 3) specific SNP signatures across phylogenetic clusters, and 4) pseudogenization of metabolic pathways. These traits correlate with host preference and, in the case of *B. ceti*, with oceanic origin.

We conclude that genome decay occurs through insertion sequence element proliferation and pseudogene formation. The extensive pseudogenization found suggests that these *Brucella* isolates from wildlife are less likely to be zoonotic. Moreover, the mechanism of pseudogenization varies according to host preference. At the same time, this gene loss is a source of genetic variation within the marine isolates and results in a signature of host-association. The impact of this phenomenon in gene content variation has been described as similar to that exerted by horizontal gene transfer in nonclonal species (Bolotin and Hershberg 2015).

How humans are intervening with this process by domestication of animals is an interesting question that is not only relevant in terms of natural history of bacterial diseases but also in terms of preventive measures such as vaccination.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Literature Cited

Al Dahouk S, et al. 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. J Microbiol Methods. 69:137–145.

Assefa S, Keane TM, Otto TD, Newbold C, Berriman M. 2009. ABACAS: algorithm-based automatic contiguation of assembled sequences. Bioinformatics 25:1968–1969.

Audic S, Lescot M, Claverie J-M, Cloeckaert A, Zygmunt MS. 2011. The genome sequence of *Brucella pinnipedialis* B2/94 sheds light on the evolutionary history of the genus *Brucella*. BMC Evol Biol. 11:200.

Barbier T, Nicolas C, Letesson JJ. 2011. *Brucella* adaptation and survival at the crossroad of metabolism and virulence. FEBS Lett. 585:2929–2934.

Bolotin E, Hershberg R. 2015. Gene loss dominates as a source of genetic variation within clonal pathogenic bacterial species. Genome Biol Evol. 7:2173–2187.
Bourg G, O'Callaghan D, Boschiroli ML. 2007. The genomic structure of *Brucella* strains isolated from marine mammals gives clues to evolutionary history within the genus. Vet Microbiol. 125:375–380.
Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S. 2000. Molecular characterization of *Brucella* strains isolated from marine mammals. J Clin Microbiol. 38:1258–1262.
Carver TJ, et al. 2005. ACT: the Artemis Comparison Tool. Bioinformatics 21:3422–3423.
Caspi R, et al. 2014. The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res. 42:D459–D471.
Chain PSG, et al. 2005. Whole-genome analyses of speciation events in pathogenic *brucellae*. Infect Immun. 73:8353–8361.
Cloeckaert A, et al. 2001. Classification of *Brucella* spp. isolated from marine mammals by DNA polymorphism at the omp2 locus. Microbes Infect. 3:729–738.
Croucher NJ, et al. 2014. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 43:e15.
Cvetnić Ž, et al. 2016. Evidence of *Brucella* strain ST27 in bottlenose dolphin (*Tursiops truncatus*) in Europe. Vet Microbiol. 196:93–97.
Dawson CE, et al. 2008. Phenotypic and molecular characterisation of *Brucella* isolates from marine mammals. BMC Microbiol. 8:224.
de Jong MF, et al. 2008. Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. Mol Microbiol. 70:1378–1396.
Ewald PW. 2004. Evolution of virulence. Infect Dis Clin North Am. 18:1–15.
Le Flèche P, et al. 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. 6:9.
Garofolo G, et al. 2014. *Brucella ceti* from two striped dolphins stranded on the Apulia coastline, Italy. J Med Microbiol. 63:325–329.
Goodhead I, Darby AC. 2015. Taking the pseudo out of pseudogenes. Curr Opin Microbiol. 23:102–109.
Greger M. 2007. The human/animal interface: emergence and resurgence of zoonotic infectious diseases. Crit Rev Microbiol. 33:243–299.
Grissa I, Bouchon P, Pourcel C, Vergnaud G. 2008. On-line resources for bacterial micro-evolution studies using MLVA or CRISPR typing. Biochimie 90:660–668.
Guzmán-Verri C, et al. 2012. *Brucella ceti* and brucellosis in cetaceans. Front Cell Infect Microbiol. 2:3.
Hernández-Mora G, et al. 2008. Neurobrucellosis in stranded dolphins, Costa Rica. Emerg Infect Dis. 14:1430–1433.
Isidoro-Ayza M, et al. 2014. *Brucella ceti* infection in dolphins from the Western Mediterranean sea. BMC Vet Res. 10:206.
Jones KE, et al. 2008. Global trends in emerging infectious diseases. Nature 451:990–993.
Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44:D457–D462.
Kohler S, et al. 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. Proc Natl Acad Sci U S A. 99:15711–15716.
Lee JJ, et al. 2014. Characterization of betaine aldehyde dehydrogenase (BetB) as an essential virulence factor of *Brucella abortus*. Vet Microbiol. 168:131–140.
Li H, et al. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–2079.
Mancilla M. 2012. The *Brucella* genomic islands. In: López-Goñi I, O'Callaghan D, editors. *Brucella*: molecular microbiology and genomics. Caister Academic Press. pp. 36–57.
Maquart M, et al. 2009. MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*. BMC Microbiol. 9:145.
Maquart M, Fardini Y, Zygmunt MS, Cloeckaert A. 2008. Identification of novel DNA fragments and partial sequence of a genomic island specific of *Brucella pinnipedialis*. Vet Microbiol. 132:181–189.
Martín-Martín AI, et al. 2009. Analysis of the occurrence and distribution of the Omp25/Omp31 family of surface proteins in the six classical *Brucella* species. Vet Microbiol. 137:74–82.
Moreno E. 1997. In search of a bacterial species definition. Rev Biol Trop. 45:753–771.
Moreno E. 1998. Genome evolution within the alpha Proteobacteria: why do some bacteria not possess plasmids and others exhibit more than one different chromosome? FEMS Microbiol Rev. 22:255–275.
Moreno E. 2014. Retrospective and prospective perspectives on zoonotic brucellosis. Front Microbiol. 5:1–18.
Moreno E, Moriyón I. 2006. The genus *Brucella*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The prokaryotes. New York, NY: Springer. pp. 315–456.
Ocampo-Sosa AA, García-Lobo JM. 2008. Demonstration of IS711 transposition in *Brucella ovis* and *Brucella pinnipedialis*. BMC Microbiol. 8:17.
OIE. 2009. Bovine brucellosis. In: OIE terrestrial manual. pp. 1–35.
Page AJ, et al. 2015. Roary: rapid large-scale prokaryote pan genome analysis. pp. 13–15.
Page AJ, et al. 2016. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genomics. 2:e000056.
Quail MA, et al. 2009. A large genome centre's improvements to the Illumina sequencing system. Nat Methods. 5:1005–1010.
Quail MA, et al. 2012. Optimal enzymes for amplifying sequencing libraries. Nat Methods. 9:10–1.
Rajashekara G, Glasner JD, Glover DA, Splitter GA. 2004. Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. J Bacteriol. 186:5040–5051.
Rajashekara G, Glover DA, Banai M, O'Callaghan D, Splitter GA. 2006. Attenuated bioluminescent *Brucella melitensis* mutants GR019 (virB4), GR024 (galE), and GR026 (BMEI1090-BMEI1091) confer protection in mice. Infect Immun. 74:2925–36.
Ronneau S, et al. 2014. *Brucella*, nitrogen and virulence. Crit Rev Microbiol. 7828:1–19.
Ruiz-Ranwez V, et al. 2013. BtaE, an adhesin that belongs to the trimeric autotransporter family, is required for full virulence and defines a specific adhesive pole of *Brucella suis*. Infect Immun. 81:996–1007.
Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.
Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.
Suárez-Esquivel M, et al. 2016. *Brucella abortus* strain 2308 Wisconsin genome: importance of the definition of reference strains. Front Microbiol. 7:1–6.
Toft C, Andersson SGE. 2010. Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet. 11:465–475.
Tsolis RM, et al. 2009. Genome degradation in *Brucella ovis* corresponds with narrowing of its host range and tissue tropism. PLoS ONE. 4:e5519.
Wattam AR, et al. 2009. Analysis of ten *Brucella* genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle. J Bacteriol. 191:3569–3579.

Wattam AR, et al. 2014. Comparative phylogenomics and evolution of the brucellae reveal a path to virulence. J Bacteriol. 196:920–930.
World Health Organization. 2014. The control of neglected zoonotic diseases. In: NZD4 organising committee, editor. WHO conference report. Geneva: WHO Press. pp. 23–35.
Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18:821–829.
Zúñiga-Ripa A, et al. 2014. *Brucella abortus* depends on pyruvate phosphate dikinase and malic enzyme but not on Fbp and GlpX fructose-1,6-bisphosphatases for full virulence in laboratory models. J Bacteriol. 196:3045–3057.

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Dwarf sperm whale is a reservoir of Brucella sp. ST27, linked to human infections

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Keywords:	Brucella ceti, Brucella, brucellosis, Kogia, dwarf sperm whale, ST27, zoonosis
Abstract:	Brucella sp. ST27 infects humans. A dwarf sperm whale Kogia sima, displaying clinical and pathological signs of brucellosis, aborted and died in the Pacific coast of Costa Rica. Brucella sp. ST27 was isolated from both, mother and calf, demonstrating that this cetacean species is a reservoir of Brucella sp. ST27

Full title: Persistence of *Brucella abortus* Lineages Revealed by Genomic Characterization and Phylo-temporal Analysis

Short title: Phylo-temporal Analysis of *Brucella abortus* Incursions

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Abstract

Brucellosis, caused by *Brucella abortus*, is a major disease of cattle and humans, with high prevalence in Costa Rica (CR). The disease was reported in CR during the beginning of the 20th century and, despite all efforts, it has not been controlled. *B. abortus* isolated in Costa Rica from bovines, humans and water buffalo were analyzed by whole genome sequencing (WGS) and associated to geographic origin, date of introduction and phylogenetic associations. Our findings are relevant from the epidemiological perspective. Following the brucellosis prevalence and the idiosyncratic settings of several middle- and low- income countries, similar scenarios could be found in other latitudes.

Chapter 4: Proposal for a suitable strategy to control brucellosis in Costa Rica

In this chapter, we discuss critical factors that should be considered to develop a suitable strategy for controlling bovine brucellosis in Costa Rica, and other countries in the region.

The control and eradication of brucellosis is a complex undertaking that requires the active involvement of all parties, including government, stakeholders, and farmers, among several (Blasco *et al.*, 2016). If the authorities and stakeholders propose eradication, then correct political decisions and sustained commitment of all relevant parties must be accomplished. The first critical factors for initiating a brucellosis control program is the understanding of the epidemiological status of the disease in the country, the identification of the circulating *Brucella* species in the livestock and the infection incidence in humans (Lubroth *et al.,* 2007). For this, adequate epidemiological data must be collected using serological tests with the highest sensitivity and specificity, as well as reliable and efficient isolation and characterization of the circulating *Brucella* strains.

Once the epidemiological units have been identified and defined, a vaccination-test- slaughter strategy has to be designed. Concomitantly, a strict individual identification, and control of movements between the epidemiological units must be achieved. It is mandatory that the administration provides the funds for the intervention costs and promotes the active involvement of all parties, including relevant stakeholders and farmers, who must receive educational information through the development of the campaign (Blasco *et al.*, 2016). An economic incentive for the brucellosis-free status should be promoted between farmers and government as well as compensation for culling with the market value of the animals to actively involve the producers (Blasco *et al.,* 2016). The current situation in Costa Rica is described in table 1.

The definition of the epidemiological unit (restricted area of intervention, regardless of administrative or national borders) and the accurate description of herd-level should be the base of the selected strategy. For this purpose, three strategies are recommended according to the herd seroprevalence of brucellosis (Blasco *et al*., 2016):

a) **Very low herd seroprevalence (≤1-4%).** A test and slaughter eradication program and the ban of vaccination could be recommended to eradicate the disease in short to medium term.
b) **Low to moderate herd seroprevalence (5-10%).** A combined eradication program based on the simultaneous application of S19 vaccination in young replacements and test and slaughter of seropositive adult animals is recommended to eradicate the disease at medium-long term.
c) **High herd seroprevalence (≥ 10%).** No matter the level of professional organization and economic resources, a mass S19 vaccination program is the only strategy to control the disease, a step strictly necessary before undertaking any other control or eradication measure.

Agglutination tests, in particular RBT, should be considered as the baseline assay for evaluating infected animals in the epidemiological units in Costa Rica (Ducrotoy *et al.,* 2016). In addition, S19 is the vaccine of choice. Up to now, this is the only approved vaccine that confers an adequate level of individual and herd protection against bovine brucellosis (Moriyón *et al.,* 2004; OIE, 2018). Considering this, the herd seroprevalence estimated in the random sampling performed during 2012-2013 (Chapter 1), only the Brunca Region is classified as low-moderate seroprevalence (4.1%), and the rest of Costa Rica possesses a seroprevalence between 10.3% and 16% (Hernández-Mora *et al.,* 2017a). Therefore, with the above possible described strategies and in accordance with the herd seroprevalence, the most feasible strategy is “c” corresponding to mass vaccination of all herds regardless the age and physiological condition (Nicoletti, 1976; Alton &Corner, 1981) before moving to other strategies.

The rationale behind mass vaccination with S19 strategy claims that, under conditions of high seroprevalence, vaccination followed by test and slaughter strategies are not applicable due to substantial economic restrictions and difficulties of controlling the circumstances in poor or remote areas. The serological interference caused by S19 vaccine could be managed with adequate time between vaccination and diagnosis and identification of the vaccinated animals (Blasco *et al*., 2016). Mass vaccination with *B. abortus* S19 was performed in the early seventies by countries

like the United States (see Anexx 1), Australia, and New Zealand, among others that have successfully controlled and eradicated brucellosis afterward (Crawford and Hidalgo, 1977). This strategy was developed as an alternative to calf vaccination with reduce doses of S19 (Nicoletti, 1976; Alton & Corner, 1981).

To avoid the problems carried out by S19 subcutaneous vaccination, the use of a reduced S19 dose by conjunctival route recommended by the OIE and FAO (OIE, 2018, Blasco *et al*., 2016) has been implemented by several authors (Corner & Alton, 1981; Corner, 1983; Nicoletti *et al.,* 1978a; Nicoletti *et at.,* 1978b; Lubroth *et al., 2*007. This vaccination strategy has several advantages; i) it diminishes the risk of abortion in pregnant animals; ii) it avoids long-term positive reactions that hamper the distinction of infected from vaccinated animals; iii) it does not require needle for application, diminishing the risk of accidental inoculations; iv) it is the cheapest vaccine on the market (table 2), finally; v) it is readily applied and less time-consuming. To decrease the overall seroprevalence, the coverage of S19 mass vaccination should be close to 100% and achieved during the shortest time possible (Blasco *et al.*, 2016).

As expected, the serological background of mass vaccinated animals living in a highly infected environment is challenging to interpret (Plommet & Fensterbank, 1976). Even by the conjunctival route, the serological response induced by S19 vaccine in adult animals is of greater intensity and duration than that induced in young replacements. Although protected, vaccinated animals produce anamnestic responses upon contact with field strains in a highly infected environment, precluding the straightforward diagnoses (Blasco *et al.,* 2016). For this reason, a serological test should be carried out only after 18 months of vaccination, once the antibody titers against LPS have lowered in the immunized animals. Test and slaughter strategy may be considered after mass vaccination in some epidemiological units with lower seroprevalence. For this, the combination of RBT with native hapten gel precipitation test has demonstrated to be useful, and therefore recommended by the OIE (Greiner *et al.,* 2009; OIE, 2018). Commonly several rounds of mass vaccination are required before the overall prevalence is lowered to the necessary prevalence levels to move to the following “b” strategy, based on the simultaneous application

of S19 vaccination in young replacements and test and slaughter of seropositive adult animals, with the economic support of the authorities. Only the calves and replacements should be vaccinated every year, following individual tagging of the immunized animals. Once the prevalence has lowered, and the disease is under control after several years of a successful vaccination, then it is possible to consider a strict intervention to move towards the eradication program, step “a”.

Parallel to the control and eradication strategies, public health and veterinary authorities must develop educational policies directed to the general public as well as farmers indicating the preventive measures such as pasteurization of dairy products, and avoiding consumption of raw milk, as well as biosafety protocols in the animal farms, to prevent reinfections (Mattar *et al*., 2017; WHO, 2014; CDC, 2017; NYDH, 2017). Also, health centers and hospitals must include brucellosis specific tests as differential diagnosis of common diseases, such as dengue, zika, chikungunya, trypanosomiasis, malaria and another fibril illness present in Costa Rica (Mattar *et al*., 2017; WHO, 2014; CDC, 2017). The use of RBT as a screening technique (Alton & Jones, 1988) following the current recommendations of the World Health Organization and the Center for Disease Control (WHO, 2014; CDC, 2017) is a straight forward inexpensive measure (WHO, 2014).

Table 1. Factors to include in the strategies of control of brucellosis in Costa Rica

	No available	Available
1. Protective vaccine		X
2. Affordable diagnostic techniques with high sensitivity and specificity		X
3. Identification of all herds	X	
4. Capacity of veterinary services to conduct the interventions on the whole population	X	
5. Availability of funds for intervention costs	X	
6. Active involvement of the breeders and other relevant stakeholders.	X	
7. Description of the brucellosis status*		X
8. Occurrence of brucellosis in humans**		X
9. Circulating *Brucella* species in the livestock*		X
10. Individual identification of the whole animal census	X	
11. Full control of the animal movements	X	
12. Funds for compensation for culling	X	
13. Sustained commitment of all relevant authorities and stakeholders	X	

*Chapter 1, **Chapter 2

Table 2. Prices per dose of commercially vaccines against brucellosis included in the OIE (2019), available in Costa Rica

	S19 Conjunctival (CZV)	**S19 Subcutaneous (CDV)**	**RB51 Single dose (MSD)**	**RB51 (Double dose)* (MSD)**
Price	$0.80	$1.6	$1.85	$3.7

CZV: CZ vaccines, Spain CDV: Diagnostic and Vaccines for animal health, Argentina, MSD: Merck Sharp and Dohme, United States.

*Protocol recommended by the fabricant https://www.msd-salud animal.com.co/productos/rb51/informacion.aspx.

CONCLUDING REMARKS

Brucellosis is an ancient zoonosis that remains prevalent in the Americas (Cardenas *et al*., 2019) (Annex 1 and 2). As expected, most of the countries have limited financial resources for generating reliable epidemiological data and implementing a brucellosis control program that involves broad vaccination coverage, wide-ranging serological testing and identification and removal of infected animals with compensation, and lack of incentives to achieve brucellosis-free certification. Instead, the epidemiological data is mostly fragmented and inconsistent, so the implemented measures are discontinuous and non-systematic (Cárdenas *et al*., 2019; Aznar *et al*., 2012; Moreno, 2002).

Unfortunately, most governments ignore the scientific literature describing the reliable diagnostic techniques as well as the efficient vaccines, vaccination strategies, and control measures. Instead, they have followed unsuitable control measures for the epidemiological conditions of the countries, and are biased by propaganda that has led to the selection of fashionable end expensive serological tests and unsuitable vaccines. Following this, most American countries have implemented brucellosis “control programs” involving voluntary actions, based on non-systematic vaccination and revaccination with RB51, S19, or both. These approaches have aggravated the problem since it has been established that RB51 vaccine does not confer adequate protection for bovine brucellosis (Moriyón *et al.,* 2004; Blasco *et al.,* 2016). Moreover, since its introduction 30 years ago, RB51 has failed to control or eradicate brucellosis in the Americas or other latitudes; and human infections with this vaccine are not trivial. Indeed, due to a lack of detectable antibodies in routine serological techniques, patients infected with rough RB51 are seldom diagnosed (Ashford, 2004). Additionally, this strain is resistant to rifampicin, one of the antibiotics used to combat human brucellosis, aggravating the zoonotic problem. Unless the RB51 strain is recovered by culture and accurately characterized, medical personnel are not aware of the infection, since traditional assays to detect antibodies against field *Brucella* strains do not work with RB51 cases (Mattar *et al*., 2017; WHO, 2014; CDC, 2017; NYDH, 2017).

In Costa Rica, the obligatory basis of the control program of brucellosis remained from 1958 until 1999, year at which the legislation handed the Brucellosis Program over to private hands on a voluntary basis, performed under the supervision of the Veterinary Services (MAG-CR, 2000). This modification diminished the control measures achieved in previous years, and aggravated the problem as already demonstrated (figure 3) (Hernández-Mora *et al*., 2017a).

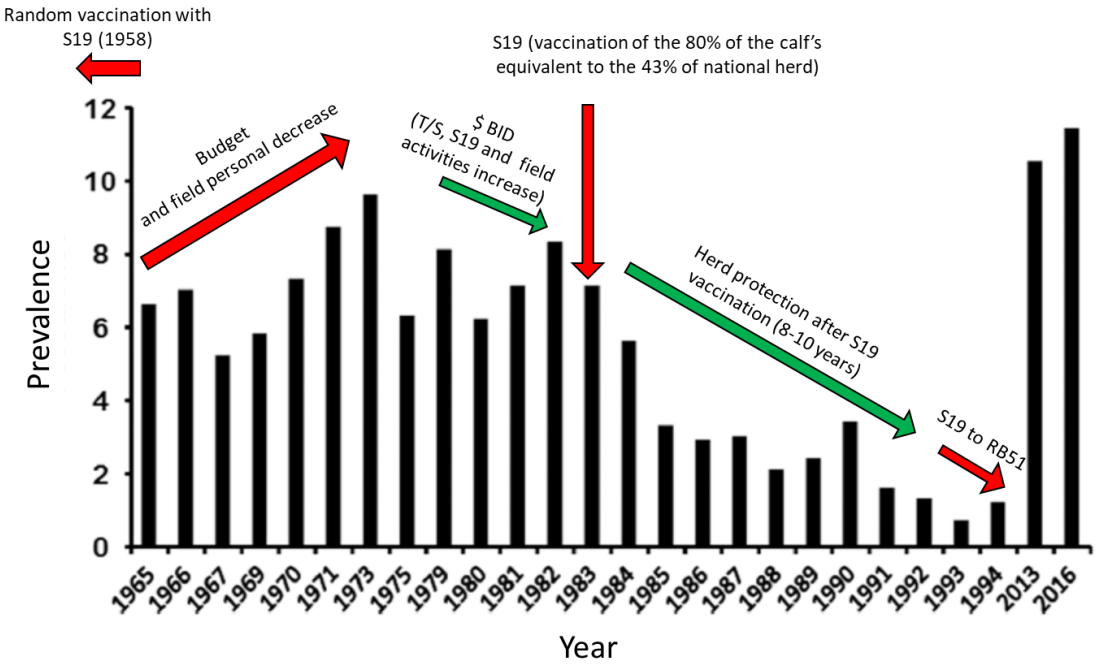

Figure 3. Official reported prevalence of brucellosis estimated by agglutination test in Costa Rica 1965 to 2016. The red arrows indicate the strategies used by the government and the green arrows indicate the years when the disease decrease its prevalence.

Presently, the program lacks: i) specific governmental financial resources, ii) field staff, iii) compulsory vaccination with S19, iv) adequate information to the producers and, v) actions for the identification of the vaccinated animals. This scenario is aggravated by the undisciplined use of RB51 instead of *B. abortus* S19 (Piagro, 1996; Hernández-Mora *et al.,* 2017a). Therefore, it is not surprising that in

terms of seroprevalence, brucellosis in cattle has increased from 2% in the nineties, to almost 11% in current years, as estimated by RBT (Piagro, 1996; Vicente *et al*., 1983; Hernández-Mora *et al*., 2017a) (figure 4).

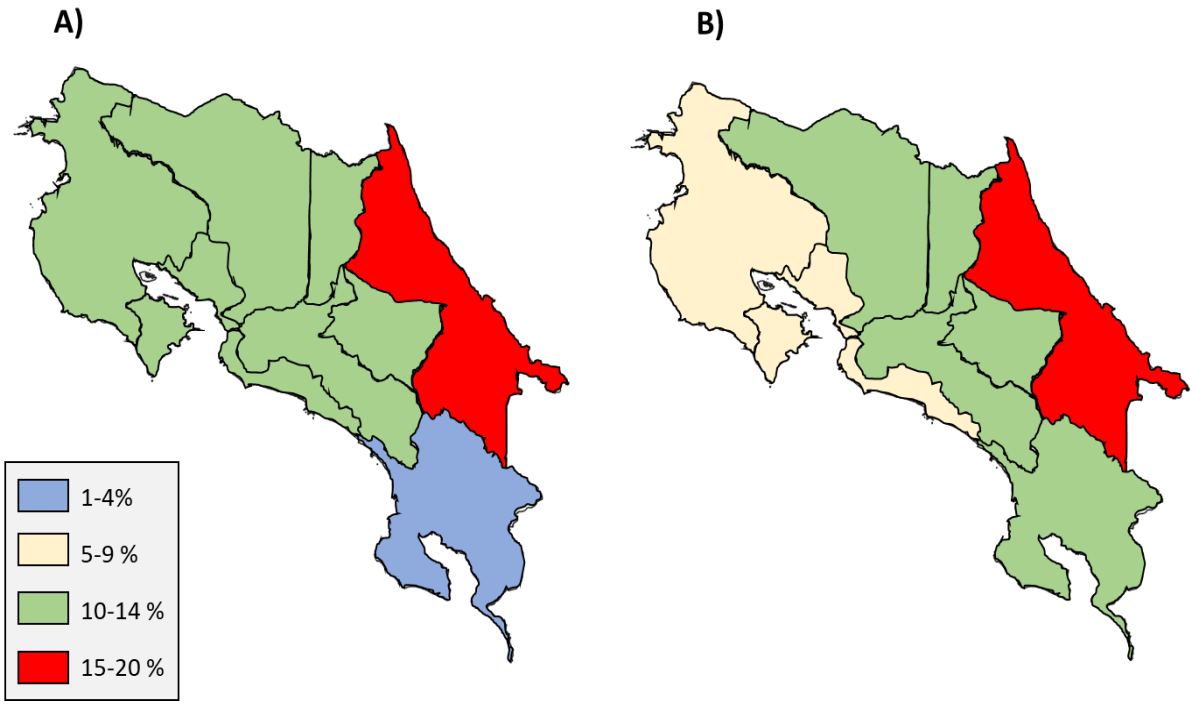

Figure 4. Herd seroprevalence of brucellosis in bovines of Costa Rica during 2012-2016. By RBT, the majority of the territory has a seroprevalence ≥ 10% using A**)** Random sampling during 2012-2013 and B) Non-random sampling during 2014-2016**.**

The increased brucellosis prevalence is worsening due to the high consumption of unpasteurized dairy products that reach up to 40 % of the consumed milk and cheese in Costa Rica (DIPOA, SENASA 2017), and the use the of febrile antigens in health centers and hospitals. As already established, this serological technique lacks diagnostic sensitivity and specificity to unambiguously identified human brucellosis cases (WHO, 2015). In consequence, several cases may course without diagnoses.

During this thesis, we have described the epidemiological status of bovine brucellosis as well as infections in other domestic animals and marine mammals.Besides,, we have made efforts to identify the *Brucella* strains that are prevalent in the territory and recommended parameters for the control of brucellosis to follow in the next years in Costa Rica. In collaboration with health authorities, we have also implemented protocols for the detection of human brucellosis in health centers and hospitals, replacing the use of febrile antigens; and instead, using RBT as screening assay. We have collaborated with different groups in the diagnosis of human brucellosis in stranded cetaceans in Costa Rica and called the attention of the risk of zoonosis and described *Brucella* species (*B. neotomae*) infecting humans, which were not considered zoonotic. Finally, we have collaborated in the standardization of the isolation, identification, and molecular diagnostic techniques that have served as a framework for reliable epidemiological studies in Costa Rica, and as a reference for studies in other countries.

While much has been accomplished, more work is still needed. Efforts to work together with the producers, cattle industry, government, and scientists, under the concept of “One Health”, should be carried out in order to finally achieve the control and eradication of brucellosis in Costa Rica. Without the long-term engagement of all parties, elimination of the disease will not be achieved, even with the best strategies and surveillance activities.

GENERAL REFERENCES

Agronegocios. (2017). El programa contra la brucelosis y tuberculosis está en marcha. Access at: http://agronegocios.com.bo/el-programa-contra-la-brucelosis-y-tuberculosis-esta-en-marcha Accessed: 6-6-2019.

Agudelo-Flórez, P., Castro, B., Rojo-Ospina, R., & Henao-Villegas, S. (2012). Canine brucellosis: Seroprevalence and risk factors in pets from eleven neighbourhoods in Medellin, Colombia Rev. *Salud pública*. 14 (4): 644-656.

Al Dahouk, S., Flèche, P.L., Nöckler, K., Jacques, I., Grayon, M., Scholz, H.C., Tomaso, H., Vergnaud, G., & Neubauer, H. 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods*. 69:137–145.

Alton, G. G., & Corner, L. A. (1981). Vaccination of heifers with a reduced dose of *Brucella abortus* strain 19 vaccine before first mating. *Aust Vet J*. 57, 548-550.

Alton G.G., Jones, L.M., Angus, R.D., & Verger, J.M. (1988). Techniques for brucellosis laboratory. INRA, France.

Ashford D.A., di Pietra J., Lingappa J., Woods C., Noll H., Neville B., Weyant R., Bragg S.L., Spiegel R.A., Tappero J.,& Perkins B.A. (2004). Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. *Vaccine*. 3;22 (25-26):3435-9.

Assefa, S., Keane, T.M., Otto, T.D., Newbold, C., & Berriman, M. (2009). ABACAS: algorithm-based automatic contiguation of assembled sequences. Bioinformatics 25:1968–1969.

Attademo, F.L.N., Silva, J.C.R., Luna, F.O., Ikeda, J., Foppel, E.F.C, Sousa G.P., Bôaviagem-Freire, A.C., Soares, R.M., Faita, T., Batinga, M.C.A., & Keid, L.B. (2018) Retrospective survey for pathogens in stranded marine mammals in northeastern Brazil: *Brucella* spp. infection in a clymene dolphin (*Stenella clymene*). *J Wildl Dis*. 2018 Jan; 54(1):151-155.

Aznar, M.N., Samartino, L.E., Humblet, M.F., & Saegerman, C. (2012). Bovine brucellosis in Argentina and bordering countries: update. *Transbound Emerg Dis*. 61(2):121-33.

Belize Agricultural Health Authority. (2011). Prevention, control and eradication of bovine brucellosis. Access at: https://members.wto.org/crnattachments/2011/sps/BLZ/11_1948_00_e.pdf. Accessed: 3-3-2019.

Berger, S. (2018). Brucellosis: Global Status. Gideon Informatics. E-Book series. Los Angeles, California, USA.

Blasco, J.M., Moreno, E., & Moriyón, I. (2016). In: Brucellosis vaccines and vaccine candidates in: Veterinary Vaccines for Developing Countries. Metwally, S and Viljoen, G J El Idrissi, A. FAO (Rome).

Borba, M.R., Stevensonc, M.A., Goncalves, V.S.P., Ferreira-Neto, J.S., Ferreira, F., Amakua, M., Telles, E.O., Santana, S.S., Ferreira, J.C.A., Lôbo, J.R., Figueiredog, V.C.F., & Dias, R.A. (2013). Prevalence and risk-mapping of bovine brucellosis in Maranhão State, Brazil. *Prev Vet Med*. 110:169–176.

Brennan, S.J., Ngelekan, M., Philibert H.M., Forbes l.B., & Allen A.L. (2008). Canine brucellosis in a Saskatchewan Kennel., *Can Vet J* 49: 703-708.

Bruha, P. (2015). The Brazilian Pet Market. Access at: https://thebrazilbusiness.com/article/the-brazilian-pet-market. Accessed: 7-2-2019.

Cannon, R.M., Roe, R.T. (1982). Livestock Disease Surveys. A field manual for veterinarians. Canberra.35.

Campos, E., Vicente, G., Ramírez, J.A., & Moreno, E. (1984). Evaluación seroepidemiológica de la brucelosis humana en poblaciones de riesgo ocupacional. *Acta Med Cost* (Costa Rica). 47:8-9.

Cardenas, L., Awada, L., Tizzani, P., Cáceres, P., & Casal J. (2019). Characterization and evolution of countries affected by bovine brucellosis (1996–2014). *Transbound Emerg Dis* 66 (3) 1280-1290.

CDC. (2017). Center for Disease Control and Prevention. Brucellosis reference guide: Exposure, testing and Prevention. Access at: https://www.cdc.gov/brucellosis/pdf/brucellosi-reference-guide.pdf Accessed: 2-3-2019.

Cervera-Hernández, M.E., Ordaz-Vázquez, A.,Torres-González, P., Chávez-Mazari, B., Soberanis-Ramos, O., Sifuentes-Osornio, J., Ponce de León, A., & Bobadilla-del Valle, M. (2016). Seroprevalence of brucellosis among dairy farm workers in Mexico. *Salud pública de México* (58) 3:366-370.

Chajon, D.E. (2015). Estudio retrospectivo sobre casos de brucelosis bovina en Guatemala durante los años 2010 al 2013, tomando como base las muestras procesadas en el Laboratorio de Microbiología de la Facultad de Medicina Veterinaria y Zootecnia. Tesis de Licenciatura Universidad de San Carlos de Guatemala. Facultad de Medicina Veterinaria y Zootecnia. Ciudad de Guatemala, Guatemala.

Chan, J., Baxter, C., & Wenman, W.M. (1989). Brucellosis in an Inuit child, probably related to caribou meat consumption. *Scand J Infect Dis*. 21(3):337-8.

Chanto. G. National Reference Center of Bacteriology (CNBR-INCIENSA) (2018). Human patients infected with brucellosis in the country 2003-2017, San José, Costa Rica.

Chikweto, A., Tiwari, K.P., Kumthekar, S., Stone, D., Louison, B., Thomas, D., Sharma, R., & Hariharan, H. (2013a). Serologic detection of antibodies to *Brucella* spp. using a commercial ELISA in cattle in Grenada, West Indies. *Trop Biomed*. 30 (2): 277–280.

Chikweto, A., Tiwari, K. P., Kumthekar, S., Langeois, Q., Gozlan, J., Lanza, M., Paterson, T., & Sharma, R. N. (2013b). Exposure to *Brucella canis* in owned and stray dogs in Grenada, West Indies. *Int J. Anim. Vet Adv* 5(2): 58-60.

CNRS-Centre National de la Recherche Scientifique. (2017). MLVA-Net for *Brucella*. http://mlva.u-psud.fr/*Brucella*/.

Cocom, E. (2016). Belize Ag Report. BLPA News (31). Access at: http://agreport.bz/blpa-news031/ Accessed: 18-1-2018.

Colman, G., Abente, A., Cristaldo, L., & Martínez, B. (2017). Seroprevalnceia de brucelosis canina (*Brucella canis*) en la ciudad de Concepción-Paraguay. *Compend. Cienc. Vet*. 07 (01): 41- 45.

Contreras, J. (2000). Brucelosis. Enfermedades de los Bovinos, Diagnóstico, Tratamiento y Control. 2da Ed. Barquisimeto, Venezuela, 475-489.

Corbel, J. (1997). Brucellosis: An Overview. 1st International Conference on Emerging Zoonoses Jerusalem, Israel. *Emerg Infect Dis* (3) 2, April–June 1997.

Corner, L. A. (1983). Three aspects of bovine brucellosis: epidemiology, the role of bulls and vaccines. N. S. W. *Veterinary Proceedings* 19, 47–48.

Corner, L. A. & Alton G. G. (1981). Persistence of *Brucella abortus* strain 19 infection in adult cattle vaccinated with reduced doses. *Res Vet Sci* 31, 342-344.

Correa, C.N.M, Gottschalk, A.F., Correa, W.M., Silva, A.S., & da Teruya, J.M. (1972). Brucellosis and leptospirosis of cattle in Sao Manuel, Sao Paulo State. Serological study 38 (2) 46-51.

Costa, M.D., Filho, M.P., Santana, E.C., Reboucas, M.P.P., & Filho, O.R.S. (1974). Contribucao ao estudo da brucelose na Bahía. II Prevalencia nos municipios de Meeiros Neto, Itanhem e Lagedao. *Bol. Inst. biol. Bahía*, 13:1-7.

Crawford, R.P., & Hidalgo, R.J. (1977). Bovine Brucellosis: An International Symposium. Texas A&M University Press.

Da Silva, J.B., Rangel, C.P., da Fonseca, A.H., de Morais, E., Vinhote, W.M., da Silva Lima, D.H., da Silva, E., Silva, N., & Barbosa, J.D. (2014). Serological survey and risk factors for brucellosis in water buffaloes in the state of Pará, Brazil. *Trop Anim Health Prod.* 2014 Feb; 46(2):385-9.

De-Miguel, M.J., Marín, C.M., Muñoz, P.M., Dieste, L., Grilló, M.J., Blasco, J.M. (2011). Development of a selective culture medium for primary isolation of the main *Brucella* species. *J Clin Microbiol*. 49:1458-1463.

De Paula Dreer, M.K., Gonçalves, D.D., da Silva Caetano, I.C., Gerônimo, E., Menegas, P.H., Bergo, D., Ruiz Lopes-Mori, F.M., Benitez, A., de Freitas, J.C., Evers, F., Navarro, I.T., & Martins, L. A. (2013). Toxoplasmosis, leptospirosis and brucellosis in stray dogs housed at the shelter in Umuarama municipality, Paraná, Brazil. *J Venom Anim Toxins Incl Trop Dis.* 25;19(1):2.

Dentinger, C.M., Jacob, K., Lee, L.V., Mendez, H.A., Chotikanatis, K., McDonough, P.L., Chico, D.M., De, B.K., Tiller, R.V., Traxler, R.M., Campagnolo, E.R., Schmitt, D., Guerra, M.A., & Slavinski, S.A. (2015). Human *Brucella canis* Infection and Subsequent Laboratory Exposures Associated with a Puppy, New York City, 2012. *Zoonoses Public Health*, 62(5), 407-14.

Dirección General de Ganadería, Bolivia. (1974). Programa nacional de control de la fiebre aftosa, rabia y brucelosis. Ministerio de asuntos campesinos y agropecuarios. La Paz, Bolivia.

DIPOA-SENASA-CR. (2017). Informe: Encuesta en 320 queseras a nivel nacional. Heredia, Costa Rica.

Ducrotoy, M.J., Conde-Álvarez, R., Blasco, J.M., & Moriyón, I., (2016). A review of the basis of the immunological diagnosis of ruminant brucellosis. *Vet. Immunol. Immunopathol*. 17, 81–102.

Duran U. (2019). Dirección general de Ganadería. Encargado de la División de Vigilancia epidemiológica. Republica Dominicana. Consultado: 25 Julio 2019

FAO. (2017). Food and Agriculture Organization of the United Nations: Cattle Population, FAOSTAT_data. Access at: http://www.fao.org/faostat/en/#search/cattle%20inventory. Accessed: 3-2-2019.

Fort, M., Baldone, V., Fuchs, L., Gimenez, H., Rojas, M., Breccia, J.D., & Oyhenart, J., (2012). Experimental infection of rabbits (*Oryctolagus cuniculus*) with *Brucella suis* biovar 1 isolated from wild hares (*Lepus europaeus*). *Vet. Microbiol*. 156, 439–442.

Foster G., Jahans K.L., Reid R.J. & Ross H.M. (1996). Isolation of *Brucella* species from cetaceans, seals and an otter.*Vet. Rec*., 138 (24), 583–586.

Furtado, M.M., Gennari, S.M., Ikuta, C.Y., Jácomo, ATdA, de Morais, Z.M., Pena, H.F.d.J., de Oliveira Porfírio, G. E., Silveira, L., Sollmann, R., Oliveira de Souza, G., Mundim Tôrres, N., & Ferreira Neto, J.S. (2015). Serosurvey of smooth *Brucella*, *Leptospira* spp. and *Toxoplasma gondii* in Free-Ranging Jaguars (*Panthera onca*) and Domestic Animals from Brazil. *PLoS ONE* 10(11): e0143816.

García-Carillo, C., Poujol, E., & Abastida, J. (1978). Aislamiento de *Brucella* en Honduras. *Zoonosis*, 20:74-77

García Carrillo, C. (1979). Aislamiento de *Brucella abortus* biotipo 1 y 4 en Nicaragua. *Bol. OSP*, 87: 132-134.

García Carrillo, C. (1981). Present Status of Bovine Brucelosis in the Americas. In: Interamerican Meeting of Directors of Animal Health. Inter-American Institute for Cooperation on Agriculture (IICA)-Pan American Zoonosis Center. Buenos Aires, Argentina.

García González, G.S., Saborido Pérez, I.M., Ramírez Lana, L., & Ponce de León Avila, I. (2012). First report of infective endocarditis in Cuba as a result of brucellosis. *Rev Cubana Med Trop*. 64 (1):65-68.

García-Juárez, G., Ramírez-Bribiesca, J.E., Hernández-Vázquez, M., Hernández-Calva, L.M., Díaz-Aparicio, E., & Orozco-Bolaños, H. (2014). Risk analysis of brucellosis in the state of Tlaxcala. *Salud publica mex* 56 (4) 355-62.

Garín, A., (2011). Program of Control/Eradication of bovine brucellosis in Uruguay. In: Proceedings of the Brucellosis 2011 International Research Conference, September 21–23, Buenos Aires, Argentina, pp 18–19.

Gil, A., Piaggio, J., Silva, M., Garin, A., Caponi, O. (2009). International Symposia on Veterinary Epidemiology and Economics proceedings, ISVEE 12: Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics, Durban, South Africa, Theme 1 - Surveillance and disease control: Brucellosis,Disease monitoring & control. Evaluation of animal disease, p 40, Aug 2009.

Gioia, G., Vinueza, R.L., Cruz, M., Jay, M., Corde, Y., Marsot, M., & Zanella, G. (2018). Estimating the probability of freedom from bovine brucellosis in the Galapagos Islands. *Epidemiol Infect*. 19: 1–3.

Giraldo Echeverri, C.A., Ruiz Cortés, Z.T., & Olivera Ángel, M. (2009). *Brucella canis* en Medellín (Colombia), un problema actual. Revista U.D.C.A Actualidad & Divulgación Científica 12 (1): 51-57.

Gonçalves, D.D., Teles, P.S., dos Reis, C.R., Lopez, F.M., Freire, R.L., Navarro, I.T., Alves, L.A., Muller, E.E., & de Freitas, J.C. (2006). Seroepidemiology and occupational and environmental variables for leptospirosis, brucellosis and toxoplasmosis in slaughterhouse workers in the Paraná State, Brazil. *Rev. Inst. Med. Trop. S. Paulo* 48(3):135-140.

González, J. (1999). Programa de brucelosis: Situación epidemiológica y estrategias para la prevención y el control / erradicación en Venezuela. Reunión consulta de expertos OPS / OMS sobre vacunas y estrategias de vacunación en los programas de control / erradicación de brucelosis. 16-18 de noviembre de 1999. Santiago de Chile, 7-8.

González-Barrientos, R., Morales, J.A., Hernández-Mora, G., Barquero-Calvo, E., Guzmán-Verri, C., Chaves-Olarte, E., & Moreno, E. (2010). Pathology of striped dolphins (*Stenella coeruleoalba*) infected with *Brucella ceti*. *J. comp. Pathol*.,142 (4), 347–352.

Greiner, M., Verloo, D. & De Massis, F. (2009). Meta-analytical equivalence studies on diagnostic tests for bovine brucellosis allowing assessment of a test against a group of comparative tests. Prev Vet Med 92, 373–381.

Grosnier, J. (1964). Les zoonoses en Haití. *Bull. Off. Int. Epiz*., 61: 417-427.

Guzmán-Verri, C., Suárez-Esquivel, M., Ruíz-Villalobos N., S. Zygmunt M., Gonnet, M., Campos E., Víquez-Ruiz E., Chacón-Díaz C., Aragón-Aranda, B., Conde-Álvarez R., Moriyón I., Blasco J.M., Muñoz P., Baker K.S., Thomson N.R, Cloeckaert, A., Moreno E. (2019). Genetic and Phenotypic Characterization of the Etiological Agent of Canine Orchiepididymitis Smooth *Brucella* sp. BCCN84.3. *Front. Vet. Sci*., 07 June 2019 https://doi.org/10.3389/fvets.2019.00175.

Hernández- Mora, G., González-Barrientos, R., Morales, J.A., Chaves- Olarte, E., Guzmán-Verri, C., Barquero-Calvo, E., De-Miguel, M.J., Marín, C.M., Blasco, J.M., & Moreno E. (2008). Neurobrucellosis in stranded dolphins, Costa Rica. *Emerg. Infect. Diseas*. 14 (9): 1429- 1433.

Hernández-Mora, G., Ruiz-Villalobos, N., Bonilla-Montoya, R., Romero Zúñiga, J.J., Jiménez-Arias, J., González-Barrientos, R., Barquero- Calvo, E., Chacó Díaz, C., Rojas, N., Chaves-Olarte, E., Guzmán- Verri, C., & Moreno, E. (2017a). Epidemiology of bovine brucellosis in Costa Rica: lessons learned from failures in the control of the disease. *PLoS ONE* 12(8): e0182380.

Hernández-Mora, G., Bonilla-Montoya, R., Barrantes- Granados, O., Esquivel-Suárez, A., Montero-Caballero, D., González-Barrientos, R., Fallas-Monge, Z., Palacios-Alfaro J.D., Baldi, M., Campos, E., Chanto, G., Barquero-Calvo, E., Chacón-Díaz, C, Chaves-Olarte, E, Guzmán Verri, C, Romero-Zúñiga,

J.J, & Moreno, E. (2017b). Brucellosis in mammals of Costa Rica: an epidemiological survey. *PLoS ONE*, 12(8), e0182644.

Hernández-Mora G., González-Barrientos, R., Palacios Alfaro, J.D., Suárez-Esquivel M., Ruiz-Villalobos N., Barquero-Calvo E., Cordero X.M., Bettoni, G., Roca, K., Guzmán-Verri C., Moreno E. (2020). *Brucella spp* ST27 in dwarf sperm whale (*Kogia sima*), Costa Rica*. Emerg Infect Dis* (submitted)

Honour, S., & Hickling, K.M.K. (1993). Naturally occurring *Brucella suis* biovar 4 infection in a moose (*Alces alces). J. Wildl. Dis.* 29(4):596-8.

Hull, N.C., & Schumaker, B.A. (2018). Comparisons of brucellosis between human and veterinary medicine. *Infect Ecol Epidemiol*. 24;8 (1):1500846.

Ibañez, A.A., Nicholls, M.J., & King, C.T. (1975). Prevalencia de la brucelosis en el ganado bovino de carne en la Región Oriental del Paraguay. Asunción, Ministerio de Agricultura y Ganadería.

Ibañez, A.A., Nicholls, M.J., & King C.T. (1977). A survey of brucellosis in beef cattle in Paraguay. *Brit Vet J*, 133 (4) 405-411.

ICA. (2019). Instituto Colombiano Agropecuario: Resolución No. 00007781 del 11 de junio 2019. Access at: https://www.ica.gov.co/portal_ica/media/video_portal/Resolucion-Brucelosis-00007781.pdf Accessed: 18-6-2019.

Ical, D. (2018). Belize makes strides in agricultura (Sede Web). Belice: News and Music Power. 27 de Julio 2018; Available at: http://belize-makes-strides-agriculture-sector/Accessed: 2-3-2019.

IICA (1980). Instituto Interamericano de Cooperación para la Agricultura. Diagnóstico de brucelosis bovina según provincias, Ecuador-1980. En G. Gómez, & E. Torres (Eds.), Primera reunión de directores de salud animal de la zona andina, RESANDINA I Bogotá, Colombia. pp. 26.

IICA/SENACSA (2017). Instituto Interamericano de Cooperación para la Agricultura: Programa de Brucelosis Bovina Paraguay. Access at: http://repositorio.iica.int/bitstream/11324/3165/1/BVE17089170e.pdf . Accessed: 5-4-2019.

Isidoro-Ayza, M., Ruiz-Villalobos, N., Pérez, L., Guzmán-Verri, C., Muñoz, P.M., Alegre, F., Barberán, M., Chacón-Díaz, C., Chaves-Olarte, E., González-Barrientos, R., Moreno, E., Blasco, J.M.,& Domingo, M. (2014). *Brucella ceti* infection in dolphins from the Western Mediterranean sea. BMC Vet Res. 10:206.

Jiménez, E. (2016). Ceniza del Turrialba afecta a 8252 animales y 261 hectáreas de cultivos. La Nación (26-05- 2016) Access at: http://www.nacion.com/sucesos/desastres/volcan_Turrialba-erupcion-afectacion-cultivosanimales_0_1563043796.html. Accessed: 8-4-2019.

Junqueira Junior, D.G., Rosinha, G.M.S, Carvalho, C.E.G, Oliveira, C.E., Sanches, C.C., & Lima-Ribeiro, A.M.C. (2013). Detection of *Brucella* spp. DNA in the semen of seronegative bulls by polymerase chain reaction. *Transbound Emerg Dis.* 60(4):376-7.

Keid, L.B., Chiebao, D.P., Batinga, M.C.A., Faita, T., Diniz, J.A., Oliveira, T.M.F.S, Ferrerira, H.L., & Soares, R.M. (2017). *Brucella canis* infection in dogs from commercial breeding kennels in Brazil. *Transbound Emerg Dis*. 64(3):691-697.

Kerby, P.J., Quiroga, J.L., McGrane, J.J., &Stagg, D.A. (1997). Field evaluation of an indirect ELISA for detection of brucellosis in lowland Bolivia. *Trop Anim Health Prod*. 29(2):65-72.

Kin, M.S, Fort, M., de Echaide S.T., & Casanave, E.B. (2014). *Brucella suis* in armadillos (*Chaetophractus villosus*) from La Pampa, Argentina. *Vet. Microbiol*. 170: (3-4): 442–445.

Kooy, P. (1970). Brucellosis treponematosis, rickettiosis and psittacosis in Surinam. *Trop. Geogr. Med.* 22:172-178.

Laroche,V., Lacombe, J., & Reyes, M.S. (1966). Quelques considerations sur la brucellose en Haití. *Bol. OSP* 60: 383-390.

Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F,, Nöckler K., Neubauer H., Guilloteau L.A., Vergnaud G. (2006) Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiol*. 6:9.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N,, Marth, G., Abecasis, G., Durbin, R. 2019. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078–2079.

Linderot de Cardona, K., De Gracia Scanapieco, A., & Braun, P.G. (2016). First results on small ruminant brucellosis and tuberculosis and caprine arthritis-encephalitis in El Salvador. *Trop Anim Health Prod*. 48(5):1083-1087.

Lopetegui, P. (1999). Programas de brucelosis: Situación epidemiológica y estrategias para a prevención y el control/erradicación en países de las Americas. Reunión de Consulta de Expertos de la OPS/OMS sobre vacunas y estrategias de vacunación en los programas de control y erradicación de las brucelosis.19-21.

Lopetegui, P. (2004). Bovine brucellosis control and eradication programme in Chile: vaccine use as a tool within the programme. *Dev Biol* (Basel).119:473-479.

Lord, V.R, & Flores, R. (1983). *Brucella* spp. from the capybara (Hydrochaeris hydrochaeris) in Venezuela: serologic studies and metabolic characterization of isolates. *J Wildl Dis*. 19 (4):308-14.

Lord, V.R., & Lord, R.D. (1991). *Brucella suis* infections in collared peccaries in Venezuela. *J Wildl Dis*. 27 (3):477-81.

Lord, V.R., Cherwonogrodzky, J.W., Schurig, G.G., Lord, R.D., Marcano, M.J., & Melendez, G.E. (1998). Venezuelan field trials of vaccines against brucellosis in swine. *Am. J. Vet. Res.* 59 (5), 546–551.

Lubroth, J., Rweyemamu M.M., Viljoen G., Diallo, A., Dungu B., & Amanfu, W. (2007) Veterinary vaccines and their use in developing countries. *Rev. sci. tech. Off. int. Epiz*, 26 (1), 179-201.

Lucero, N. E., Ayala, S.M., Escobar, G.I., & Jacob, N.R. (2008). *Brucella* isolated in humans and animals in Latin America from 1968 to 2006. *Epidemiol. Infect*. 136, 496–503.

MAGA (2011). Ministerio de Agricultura Ganadería y Alimentación Guatemala: Programa de brucelosis y tuberculosis. (2011). Access at: http://visar.maga.gob.gt/?page_id=919. Accessed: 8-6-2019.

MAGA (2018) Ministerio de Agricultura Ganadería y Alimentación Guatemala: Reglamento para el control progresivo y erradicación de la brucelosis. Acuerdo gubernativo. Access at: https://visar.maga.gob.gt/?p=12186.Accessed: 8-6-2019.

MAG- CR. (1978). Ministerio de Agricultura y Ganadería. Dirección de Salud Animal. Proyecto Salud Animal MAG-BID. San José, Costa Rica.

MAG- CR (1990). Ministerio de Agricultura y Ganadería. Informe sobre brucelosis. Archivos de informes semestrales y anuales. Dirección de Salud animal. San José, Costa Rica.

MAG-CR. (2000). Ministerio de Agricultura y Ganadería. Reglamento sobre el control de la brucelosis en los animales. Decreto No. 28514-MAG. La Gaceta No 53, San José, Costa Rica. 2000. Access at http://www.ecolex.org/fr/details/legislation/decreto-no-27946mag-reglamento-sobre-el-control-de-la-brucelosis-en-losanimales-lex-faoc017344/ Accessed 5-3-19.

MAG-CR. (2008). Ministerio de Agricultura y Ganadería. Reglamento para la intervención de la brucelosis bovina. Decreto 34858-MAG-2008. La Gaceta No 230, San José, Costa Rica. Access at: http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=64425&nValor3=74734&strTipM=TC. Accessed: 4-5-2017.

MAG-CR (2018). Ministerio de Agricultura y Ganadería: Reglamento sobre el control de la brucelosis en los animales. Decreto No.34858-MAG. La Gaceta No.154, San José Costa Rica. 2018. Access at: http://www.mag.go.cr/legislacion/2008/de-34852.pdf. Accessed: 5-319.

MAG-El Salvador. (2015). Ministerio de Agricultura y Ganadería: Solicitud de realización de pruebas diagnósticas de brucelosis. Access: http://www.mag.gob.sv/direccion-general-de-ganaderia/servicios-veterinarios/solicitud-de-realizacion-de-pruebas-diagnosticas-de-brucelosis-y-tuberculosis/. Accessed: 22-6-19.

MAG-El Salvador. (2018). Ministerio de Agricultura y Ganadería: Número de muestras y animales positivos de Brucelosis bovina del 2014 al 2018. Access at: www.mag.gob.sv. Accessed: 7-8-19.

MAGFOR. (2009). Ministerio Agropecuario y Forestal. Nicaragua: Acuerdo Ministerial No 008-2009. Access at: http://extwprlegs1.fao.org/docs/pdf/nic90609.pdf Accesed: 8-6-2019.

MAG-Paraguay. (1976). Ministerio de Agricultura y Ganadería- Paraguay: Programa Nacional de Salud animal. Brucelosis, tuberculosis, rabia en bovinos. Primera etapa. Asunción, Paraguay.

Mancilla M, Ulloa M, López-Goñi I, Moriyón I, María Zárraga A. 2011. Identification of new IS711 insertion sites in *Brucella abortus* field isolates. *BMC Microbiol*. 11:176.

Martínez, P. (2013). Brucelosis humana: situación epidemiológica en Chile, 2001-2010*. Rev Chilena Infectol.* 30 (6): 653-659.

Martins, G., Penna, B., Hamond, C., Leite, R.C., Silva, A., Ferreira, A., Brandão, F., Oliveira, F., & Lilenbaum, W. (2012). Leptospirosis as the most frequent infectious disease impairing productivity in small ruminants in Rio de Janeiro, Brazil. *Trop Anim Health Prod* 44:773–777.

Maquart, M., Le Flèche, P., Foster, G., Tryland, M., Ramisse, F., Djønne, B., Al Dahouk, S., Jacques, I., Neubauer, H., Walravens, K., Godfroid, J., Cloeckaert, A., & Vergnaud G. (2009) MLVA-16 typing of 295 marine mammal *Brucella* isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*. BMC Microbiol. 9:145.

Mattar, S., Tiquea, V., Miranda, J., Montes, E., & Garzona, D. (2017). Undifferentiated tropical febrile illness in Cordoba, Colombia: Not everything is dengue*. J Infect Public Health.* 10 (5):507-512.

May-Collado LJ, Amador-Caballero M, Casas JJ, Gamboa-Poveda MP, Garita Alpízar F, Tim Gerrodette, González-Barrientos R, Hernández-Mora G, et al. Palacios D.M., Palacios Alfaro J.D., Pérez B., Rasmussen K, Trejos-Lassso L., & Rodríguez-Fonseca J. (2017). Ecology and conservation of Cetaceans of Costa Rica and Panama In: Advances in Marine Research in Latin America: Technological Innovation in Ecology and Conservation Springer eds. pp 293-319.

McMeekin. 2018. Bolivia's canine citizens. Access at: http://www.bolivianexpress.org/blog/posts/bolivias-canine-citizens . Accessed: 7-2-2019.

Messier, V., Le´ vesque, B., Proulx, J.F., Rochette, L., Serhir, B., Couillard, M., Ward, B.J., Libman, M.D., Dewailly, E., & Dery, S. (2012). Seroprevalence of Seven Zoonotic Infections in Nunavik, Quebec (Canada). *Zoon Pub Health*. 59: 107–117.

MediVet-SENASA. (2016) Vacuna antibrucélica. Dirección de Medicamentos Veterinarios. Access at: http://www. senasa.go.cr/medivet/WebForm2.aspx?id=AR-48-64-51-5680. Accessed: 3-3-2019.

MIDA. (2018). Ministerio de Desarrollo Agropecuario: Resolución No. OAL-110-ADM-2018. Gaceta Oficial Digital. 29 de Agosto 2018 Access at: https://www.gacetaoficial.gob.pa/pdfTemp/28600/GacetaNo_28600_20180829.pdf Accessed 5-8-19.

Ministerio de Economía y Hacienda. (1953). Anuario Estadístico de Costa Rica. Dirección General de Estadísticas y Censos. San José, Costa Rica. http://biblioteca.ccp.ucr.ac.cr/handle/123456789/1303.

Ministerio de Economía y Hacienda (1956) Anuario Estadístico de Costa Rica. Dirección General de Estadísticas y Censos. San José, Costa Rica. Access at: http://biblioteca.ccp.ucr.ac.cr/handle/123456789/1299. Accessed: 3-5-19.

Ministerio de Salud Argentina (2013). Guía para el equipo de salud No.12. Av. 9 de Julio 1925 (C1073ABA), Cdad. Autónoma de Bs. As., República Argentina. Access at: http://www.msal.gob.ar/images/stories/bes/graficos/0000000304cnt-guia-medica-brucelosis.pdf. Accessed: 4-4-19.

Morales-Otero, P. (1949). A short note on the epidemiology of brucellosis in Puerto Rico. Puerto Rico. *PR J Pub. Hlth. Trop. Med*. 24: 349-354.

Moreno E. (2002). Brucellosis in Central America. *Vet Microbiol*. 90:31-38. & Gall, D. (1998). Comparison of different serological assays for the differential diagnosis of brucellosis. In International Atomic Energy, Diagnosis and epidemiology of animal diseases in Latin America IAEA-TECDOC 1055. Viena;153-161.

Moriyón, I., Grilló, M.J., Monreal, D., González, D., Marín, C., López-Goñi, I., Mainar-Jaime, R.C., Moreno, E., & Blasco, J.M. (2004) Rough vaccines in

animal brucellosis: structural and genetic basis and present status. *Vet Res*. 35(1):1-38.

Murray, E., & Fow, O. (1998). Medicine and Surgery of South American Camelids. Llama, Alpaca, Vicuña, Guanaco. Second Edition. lowa State University Press. USA.

Nicoletti, P. (1976). A preliminary report on the efficacy of adult cattle vaccination using Strain 19 in selected dairy herds in Florida. *In Proc Annu Meet US Anim Health Assoc*, 91–106.

Nicoletti, P., Jones, L. M. & Berman, D. T. (1978a). Adult vaccination with standard and reduced doses of *Brucella abortus* strain 19. Vaccine in a dairy herd infected with brucellosis. *J Amer Vet Med Assoc.* 173, 1445–1449.

Nicoletti, P., Jones, L. M. & Berman, D. T. (1978b). Comparison of the subcutaneous and conjunctival route of vaccination with *Brucella abortus* strain 19 vaccine in adult cattle. *J Am Vet Med Assoc* 173, 1450–1456.

Ocampo-Sosa, A.A., García-Lobo, J.M. 2008. Demonstration of IS711 transposition in *Brucella ovis* and *Brucella pinnipedialis. BMC Microbiol.* 8:1–10.

OIE (2009). World Organization for Animal Health. Bovine brucellosis: In manual of diagnostic tests and vaccines for terrestrial animals. 12 rue de Prony 75017. Paris.

OIE (2018). World Organization for Animal Health. Brucellosis (*Brucella abortus, B melitensis and B. suis*): In manual of diagnostic tests and vaccines for terrestrial animals 12 rue de Prony 75017. Paris.

OIRSA, (2014). Organismo Internacional Regional de Sanidad Agropecuaria. Informe final Consultoria regional para elaborar un estudio sobre la situación sanitaria de la brucelosis bovina, la tuberculosis y la enfermedad de Newcastle. STF. Access at:

https://www.standardsfacility.org/sites/default/files/STDF_PG_358_Estudio_Situacion_Sanitaria.pdf. Accessed 4-11-19

Oliveira, A, M., Ristow, P., I. Ko A., & Di-Lorenzo C. (2012). Serological trail of *Brucella* infection in an urban slum population in Brazil. *J Infect Dev Ctries* 6(9): 675–679.

Omer, M.K., Skjerve, E., MacMillan, A.P., Woldehiwet, Z. Comparison of three serological tests in the diagnosis of *Brucella* infection in unvaccinated cattle in Eritrea. *Prev Vet Med*. 2001; 48:215-222.

Ontario Agency for Health Protection and Promotion (Public Health Ontario). (2016). Reportable disease trends in Ontario, 2014. Toronto, ON: Queen's Printer for Ontario.

Ontario Agency for Health Protection and Promotion (Public Health Ontario). (2018). Factors affecting reportable diseases in Ontario (1991-2016). Toronto, ON: Queen's Printer for Ontario.

Oseguera, Montiel D., Frankena, K., Udo, H., Keilbach-Baer N.M., & van der Zijpp A. (2013). Prevalence and risk factors for brucellosis in goats in areas of Mexico with and without brucellosis control campaign. *Trop Anim Health Prod*. 45(6):1383-9.

Palisade Corporation. Risk analysis using Monte Carlo simulation, 2017. http://www.palisade.com/risk/.

Page, A.J., Taylor, B., Delaney, A.J., Soares, J., Seemann, T., Keane, J.A., Harris, S.R. 2016. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb Genomics*. 2: e000056.

Pedersen, K., Bauer, N.E., Olsen, S., Arenas-Gamboa, A.M., Henry, A.C., Sibley, T.D., & Gidlewski, T. (2017). Identification of *Brucella* spp. in feral swine (*Sus scrofa*) at abattoirs in Texas, USA. *Zoon Pub Health*. 64(8):647-654.

Pelaiz, A.J. (1950). Estado actual de la brucelosis bovina en Cuba. In: Inter-American Congress on Brucellosis. 3: 48-58, PAHO/WHO, Washington.

Pérez-Román, C., Nema-Vidaurre, J., & Fonseca-Araya, E. (1984). Brucelosis en personal del matadero de Nicoya,Guanacaste. *Acta Méd Cost* (Costa Rica) 27: 41-44.

PIAGRO. (1996). Programa de información agropecuaria: Brucelosis y tuberculosis. Taller de evaluación y perspectivas de los programas oficiales de brucelosis, tuberculosis y leptospitosis. Ministerio de Agricultura y Ganadería, San José, Costa Rica. 1996. Access at http://www.mag.go.cr/bibliotecavirtual/L73-2398.pdf. Accessed 2-3-19.

Pisani, A., Vacarezza, M., & Tomasina, F. (2017). Estudio de 14 casos de brucelosis en trabajadores de un frigorífico como enfermedad profesional. Uruguay 2009-2010. *Rev. Med Urug,* 33(3): 168-173.

Plommet, M. & Fensterbank, R. (1976). Vaccination against bovine brucellosis with a low dose of strain 19 administered by the conjunctival route. III. Serological response and immunity in the pregnant cow. *Ann Rech Vet* 7, 9–23.

Poester, F.P., Goncalves, V.S.P., & Lage Pereira, A. (2002). Brucellosis in Brazil. *Vet. Microbiol* 90: 55-62.

Polanco González, J.M. & Rizo Castro, D.S. (2006). Estudio Epidemiológico de la prevalencia de Brucelosis bovina en la zona seca del municipio de San Pedro de Lóvago, Chontales. Tesis. Universidad Nacional Agraria. Facultad de Ciencia Animal. Departamento de Veterinaria, Managua, Nicaragua.

Porto, C.E. (2015). Capa descargada de http://tapiquen-sig.jimdo.com. Tapiquén. Orogénesis Soluciones Geográficas. Porlamar, Venezuela.

Procuraduría General de la República. (2010). Control de ganado bovino, prevención y sanción de su robo, hurto y receptación. Sistema costarricense de información jurídica. Ley No 8799. La Gaceta No 88, San José, Costa

Rica. Access at: http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=67888&nValor3=80618&strTipM=TC.Accessed 5-9-19.

Procuraduría General de la República. Reglamento de vigilancia de la salud. Sistema costarricense de información jurídica. Norma 37306-S. La Gaceta No 203, San José, Costa Rica. 2012. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&param2=18&nValor1=1&nValor2=73471&nValor3=90176&strTipM=TC&lResultado=180&nValor4=1&strSelect=sel.

Procuraduría General de la República. Ley de Bienestar de los Animales. Sistema costarricense de información jurídica. Ley No 7451. La Gaceta No 236, San José, Costa Rica. 1994. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=24319&nValor3=25739&param2=1&strTipM=TC&lResultado=2&strSim=simp.

Procuraduría General de la República. Ley General del servicio nacional de salud animal. Sistema costarricense de información jurídica. Ley No 8495. La Gaceta No 93, San José, Costa Rica. 2006. http://www.pgrweb.go.cr/scij/Busqueda/Normativa/Normas/nrm_texto_completo.aspx?param1=NRTC&nValor1=1&nValor2=57137&nValor3=80913&strTipM=TC.

Quail, M.A., Kozarewa, I., Smith, F., Scally, A., Stephens, P.J, Durbin, R., Swerdlow, H., &Turner, D.J. 2008. A large genome centre's improvements to the Illumina sequencing system. *Nat Methods*. 5: 1005–1010.

Quail M.A., Otto, T.D., Gu, Y., Harris, S.R., Skelly, T.F., McQuillan, J.A., Swerdlow, H.P., Oyola, S.O. 2012. Optimal enzymes for amplifying sequencing libraries. *Nat Methods*. 9:10–1.

Quirós D. El primer caso de fiebre de Bruce en Costa Rica. Gaceta Médica de Costa Rica. 1915; 11:241-244.

Quirós-Quirós, E. (2006). Historia de la ganadería bovina en Costa Rica. Corporación Ganadera (CORFAGA). Archivos Institucionales. Available at: http://corfoga.org/carne-de-costa-rica/historia-de-la-ganaderia%20/.

Recio, P. (2015). SENASA confirma brote de brucelosis en fincas cercanas al volcán Turrialba. La Nación (16-03-2015) Access at: http://www.nacion.com/nacional/salud-publica/Senasa-confirma-brucelosis-cercanas-Turrialba_0_1475652544.html.Accessed 4-6-2016.

Reid, I. (2011). CVMA-Hill. Canada´s Pet Wellness report: A snapshot of Canada's pet health and wellness. Canadian Veterinary medical Association & Hills Science Diet. Access at https://www.canadianveterinarians.net/documents/canada-s-pet-wellness-report2011 Accessed 7-2-2019 .

Reyes-Knoke M.A., &, Rice D.A. (1983). Serological prevalence of bovine brucellosis in El Salvador. *Trop Anim Health Prod*. 15(1):49-50.

Rice, D.A., Calderón, J.E., & Miron C.A. (1979). A serological survey of swine sera for antibodies to *Brucella* spp. in El Salvador. *Trop Anim Hlth Prod*. 11 (1) 36.

Román-Ramírez, D.L., Martínez-Herrera, D.I., Villagómez-Cortés, J.A., Peniche-Cardeña, A.E. de J., Morales-Álvarez, J.F., & Flores-Castro, R. (2017). Epidemiología de la brucelosis caprina en la Zona Centro del Estado de Veracruz. *Gac Med Mex*.153:26-30.

Russo A.M., Mancebo O.A., Monzón C.M., Gait J.J., Casco R.D. & Torini de Echaide S.M. (2016). Epidemiología de la brucelosis caprina y ovina en la provincia

de Formosa, ArgentinaEpidemiology of caprine and ovine brucellosis in Formosa province. *Rev Argent Microbiol.* 48 (2) 147-153.

Samartino L.E (2002). Brucellosis in Argentina. *Vet Microb*. 90: 71-80.

Secretaría de Agricultura y Ganadería (1997). Reglamento de Control y Erradicación de la Brucelosis y la Tuberculosis Bovina. Acuerdo No.1735-97. La Gaceta 23 de mayo 1998, Tegucigalpa, Honduras Access at: http://extwprlegs1.fao.org/docs/pdf/hon16011.pdf Accessed: 7-8-19.

Secretaria de Defensa Agropecuaria. (2017). Ministério da Agricultura, Pecuária e Abastecimento: INSTRUÇÃO NORMATIVA Nº 10, DE 3 DE MARÇO DE 2017. Access at http://www.in.gov.br/materia/-/asset_publisher/Kujrw0TZC2Mb/content/id/19124587/do1-2017-06-20-instrucao-normativa-n-10-de-3-de-marco-de-2017-19124353. Accessed 3-5-19.

SENACSA. (2017). Resolución No. 2017-1093. Vacunación contra la brucelosis bovina, en todo el territorio nacional correspondiente a la categoría desmamantes hembras (Carimbo 7) y su registro. Gobierno Nacional de Paraguay. Access at: http://senacsa.gov.py/application/files/2714/9994/9872/SENACSA-RES-2017-1093_PRY-DEC-2017-7426.pdf. Accessed 16-9-19.

SENACSA (2018). Manual de Salud Pública y Zoonosis. Servicio Nacional de Calidad y Salud Animal. Gobierno Nacional. 23-25. Access at: http://www.senacsa.gov.py/application/files/3614/7275/6080/SENACSA-Manual_Salud_Publica_2016_ES.pdf. Accessed 4-7-2019.

SENASA- Argentina (2002). Resolución 1048/2002: Abrogación de la inscripción provisional de la vacuna de brucelosis bovina RB51 y su certificación de uso y comercialización. *Bol Of* 31/12/2002. No. 30058, 19.

SENASA- Argentina (2015). Servicio Nacional de Salud Animal: *Brucella ovis*. Access at: http://www.senasa.gob.ar/cadena-animal/ovinos/produccion-

primaria/sanidad-animal/enfermedades-y-estra-sani/*Brucella*-ovis Accessed 3 -3-19.

SENASAG. (2012). Laboratorios de Diagnóstico Veterinario. Access at http://www.senasag.gob.bo/laboratorio/laboratorios-oficiales-del-senasag.html Accessed 6-6-2019.

SENASAG. (2014). Área Nacional de Programas de Sanidad Animal. Access at http://www.senasag.gob.bo/programas-de-sanidad-animal.html. Accessed: 6-6-2019.

SENASICA (2019). Vacunas utilizadas en la Campaña Nacional contra la brucelosis en los animales. Access at https://www.gob.mx/pronabive/articulos/vacunas-utilizadas-en-la-campana-nacional-contra-la-brucelosis-en-los-animales?idiom=es. Accessed 8-9-2019.

Sequeira, A., Campos, E., Mendoza, L., San-Roman, M.A., & Moreno, E. (1984). Identificación de especies y biotipos de *Brucella* aisladas en Costa Rica. Turrialb. 34:525-526.

Serrano-Machuca, J.J., Villarreal Ríos, E., Galicia Rodríguez, L., Vargas Daza, E.R.,Martínez González, L.,& Mejía Damián, A.F. (2009). Detección de anticuerpos circulantes en donantes de sangre en México. *Rev Panam Salud Publica*/Pan Am J Public Health 26(4), 355.

SESA. (2008). Servicio Ecuatoriano de Sanidad Agropecuaria: resolución No. 25 R.O.No.376 Registro Oficial Access at: http://www.agrocalidad.gob.ec/wp-content/uploads/pdf/sanidad-animal/02-control zoosanitario/Resoluci%C3%B3n%20025.pdf Accessed 3-6-19.

Shury, T. K., Nishi, J.S., Elkin, B.T., & Wobeser, G. A. (2015). Tuberculosis and brucellosis in wood bison (Bison Bison Athabascae) in Northern Canada: A renewed need to develop options for future management. *J. Wildl. Diseas*, 51(3), 543–554.

Sohn, A.H., Probert, W.S., Glaser, C.A., Gupta, N., Bollen, A.W., Wong, J.D., Grace, E.M., & McDonald, W.C. (2003). Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp. *Emerg. infect. Dis*., 9 (4), 485–488.

Solorio-Rivera, J.L., Segura-Correa, J.C., & Sánchez-Gil, L.G. (2007). Seroprevalence of and risk factors for brucellosis of goats in herds of Michoacan, Mexico*. Prev Vet Med.* 14;82(3-4):282-90.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690.

Statista (2017). Number of dogs in the United States 200 to 2017. Available at: https://www.statista.com/statistics/198100/dogs-in-the-united-states-since-2000/. Acceded 7-2-2019.

Suárez-Esquivel, M., Baker, K.S., Ruiz-Villalobos, N., Hernández-Mora, G., Barquero-Calvo, E., González-Barrientos, R., Castillo-Zeledón, A., Jiménez-Rojas, C., Chacón-Díaz, C., Cloeckaert, A., Chaves-Olarte, E., Thomson, N.R., Moreno, E., & Guzmán-Verri, C. (2017a). *Brucella* genetic variability in wildlife marine mammals' populations relates to host preference and ocean distribution. *Genome Biol Evol* 9 (7):1901-1912.

Suárez-Esquivel, M., Ruiz-Villalobos, N., Jiménez-Rojas, C., Barquero-Calvo, E., Chacón-Díaz, C., Víquez-Ruiz, E., Rojas-Campos N, Baker K.S, Oviedo-Sánchez G., Amuy E., Chaves-Olarte E., Thomson N.E., Moreno E., & Guzmán-Verri, C. (2017b). *Brucella neotomae* infection in humans, Costa Rica. *Emerg Infect Diseas*, 23(6), 997-1000.

Suárez-Esquivel, M., Hernández-Mora G., Ruiz-Villalobos, N., Rojas-Campos N., Barquero-Calvo E., Oviedo-Sánchez G., Foster J.T., Ladner J.T., Chacón-Díaz C., Chaves-Olarte E., Baker K.S., Thomson N.R., Moreno E., & Guzmán-Verri C. (2020). Phylo-temporal Analysis of *Brucella abortus*

Incursions in Costa Rica. Sometido a PLOS Neglected Tropical Diseases (submitted).

Sux, Blanco, M. (2005). Brucelosis en Alpacas (Lama pacos) en las comunidades del cantón Ulla, Ulla, Provincia Franz Tamayo del Departamento de la Paz. Tesis de grado Medicina Veterinaria y Zootecnia. Universidad Católica Boliviana. La Paz Bolivia. 2005. Access at:https://scholarsarchive.byu.edu/cgi/viewcontent.cgi?article=6435&context=etd. Accessed 31-08-2019.

Szyfres, B., Blood, B.D., & Moya, V. (1959). Estado actual de la brucelosis en la América Latina. *Bol Oficina Sanit. Panam*. 46:48-64.

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J.P., Frankena, K. (2001). WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. *Vet Rec*. 2001; 148:567-572.

Tuemmers, C., Lüders, C., Rojas, C., Serri, M., Castillo, C., & Espinoza, R. (2013). Detection of *Brucella canis* by immunochromatography method in vague dogs captured in Temuco city, Chile, 2011. *Rev Chilena Infectol*. 30 (4): 395-401.

Turvey, S., Hui, C., Tyrrell, G., & Singh, A.E. (2017). *Brucella suis* as a cause of late prosthetic joint infection: A case report and review of the literature. *JAMMI*.69–74.

USDA-United States Department of Agriculture. (2018). National Brucellosis Eradication Program. Available at: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/cattle-disease-information/national-brucellosis-eradication. Accessed 20-10-2019.

Vargas, F. (2000). Inmunología de la brucelosis y uso de la vacuna RB51 en el programa de control y erradicación de la enfermedad. Enfermedades de los Bovinos: Diagnóstico, Tratamiento y Control, editado por Contreras, J., Segunda Edición. Barquisimeto, Venezuela, 490-515.

Vargas, F. J. (2002). Brucellosis in Venezuela. *Vet Microbiol* 90: 39–44.

Vargas-Chiarella, C.R., Cárdenas-Canedo, S.L., & Escobar Camach, J.R. (2017). Brucellosis in Cochabamba, Bolivia. First regional prevalence study. *Gac Med Bol* 40: 21-23.

Vargas-Ulate T. (2004). Geografía de Costa Rica. Editorial Tecnológica, Cartago, Costa Rica. 2004; 124.

Vicente, G.S., Kirschbaum, A., & Ureña, M. (1983). Consideraciones sobre la vacunación antibrucelosa en Costa Rica 1978-1982. Ciencias Veterinarias (Costa Rica). 123-127.

WAHIS- World Animal Health Information System. (2018a). Info by country/territory. Access at: http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home. Accessed: 5-3-2019.

WAHIS-World Animal Health Information System. (2018b). Human cases of zoonoses for the selected country. Access at: https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Zoonoses Accessed: 14-6-2019.

Wall, T. (2018). Colombian pet market may grow to US 251 million in 2018. Access at: https://www.petfoodindustry.com/articles/7676-colombian-pet-market-may-grow-to-us251-million-in-2018?v=preview Accessed: 7-2-2019.

Wattam, A.R., Williams, K.P., Snyder, E.E., Almeida, N.F. Jr., Shukla, M., Dickerman, A.W., Crasta, O.R., Kenyon, R., Lu, J., Shallom, J.M., Yoo, H., Ficht, T.A., Tsolis, R.M., Munk, C., Tapia, R., Han, C.S., Detter, J.C., Bruce, D., Brettin, T.S, Sobral, B.W., Boyle, S.M., Setubal, J.C. 2009. Analysis of ten *Brucella* genomes reveals evidence for horizontal gene transfer despite a preferred intracellular lifestyle. *J. Bacteriol*. 191:3569–79.

Whatmore, A.M., Dawson, C., Muchowski, J., Perrett, L.L., Stubberfield, E., Koylass, M., Foster, G., Davison, N.J., Quance, C., Sidor, I.F., Field, C.F., St. Leger,

J. (2017). Characterisation of North American *Brucella* isolates from marine mammals. *PLoS ONE* 12(9): e0184758.

WHO. (2014). World Health Organization. The Control of Neglected Zoonotic Diseases: from advocacy to action. Report of the 4th International meeting. Geneva, Switzerland 19-20 Nov 2014. ISB 878 92 4 1508568.

Willis E. A. (2003). Seascape with monkeys and guinea-pigs: Britain's biological weapons research programme, 1948–54, Medicine, Conflict and Survival, 19:4, 285-302.

WPA-World Animal Protection. (2016). Estudio nacional sobre tenencia de perros en Costa Rica 2016. Access at: https://issuu.com/wspalatam/docs/estudioperros-web-singles. Accessed: 7-2-2019.

Zambriski J.A., Saito, M., Nydam, D.V., Reyes-Garay, H.A., Castillo, R., Cepeda, D., Cespedes, Zambrano M.J., García-Vara, P., Maves, R.C., Solano, M., Torrico, F., Gilman, R.H. (2010). Assessment of *Brucella melitensis* disease burden in lactating goats in Mizque, Bolivia. 14th International Congress on Infectious Diseases (ICID) Abstracts. 34.020 https://doi.org/10.1016/j.ijid.2010.02.1842.

Zavala M., Morales S. (2016). Seroprevalencia de anticuerpos contra *Brucella canis* en perros del Distrito de Pucusana, Lima, Peru. *Rev Invest Perú*. 27 (2).

Zelaya B., Lepe-López, M., Muñoz, A., Cutzán, M., Paniagua, J., & Escobar, J. (2017). Monitoreo serológico de Brucelosis Bovina en Guatemala: reactores positivos a la prueba de Rosa de Bengala durante el periodo 2010-2015. *Rev. Electrón*. Vet.18 :12.

Zeledón-Alvarado, J. (1940). Primera historia clínica de brucelosis humana en Costa Rica. *Rev. Med.* 72:153-167.

Zerbino D.R., & Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res*. 18:821–829.

Annex 1. Brucellosis in the Americas

Brucellosis is a zoonotic disease that is widespread throughout the world. According to the World Health Organization, this bacterial disease is among the seven most frequently neglected zoonotic diseases. It has been estimated that in low-income countries, there are approximately five and twelve million new human brucellosis cases per year (Hull and Schumaker, 2018). Except for *B. inopinata, B. microti, B. papionis,* and *B. vulpis*, all other accepted *Brucella* species are present in the American continent, primarily associated with their respective preferred mammal host (figure 5). According to the Food and Agriculture Organization of the United Nations (FAO) the total number of susceptible hosts in the American continent by 2017 correspond to 516 million bovines, 180 million pigs, 81.3 million sheep, 37 million goats, 1.3 million buffaloes and close to 150 million dogs in Canada, USA, Brazil, Argentina, Colombia, Chile, Bolivia and Costa Rica (FAO, 2017; Statista 2017; Reid 2011; Bruha 2015; Wall 2018; McMeekin, 2018; WPA 2016).

Therefore, the economic impact of animal brucellosis and the associated zoonotic disease is relevant, thus, the situation requires the application of suitable control and eradication programs.

This section describes the status of animal and human brucellosis in the Americas, covering mainly a time span of 85 years, from 1934 to 2019. A significant amount of the information collected does not come from scientific journals, but from information scattered in national reports issued by Animal and Public Health authorities of each country. During the eighties, Latin American countries had military conflicts, critical economic growth, political upheaval against authoritarian regimes, and economic recession.

Therefore, the activities devoted to the control of brucellosis during those years diminished, and the access to the information was limited, and in many cases not accurate.

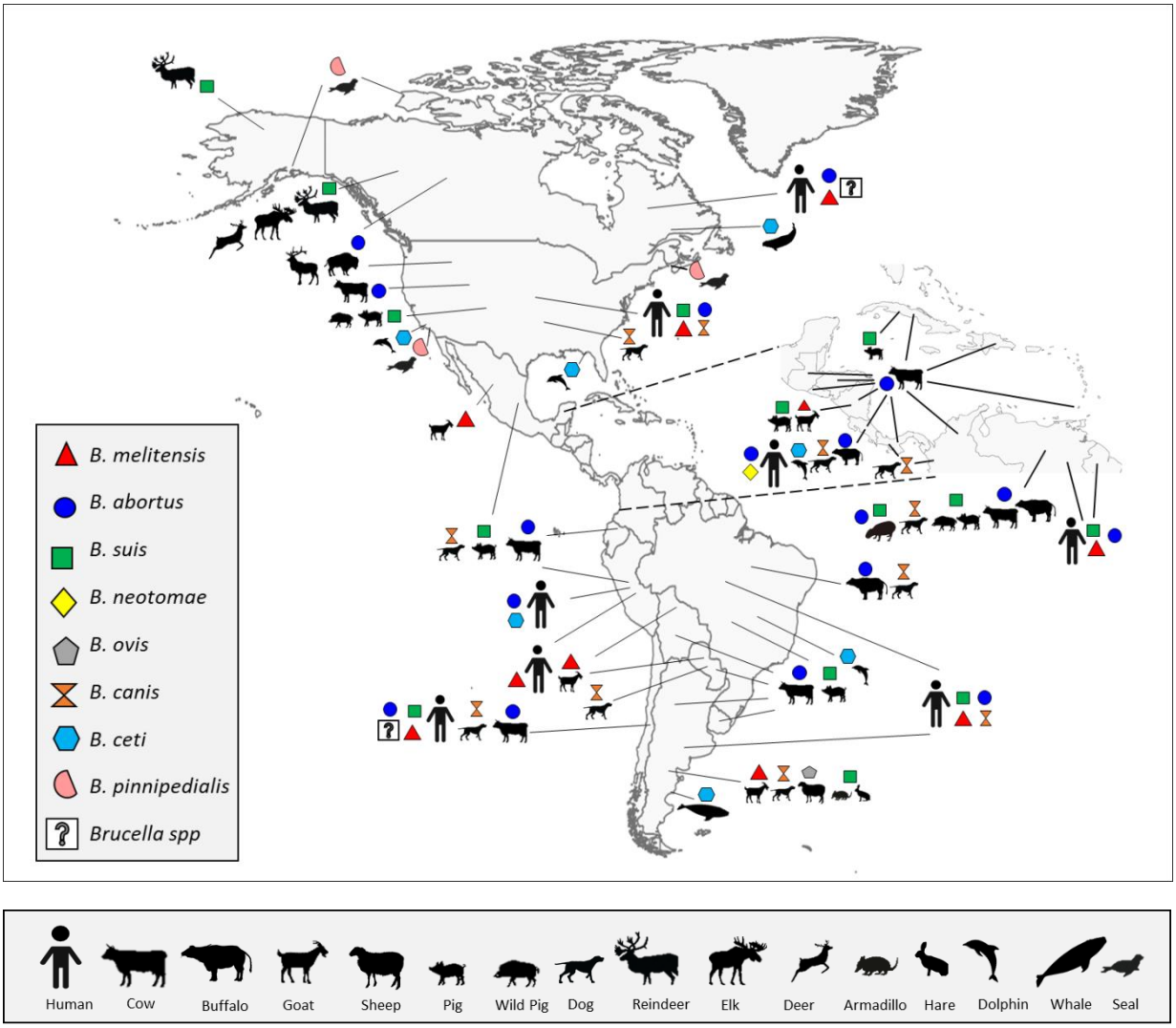

Figure 5. Reported species of *Brucellae* in humans, domestic and wildlife animals from the Americas during 2006 to 2018 based on the information of CDC (2017); Porto (2015); Shury *et al., (*2015); WAHIS (2018a); WAHIS (2018b).

Brucellosis in northern American countries

Canada

Several attempts to control and to eradicate bovine brucellosis were carried out in Canada since the second half of the XX century. The brucellosis vaccination campaign in cattle started in 1950, with the establishment of the Federal-Provincial Calfhood Vaccination Program. At that time, the national herd seroprevalence of bovine brucellosis was estimated at 9%. The Federal Department of Agriculture granted exclusive use of *B. abortus* S19 vaccine, and they were responsible for the management and control of the vaccine. After six years of S19 calfhood vaccination, the herd seroprevalence decreased by 4.5%, and then the test and slaughter program started in 1957. Individual identification and testing was mandatory, and infected herds were quarantined. Diagnosis by serum agglutination test was mandatory for all animals. Positive reactors were slaughtered following economic compensation. The compensation rates were adjusted according to the animal's age, and affected owners received compensation for the value of the carcasses. A total of 687 control areas were established in the country in 1957. These control areas were certified for three years when the infection rate was reduced below 1% of the cattle population and 5% of the herds. The brucellosis-free areas were certified for five years when the infection rate was below 0.2 % of the cattle in the area and 1% of the herds (Crawford and Hidalgo, 1977).

The surveillance in farms was carried out by using a combination of the milk ring test and testing of cattle at markets, started in 1960. The objective was to locate infected herds and to reduce the number of required tests to certify brucellosis-free areas. The milk ring testing was done by collecting milk and cream samples three times per year from each herd. When positive herds were detected, the origin of infection was traced, and all animals were subjected to a blood test (Crawford and Hidalgo, 1977). At markets, the female cattle over 24 months of age assigned for slaughter were tested by agglutination, and positive animals traced to the herd of origin for surveillance.

Despite all the efforts, an increased brucellosis incidence in a few Canadian provinces was detected in 1974. Most *Brucella* infections occurred after purchasing

cows from untested herds (Crawford and Hidalgo, 1977). All herds adjacent to an infected herd were tested and quarantined. In cases in which brucellosis was established, the herd was depopulated with economic compensation and replaced with brucellosis-free animals.

In 1976, following S19 vaccination, testing, and slaughter, with compensation, the individual brucellosis seroprevalence was below 2%, with a total bovine population of 14 million animals. Finally, in 1989, the country was declared free of bovine brucellosis, and vaccination was banned (Crawford and Hidalgo, 1977; García Carrillo, 1981; FAO, 2017).

B. abortus was limited to one or more zones in the territory during 2007, suspected but not confirmed and from 2008 onwards absent in domestic animals. In wildlife, *B. abortus* was suspected but not confirmed from 2009 to 2011. In bison (*Bison bison athabascae*) and in elk (*Cervus Canadensis*), *B. abortus* was only limited to one or few zones from 2012 to 2018 (WAHIS, 2018a, Shury, 2015). *B. suis* biovar 4 was absent in domestic animals during 2006 to 2018, but reported in wildlife during 2009 and 2018, mainly in caribou and reindeer (*Rangifer tarandus*) (WAHIS, 2018a). In the past *B. suis* biovar 4 had been reported in moose (*Alces alces)* (Honour and Hickling, 1993). In 2008, *B. canis* was isolated from kennels in Saskatchewan province (Brennan *et al.*, 2008). *B. pinnipedialis* and *B. ceti* infecting marine mammals were also characterized recently (Whatmore *et al*., 2017). *B. melitensis* has never been reported in domestic animals or wildlife in Canada (WAHIS, 2018a).

In 1928, human brucellosis was included as a notifiable disease, and since then, a total of 6357 cases were reported until 2011. Most of the cases were hunters, veterinarians, farmers, abattoir workers, and laboratory personnel (Ontario Agency, 2016; Berger, 2018). Presently, the rate of *Brucella* sp. infection in humans nationwide in Canada range from 0.2 to 0.5 per 1000000 inhabitants (Ontario Agency, 2016). During 2004, in the Nunavk region, less than 1% of seroprevalence was detected in indigenous people (Messier *et al.,* 2012). These and other habitants of the Arctic regions like Inuits are of concern, since they eat raw caribou meat and raw skin and blubber from belugas and other cetaceans, which have been shown to

be a source of infection with *B. suis* biovar 4 and *B. ceti,* respectively (Chan *et al*., 1989; Whatmore *et al*., 2017). Intermittent human brucellosis cases caused by *B. suis* biovar 4 have been detected in Canada (Turvey *et al.,* 2017). From 2003 to 2014, the infection rate in Ontario ranged from 0.2 to 0.8 per 1000000 inhabitants (Ontario Agency, 2016). Of those cases, 44% were attributable to imported cases of *B. melitensis*, 7% to *B. abortus*, 10% to other *Brucella* species, and 39% did not specify the *Brucella* species found. Most of the cases (76.2%) were attributed to imported cases from travelers from Mexico, Central America, South America, Mediterranean countries, Africa, Middle East, and Asia. In the remainder of the cases (23. 8%), the risk exposure was unknown (Ontario Agency, 2018) (figure 6). Other sources reported between 4 and 19 human patients from 2005 to 2016 (figure 7) (WAHIS, 2018b).

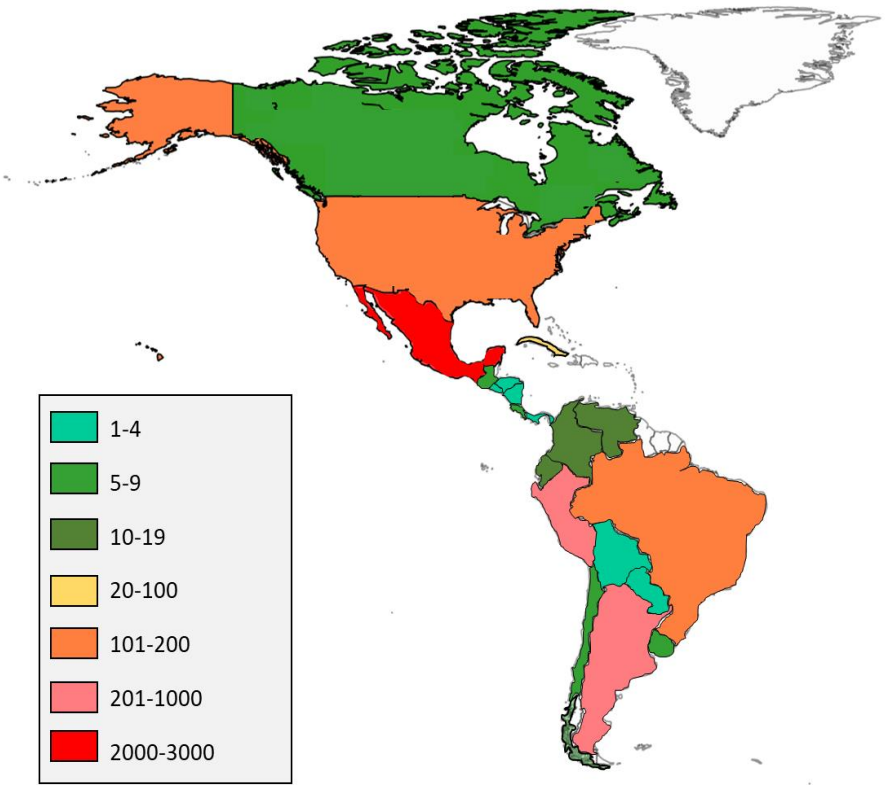

Figure 6. Human infection rate in America between 2005-2018

(Porto, 2015, WAHIS, 2018b)

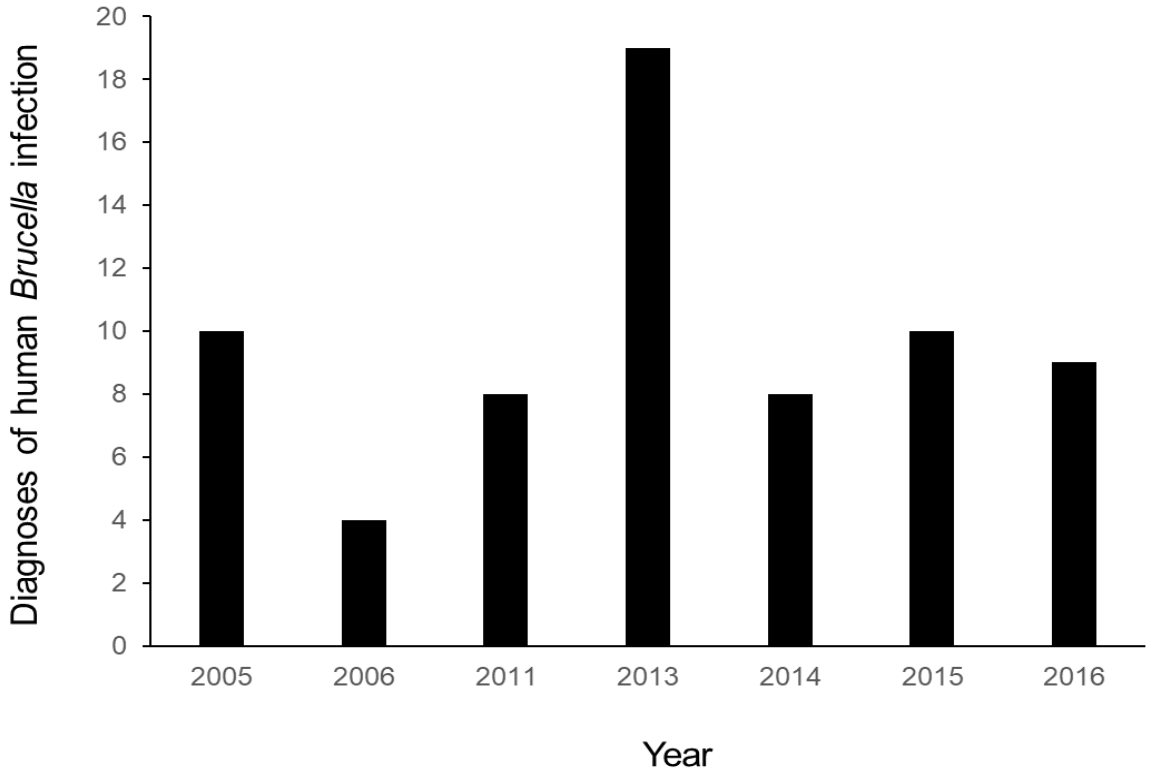

Figure 7. Brucellosis human cases officially reported in Canada between years 2005-2016 (WAHIS, 2018b)

United States

The first attempts to control bovine brucellosis in the United States started in 1934, with the Cooperative State-Federal Brucellosis Eradication Program (Crawford and Hidalgo, 1977). By 1940, 17 states, including 209 counties, were certified for reducing brucellosis herd seroprevalence to less than 5% and less than 1% seroprevalence of cattle. In 1941, individual identification, control of animal movements, S19 vaccination (3 to 6 months of age), and serological testing were introduced for calves in 39 states. In 1942, North Carolina was the first state to achieve modified certified status, starting from an individual seroprevalence (estimated by agglutination tests) of 11.5% in 1934-1935 to 5% in 1937 and to 2.4% in 1941 (Crawford and Hidalgo, 1977; García Carrillo, 1981). The efforts were dampened during World War II, and by 1946 the number of reactors among individual cattle tested increased to 5%, despite the use of S19 vaccine; mainly because there was an increased number of susceptible non-vaccinated cattle

introduced into the herds (Crawford and Hidalgo, 1977). In 1947, the United States Livestock Sanitary Association adopted an eradication program on a national basis, approved by USDA. A frequent screening of dairy herds with milk ring testing was initiated in 1952. By 1954, it was estimated that 26% of the national herds were positive for brucellosis. During that year, additional funds were available to eradicate brucellosis with new efforts involving state by state planning using Rose Bengal Test (RBT), elimination of reactors, and broader S19 vaccination. By 1960, greater emphasis was given to surveillance activities, and many states achieved the brucellosis-free status, following serological testing of beef and dairy cattle, including the milk ring testing (Crawford and Hidalgo, 1977; García Carrillo, 1981). In addition, two programs were established: firs, market cattle identification (MCI) for animals being tagged for slaughter, and tracing of positive animals to the herd of origin; second was livestock market cattle testing, for monitoring the total population for brucellosis in areas where the number of infected herds was low (Crawford and Hidalgo, 1977; García Carrillo, 1981). However, the market cattle testing showed some weaknesses. It provided limited information to specific areas but did not include the surrounding farms.

With a population of nearly 108 million bovines and despite the success achieved in previous years for lowering the brucellosis herd seroprevalence, the government decided to decrease calf vaccination with S19 in 1964. The main argument was the high costs and “low” probability of infection in areas with reduced brucellosis individual seroprevalence (Crawford and Hidalgo, 1977; García Carrillo, 1981; FAO, 2017). Due to this measure, calf vaccination decreased from 7 million to 3.8 million and reached a minimum in 1975. Brucellosis increased from 12000 positive herds in 1971 to 16000 positive herds in 1975, revealing that the decrease of calf vaccination was premature. Calf vaccination was recommended once more in 1979, even though the individual seroprevalence was below 1% in most states. From that year on, the vaccination began to increase up to 5 million S19 doses.

According to Crawford and Hidalgo (1977), the progress of the campaign was obtained following these activities: 1) The individual identification of the animals and the compulsory control of animal movements, 2) The establishment of the National

Commission and committees in the different states, which included representatives of the agricultural and livestock sectors, the food-producing industries, scientific and educational institutions, industry associations and physicians; 3) the oral and written press with active participation; 4) the production of printed matter broadly distributed; 5) information and literature material for cattle owners; 6) the compulsory action followed by most of the owners in the regions; 7) the standard diagnostic techniques which included the simplest and less expensive ones, such as card tests and RBT; 8) slaughter of the reactors with partial compensation to the owners; 9) payment of indemnity to the owners after depopulation of reactor problematic herds; 10) the use S19 by all states for over forty years with satisfactory results (Crawford and Hidalgo, 1977; García Carrillo, 1981). National eradication in cattle was mostly achieved by the early 2000s with now only an occasional spillover case in cattle in the Greater Yellowstone Area, but none elsewhere since 2010 (USDA, 2018).

According to the World Animal Health Information System (WAHIS, 2018a), *B. melitensis* is currently absent in the USA in domestic animals and wildlife. As a direct consequence of important anthropogenic interventions, (winter-feeding of wildlife, which increased the animal density and the frequency of contacts) *B. abortus* has been reported in free-ranging elk (*Cervus elaphus)* and bison (*Bison bison)* in the Yellowstone Park region since the last decade (WAHIS, 2018a). The individual seroprevalence in pigs and feral pigs from Texas and Georgia was 13% and 22%, in 2015 and 2017, respectively (Pedersen *et al*., 2017). In wildlife *B. suis,* biovar *4* is endemic in wild caribou (*Rangifer tarandus*) herds in Alaska. *B. suis biovar* 1 is endemic in feral swine in several states, and it is reported as a pathogen limited to one or several zones in the country (WAHIS, 2018a). *B. ceti* and *B. pinnipedials* infecting marine mammals in the Atlantic and Pacific of the United States have also been reported (Whatmore *et al.,* 2017).

From 1993 to 2010, the Center for Disease Control reported 1971 human cases in the United States. California, Texas, Arizona, and Florida are the states with the higher number of cases, close to 56.5% of all reports. Approximately 70 to 75% of U.S brucellosis human cases are due to *B. melitensis* and *B. abortus*, from tourist or migrants that consume unpasteurized dairy products from countries of the

Mediterranean Basin (Portugal, Spain, Southern France, Italy, Greece, Turkey and North Africa) as well as Mexico, Central and South America, Eastern Europe, Asia, Africa, the Caribbean and the Middle East. Feral swine hunters that manage infected carcasses or consume raw or undercooked pork are also at risk for food-borne exposure to brucellosis (*B. suis*) as well as owners who buy dogs as pets from infected kennels (*B. canis*) (CDC, 2017; Dentinger *et al*., 2015). According to WAHIS (2018b), an average of 118 human patients were reported yearly between 2005 and 2018 (figure 8).

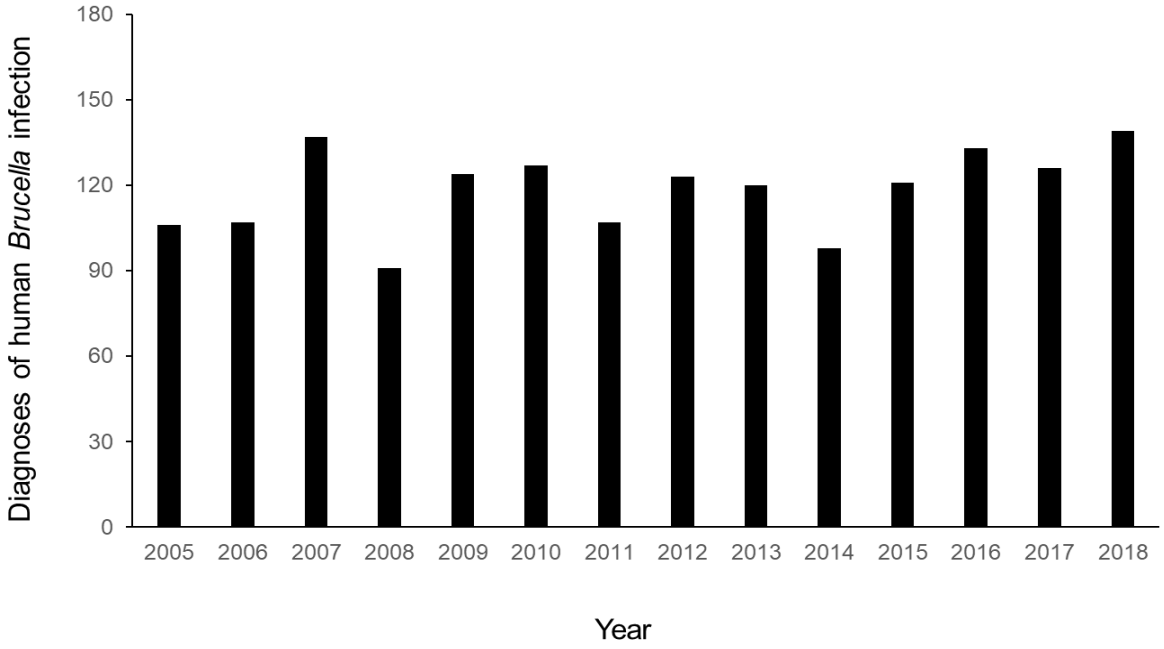

Figure 8. Brucellosis human cases officially reported in the United States between years 2005-2018 (WAHIS, 2018b)

Mexico

Even though many National Brucellosis Programs had been proposed in Mexico before 1970, it was not until 1971 that a more or less well-established national campaign was implemented, with an estimated individual seroprevalence of 14% in a population of 22.2 million bovines (García Carrillo, 1981; FAO, 2017). Three stages were implemented; first, the Department of Animal Health conducted a serological survey for the exportation of cattle in 1968 and 1969, and decided to

survey selected areas. Second, on a voluntary basis, there were efforts of test, slaughter, and quarantine reactor farms. Third, vaccination with S19 vaccine of female calves between three and six months of age (Crawford and Hidalgo, 1977; García Carrillo, 1981). The campaign included surveillance in 26 Mexican states (representing 9.5% of the country) at the slaughterhouses, pasteurization plants, and milk collection in dairy farms (Crawford and Hidalgo, 1977). As expected, due to its voluntary basis the program failed, mainly due to the absence of compensation for the slaughtered animals as well as other coordinated actions, such as following up of the cases, identification of the vaccinated animals, and regular testing of the herds. In addition, other factors delayed the progress of the control program in some areas, including: i) the absence of a professional veterinary service represented by a lack of human and material resources needed for the program; ii) lack of economic resources to compensate or indemnity the owners for cattle slaughter; iii) the absence of vaccines and diagnostic tests when needed; iv) poor identification system to control movement of bovines from one region to another, and finally; vi) the social, economic, and cultural level of the owners which made it difficult to execute the program (Crawford and Hidalgo, 1977).

Currently, the campaign has been partially supported by the National Campaign against Brucellosis in Animals NOM-041-ZOO-1995, and it is of nationwide application, on a compulsory basis. However, the coverage is very low. The Secretariat coordinated with state governments, producers, and industry and sectors linked to livestock farming, the financing mechanisms, and actions to compensate the owners of brucellosis reactor cattle slaughtered. Vaccinations are based on the use of a full dose of S19 vaccine in calves only from 3 to 6 months of age, and a reduced dose S19 vaccine for females older than 6 months and pregnant cows, both applied subcutaneously (SENASICA, 2019). Like in other countries, RB51 vaccine is also used, in Mexico, however, its efficacy under a high prevalence of circulating bacteria is debatable due to a low protective performance (Moriyón *et al.*, 2004).

The state of Sonora claims (with no clear and accessible epidemiological data), to be free of brucellosis caused by smooth *Brucella* species. Also, 31% of the

Mexican national territory has been declared in the “eradication phase” (Baja California Sur, Campeche, Colima, Guerrero, Nayarit, Quintana Roo and Yucatán, as well as some regions of Aguascalientes, Baja California, Chiapas, Guanajuato, Huasteca region, Hidalgo, and Puebla), even though several of these states and regions have reported high seroprevalence, with cases of bovine and human brucellosis. In addition, no solid epidemiological data for cattle brucellosis is available in most of these regions (García-Juarez *et al*., 2014). Moreover, during 2014, Mexico had the largest number (5514) of reported outbreaks in animals worldwide, of which 5174 (93.9%) were due to *B. abortus* and 340 (6.1%) to *B. melitensis*, with no reported cases for *B. suis* (Hull and Schumaker, 2018). As expected, bovine brucellosis continues to be highly prevalent in Mexico, and failures in the control measures are evident.

B. melitensis is also highly prevalent in both sheep and goats. During 2009-2012, an individual seroprevalence of 0.52% was reported in Veracruz (Román Ramírez, 2017). Higher seroprevalence of 9.8% and 11% was reported in Michoacán in 2007 and 2013, respectively (Solorio-Rivera *et al.,* 2007; Oseguera *et al*., 2013). During 2013, a seroprevalence of 38% was described in Jalisco (Oseguera *et al.,* 2013), and in 2014, a seroprevalence of 66.8% in Huanantla, Tlaxcala (García-Juarez *et al.,* 2014).

Official data issued by the Animal Health authorities at WAHIS (2018a) report that in Mexico, *B. melitensis* is mainly restricted to goats and sheep, while *B. abortus* is mainly detected in bovines. Likewise, *B. suis* is present in swine and suspected in wildlife animals, but not confirmed. However, the absence of systematic surveillance and epidemiological data regarding the isolation and characterization of the *Brucella* strains in the country precludes any significant conclusions.

Human blood donors from the Northeastern area of Mexico had seroprevalence of 0.71% during 2009 (Serrano Machuca *et al*., 2009). In 2010-2012, in Ixtenco, Tlaxcala, the reported seroprevalence of housewives from rural areas, was 1.51%. The most relevant risk factors reported were related to traditional socio-cultural aspects, such as low educational level, goat production units, sanitary deficiencies, and unpasteurized dairy products (García-Juarez *et al*., 2014). During

2016, a survey of dairy farmworkers in Hidalgo, described a seroprevalence of 18.1%, primarily been the calf caretakers (45.4%) (Cervera *et al*., 2016). According to WAHIS (2018b), Mexico is the country with the highest number of reported human cases of the Americas, with an average of 2587 human brucellosis cases per year from 2005 to 2018 (figure 9).

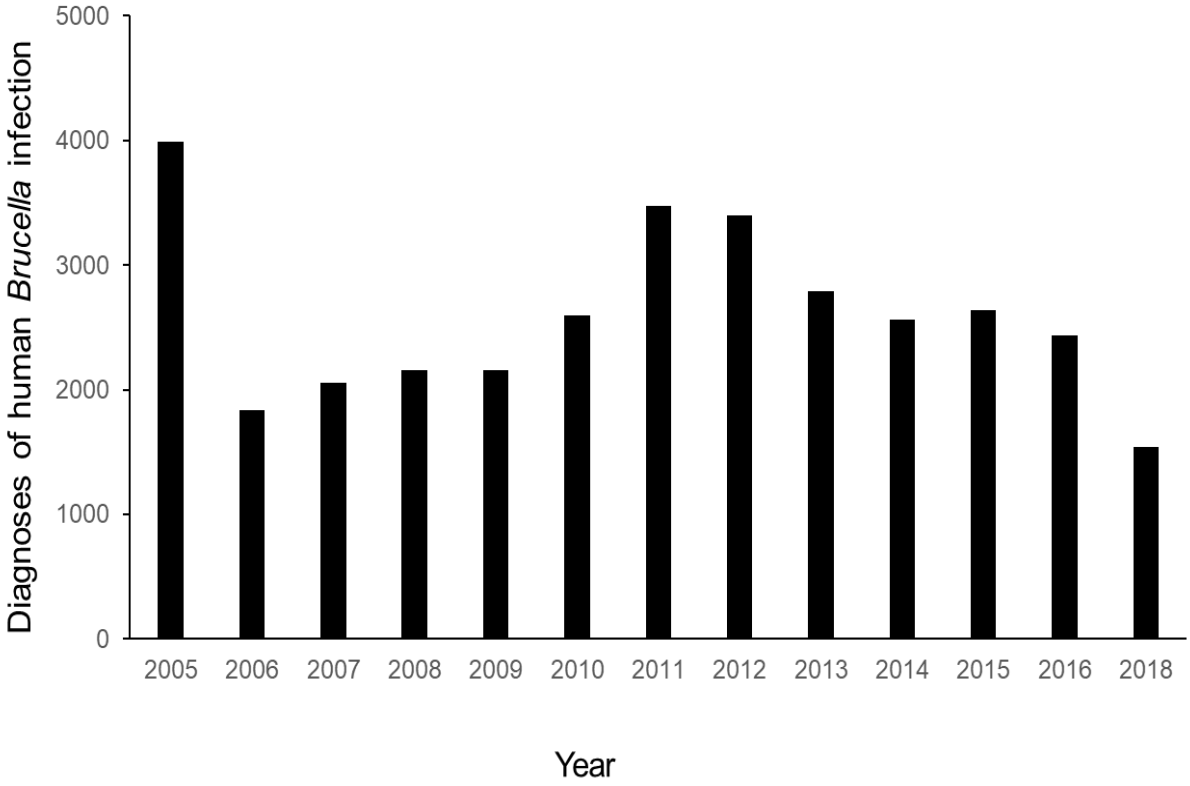

Figure 9. Brucellosis human cases officially reported in Mexico between years 2005-2018 (WAHIS, 2018b)

Guatemala

In 1981, the seroprevalence of brucellosis in Guatemala ranged from 1% to 20%, and it was considered an endemic zoonosis. Different strategies have been implemented since then, including test and slaughter and vaccination of calves with S19, with unknown coverage (García Carrillo, 1981). In 1997, a low- prevalence of *B. abortus*, *B. suis,* and *B. melitensis* was reported (Corbel, 1997). Sporadic vaccination with Rev 1 in goats was used (Moreno, 2002).

Using RBT in 31038 routine bovine samples, the estimated national bovine brucellosis prevalence ranged from 4.8% to 9.8%, from 2008 to 2015, been Peten, Izabal, and Escuintla were the most affected provinces (Chajon, 2015; Zelaya *et al*., 2017). In 2010 a study of 19733 samples indicated a RBT individual seroprevalence of 1.95% (OIRSA, 2014). Moreover, in 2011, a prevalence of infected herds was estimated to be 85.4% for a total of 2532 farms from 22 provinces (MAGA, 2011).

During 2011, the Ministry of Agriculture, livestock and food, described the progressive control of farms and reported areas “free” of the disease. There was no compensation for the slaughter of positive animals. The basis of control was the elimination of the reactors from the herd (MAGA, 2011). However, no systematic epidemiological data regarding the status of the disease in Guatemala is available.

Currently, the progressive control program of brucellosis includes test and slaughter in different ruminant species (*B. taurus, B. indicus, B. frontalis, B. javanicus* and *B. grunniens*, *Bison bison*, *B. bonasus* and *Bubalus bubalis*). Vaccination is performed either with S19 or RB51, with no clear criterion regarding the immunization strategy. Dairy products, as well as their mobilization, are subject to governmental veterinary controls. Bovines in slaughterhouses are tested for *Brucella* infection, as well as bacteriological studies are performed after reports of abortion. Movements of positive herds are not allowed, and the only final destination is slaughter. Animals that attend exhibitions, fairs, markets, national or international livestock auctions, must come from farms free of the disease (MAGA, 2018).

B. abortus was reported from 2006 to 2017 in bovines and other domestic animals like buffaloes and is suspected but not confirmed in wildlife from 2009 to 2017 (WAHIS, 2018a). No attempts at isolating *B. melitensis* and *B. suis* have been performed in the country, and therefore was not reported from 2006 to 2018 in domestic animals or wildlife.

According to WAHIS (2018b), a total of 26 human patients with brucellosis were reported in Guatemala between 2005 and 2012 (figure 10).

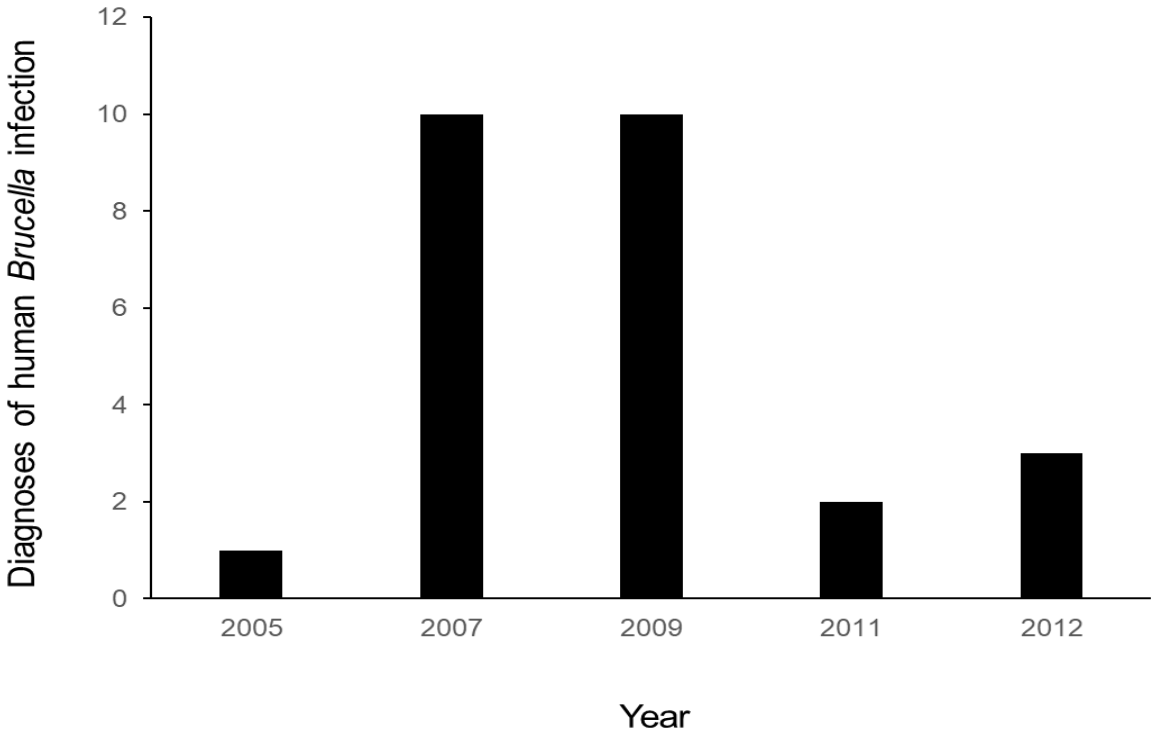

Figure 10. Brucellosis human cases officially reported in Guatemala between years 2005-2012 (WAHIS, 2018b)

Belize

In 1980, the reported individual prevalence of brucellosis was 0.1% in 8133 bovines (García Carrillo, 1981). Since then, the National Bovine Brucellosis Program is mandatory (FAO, 2017). Up to now, vaccination against bovine brucellosis is prohibited, all animals are individually identified and strict control of animal movements is performed (Belize Agricultural Health Authority, 2011).

Bovines were tested by agglutination assay, and all reactor animals were slaughtered with financial compensation by the Ministry of Agriculture and by the producer´s association. The Belize animal health authorities declared brucellosis-free areas when individual serological prevalence was less than 0.1%. Animals introduced into these zones came from brucellosis-free herds. The free areas established quarantines before the entry of new animals into brucellosis free herds,

with two consecutive serological tests separated by 30 days. Milk was verified by ring test on a regular basis. Susceptible animal species, such as equines and canines, were forbidden to enter the production units (Belize Agricultural Health Authority, 2011).

The current bovine population of Belize is 113122 animals (FAO, 2017). During 2012, an individual seroprevalence of brucellosis of 0.2% was reported (OIRSA, 2014). In 2016, the government declared the country “free of brucellosis” (claiming less than 0.01% of individual prevalence) (Cocom, 2016; FAO, 2017). According to the Minister of Agriculture, this condition was finally achieved in 2018. No human cases have been reported by FAO from 2005 to 2018 (WAHIS, 2018b), and *Brucella* organisms have not been reported in domestic animals or wildlife (WAHIS, 2018a). However, no clear epidemiological data is available certifying the brucellosis-free status. Moreover, no attempts to isolate *B. melitensis, B. abortus* and *B. suis* have been carried out (Ical D, 2018), in spite of the fact that neighboring countries such as Mexico and Guatemala have brucellosis.

El Salvador

National individual seroprevalence of bovine brucellosis in El Salvador during 1975, 1977, and 1983 was estimated to be 1.08%, 1.95%, and 1%, respectively (García Carrillo, 1981; Reyes-Knoke & Rice, 1983). In 1979 an individual seroprevalence of 1.2% in adult swine was reported (Rice *et al.,* 1979). The program for the control and eradication of bovine brucellosis consisted in the certification of free herds based on serological diagnoses. Vaccination with S19 was sporadic, and no compensation for the slaughter of positive animals was granted. According to the Regional International Agency of Agricultural Health (OIRSA, 2014), the estimated herd seroprevalence during 2010-2013 was 7.5% and individual seroprevalence of 1.17%. However, according to the Ministry of Agriculture and Livestock, the national prevalence from 2014 to 2018 among 13340 animals sampled in all the territory was 1.4% (MAG-El Salvador, 2018). However, no publication or governmental epidemiological data is available.

Currently, the brucellosis program in El Salvador is voluntary, and the main objective is to certify herds free of brucellosis (MAG- El Salvador, 2018). The vaccine used is RB51, and the government sells it with an average cost of $1.00 per vaccine doses (MAG- El Salvador, 2015). Positive animals are marked for slaughtered with no compensation (MAG- El Salvador, 2018).

B. melitensis and *B. suis* are not reported in domestic animals or wildlife from 2006 to 2018 (WAHIS, 2018a). *B. abortus* was reported from 2006 to 2017 in domestic animals, and there is no information available in wildlife. However, no information on attempts to isolate and characterize these *Brucellae* strains is available. In 2016, a national survey detected 1% of antibodies on goat and no reaction in sheep using RBT (Linderot *et al*., 2016). Human and animal cases due to *B. abortus* and *B. suis* have been reported (Rice *et al.,* 1979; Corbel, 1997). From 2010 to 2016, nine human brucellosis cases were reported in El Salvador (figure 11) (WAHIS, 2018b), indicating the presence of this bacteria in the territory.

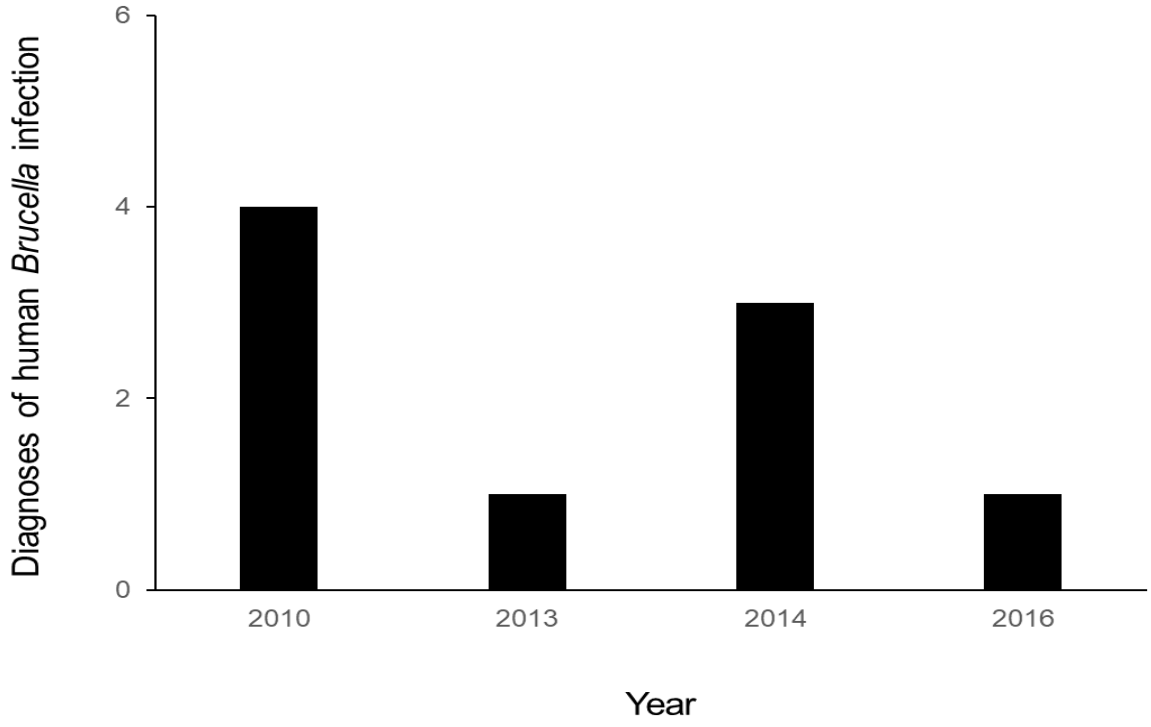

Figure 11. Brucellosis human cases officially reported in El Salvador between years 2010-2016 (WAHIS, 2018b)

Honduras

In 1972 the herd prevalence of brucellosis in Choluteca (8.72%), Morazán (7.7%), Comayague and La Paz (5.9%) and Santa Bárbara (4.1%) was reported, with an average of 6.6% (García Carrillo, 1981). The governmental strategy used was test and slaughter, with optional vaccination of calves with S19 when the herd prevalence was equal or higher than 5%. The country program began in 1977 with Area I, which included San Pedro de Sula and Choloma with herd prevalences of 25% to 48%, respectively (García Carrillo, 1981). During the same year, the infection with *B. abortus* bv1 was confirmed at the Pan American Zoonoses Center (García-Carrillo *et al.*, 1978).

During 1997, actions for the control and eradication of the brucellosis were established by the Ministry of Agriculture and Livestock of Honduras. The same year the presence of *B. abortus* was detected, and a high brucellosis individual prevalence was reported in the swine population (Corbel, 1997). The actions were mandatory nationwide, with test and slaughter to be executed progressively, giving priority to the areas and herds with the highest risk. Calf vaccination with S19 was performed by government veterinarians, and each animal identified by tagging. The governmental animal authorities (SENASA) paid for sampling and surveillance, but the diagnoses costs were covered by the owners. Diagnosis was only performed in cattle older than 12 months, except for males and tagged animals. Mobilization was allowed only for animals coming from “brucellosis-free” herds. Positive herds were subjected to quarantine. Only serologically negative animals were allowed in exhibitions. Compensation was established at the beginning of the program but not anymore (Secretaría de Agricultura y Ganadería, 1997). Milk from positive herds was pasteurized or boiled. During 2014, an individual seroprevalence of 0.11% was reported by the authorities, however, only 0.51% of the national bovine population was reported under control of the Brucellosis National Program (OIRSA, 2014).

B. melitensis and *B. suis* were officially reported as absent from 2006 to 2018 (WAHIS, 2018a). However, no published or governmental information on attempts to isolate or identify these strains is available. *B. abortus* was reported in the territory

from 2010 to 2014 and limited to certain infected zones from 2014 to 2017. However, due to the small size of the country, brucellosis is considered prevalent in all areas. There is no information regarding wildlife (WAHIS, 2018a). From 2006 to 2016, a total of 39 human patients were reported as infected with brucellosis in Honduras (figure 12) (WAHIS, 2018b).

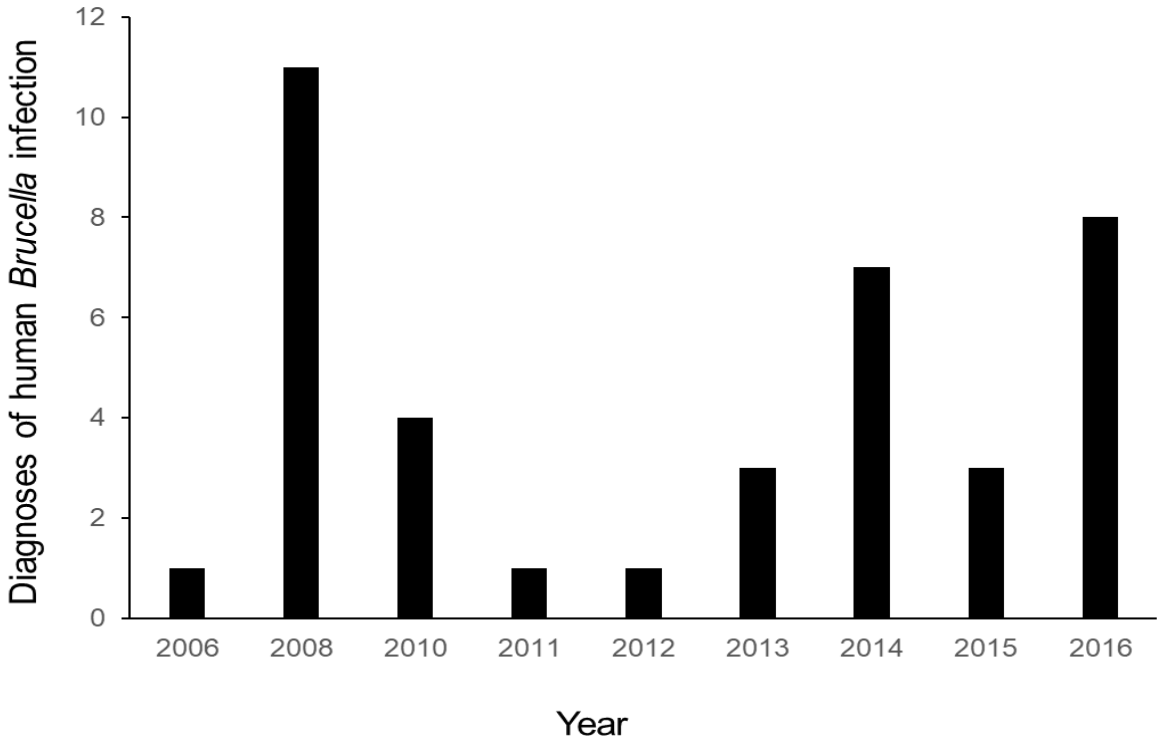

Figure 12. Brucellosis human cases officially reported in Honduras between years 2006-2016 (WAHIS, 2018b)

Nicaragua

In 1976, the Ministry of Agriculture started the Brucellosis Control and Eradication Program by test and slaughter in some areas. In herds with a high number of animals (>500), sacrifice was precluded, and unrestricted vaccination with S19 of calves aged between 3 to 6 months was carried out (García Carrillo, 1981). In 1977, *B. abortus* biovar 1 and 4 were isolated in Nicaragua. In 1979, the estimated nationwide brucellosis individual seroprevalence was 2% within a cattle population of 2.7 million animals (García Carrillo, 1979; García Carrillo, 1981; FAO, 2017).

Currently, some regulations are executed, giving priority to dairy cattle. The diagnostic tests include RBT, rivanol, CFT, and milk ring test. The positive animals (females and males older than 6 months) are slaughtered after the diagnoses with no compensation. Farms adjacent to the positive herds are regularly screened by serological tests. The Animal Health authorities declared brucellosis-free farms when herds tested negative for 6 months. An area is declared free of brucellosis when its herd prevalence is less than 0.2%. Mobilization of the animals is restricted to those that tested negative within 60 days. Animals that participate in fairs and exhibitions should come from brucellosis-free farms (MAGFOR, 2009). Owners are required to cover all diagnostic tests, tags, maintenance, and certifications. Since 2009, vaccination against brucellosis is not allowed in Nicaragua (MAGFOR, 2009), though some unofficial vaccination with RB51 vaccines is carried out. From 2008 to 2013 the reported individual seroprevalence of brucellosis ranged from 2.55% to 1.28% (OIRSA, 2014).

In 2016, a serological survey was performed in 1047 bovines from 170 farms in San Pedro de Lovago. The estimated individual seroprevalence via RBT and Rivanol test was 0.29% and 0.19%, respectively (Polanco & Riso, 2006).

B. melitensis was reported in 2006, *B. abortus* from 2006 to 2018, and *B. suis* was present in 2006 and 2012. However, no clear attempts to isolate or characterize the bacteria or epidemiological studies have been carried out. There is no information on *Brucella* species in wildlife (WAHIS, 2018a). Sixteen cases of human brucellosis were reported from 2005 to 2017 (figure 13) (WAHIS, 2018b), indicating the presence of *Brucella* organisms in the territory.

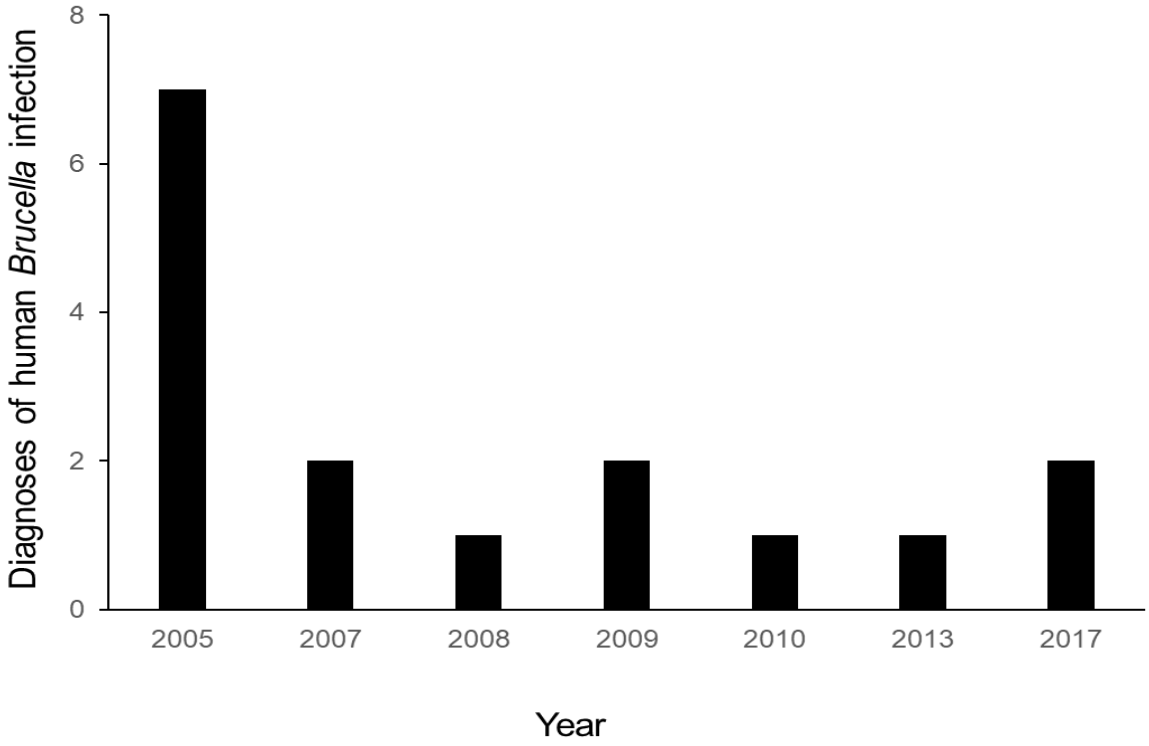

Figure 13. Brucellosis human cases officially reported in Nicaragua between years 2005-2017 (WAHIS, 2018b)

Costa Rica

In 1950, the Costa Rican National Animal Health Service aimed to control the disease through intervention measures in cattle (MAG-CR, 1978). At that time, reports of epidemic abortions, S19 vaccination, and agglutination diagnostic tests were the only strategies followed. In 1958, the serological diagnosis of brucellosis in bovine herds became mandatory and a national campaign for the control and eradication of the disease started on a voluntary basis with S19 calf vaccination, and elimination of the positive reactor animals with no compensation (Piagro, 1996).

An official governmental program to control and eradicate brucellosis started in 1976 (Vicente, 1983). The strategy consisted in S19 calf vaccination combined with test and slaughter. By 1980, the coverage of vaccination reached close to 43% of the bovines (Vicente, 1983). During 1999, S19 vaccine was replaced by RB51,

and the national program was changed from a compulsory to a voluntary basis with supervision by the authorities (MAG-CR, 2000). The results of the national survey performed during 2012 described a herd prevalence of 10.5 % nationwide, using RBT. This estimated herd prevalence was supported by routine testing of close to 500000 sera of bovines in 2016 and 2017 (Hernández Mora *et al*., 2017a). In 2018, the control program returned to being compulsory (MAG-CR, 2018). By the end of 2018 and 2019, field trials had been conducted in order to define more suitable control strategies for the country and the most affordable options for vaccination, including conjunctival vaccination with S19 as described before by different countries including The United States (Plommet & Fensterbank, 1976).

At least five *B. abortus* clusters, determined by whole-genome sequence analysis, have been found to affect humans and bovines (including water buffaloes) (Suárez-Esquivel *et al*., 2019). *B. canis* in dogs, *B. ceti* in dolphins and *B. neotomae* in humans are present in the country (Hernández-Mora *et al*., 2008; Hernández Mora *et al.,* 2017b; Suárez *et al.,* 2017a; Suárez-Esquivel, 2017b). In addition, a new smooth species named *Brucella* BCCN 84.3 was isolated from a dog (Guzmán-Verri *et al.,* 2019).

B. melitensis and *B. suis* have not been currently detected in Costa Rica (Hernández Mora *et al.,* 2017b). According to the National Reference Center of Bacteriology (CNBR-INCIENSA), a total of 111 human brucellosis cases were reported in Costa Rica from 2003 to 2017 (figure 14). *B. abortus* was the species isolated in most human patients (Hernández Mora *et al*., 2017b; Chanto, 2018). *B. neotomae* was reported in two human brucellosis cases (Suárez-Esquivel, 2017b).

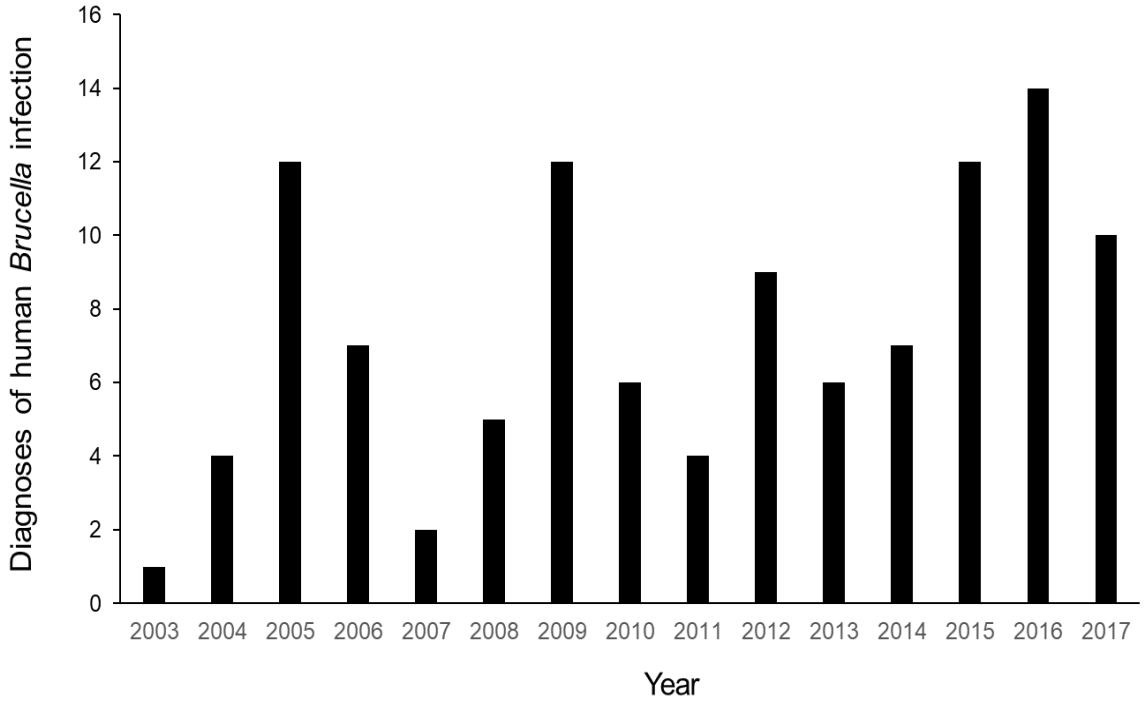

Figure 14. Brucellosis human cases officially reported in Costa Rica between years 2003-2017 (WAHIS, 2018b)

Panama

By 1970, the estimated individual seroprevalence ranged between 2% and 4.7% in a bovine population of 1.1 million bovines (García Carrillo, 1981; FAO 2017). Then, a strategy of test and slaughter with no vaccination was implemented until the present day. The Ministry of Agricultural Development of Panama (MIDA) annually performs epidemiological surveillance for the control and eradication of bovine brucellosis. Diagnosis includes the RBT and iELISA, and the reactors are sent to the slaughterhouses with compensation to the owners (MIDA, 2018). Monitoring includes serological testing at slaughterhouses, dairy processing plants, and selected farms. After testing, farms and areas may be declared free of brucellosis. Alternatively, infected farms with brucellosis are declared in quarantined, and the mobilization of animals comes under official control (MIDA, 2018).

During 2008, 2 samples resulted positive in RBT from 151585 animals tested nationwide as well as 7 samples out of 119699 bovines tested in 2010. No positive samples resulted in RBT during 2011 and 2012 out of 79879 and 92902 animals

tested (OIRSA, 2014). Beside sporadic official reports, no published epidemiological data are available in this country. After 2008, no isolation of *Brucellae* have been obtained, nor characterization of *Brucella* species and strains are documented either (OIRSA, 2014). According to the official data submitted to WAHIS (2018a), from 2006 to 2018, *B. melitensis* and *B. suis* are reported absent in domestic animals. *B. abortus* was present nationwide from 2006 to 2012 and was reported causing 59 outbreaks involving the seven provinces (Berger, 2018). There is no information on these *Brucellae* species in wildlife (WAHIS, 2018a). A total of 14 human brucellosis cases were reported from 2005 to 2014 (WAHIS, 2018b), indicating the presence of this organism in the territory (figure 15).

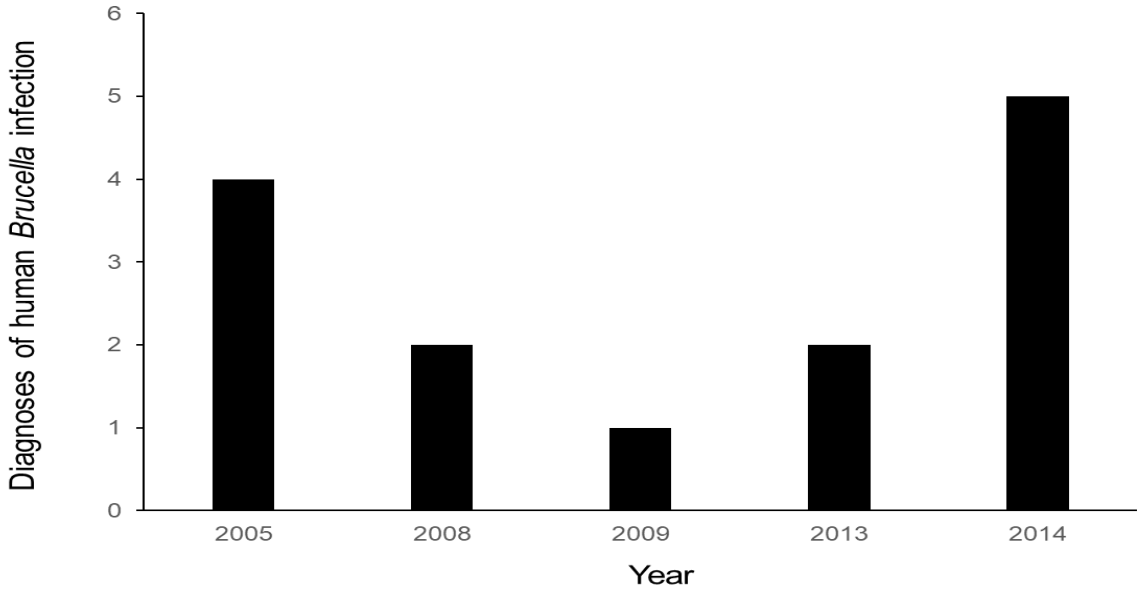

Figure 15. Brucellosis human cases officially reported in Panama between years 2005-2014 (WAHIS, 2018b)

Brucellosis in the Andean Area countries (Bolivia, Peru, Ecuador, Colombia and Venezuela)

Bolivia

Bolivia made efforts to control and eradicate brucellosis since the decade of 1970; however, they were unsuccessful (Agronegocios, 2017; Dirección General de Ganadería, Bolivia. 1974). In 1978, the bovine prevalence using the milk ring test in dairy herds at the Department of Santa Cruz and in Cochabamba were 30.5% and 1.06%, respectively (García Carrillo, 1981). In 1997, the estimated herd seroprevalence in cattle was 10% (Kerby *et al*., 1997), and it was reported the presence of *B. abortus* in bovines. *B. melitensis* has been detected in small ruminants and *B. suis* in swine with low and sporadic incidence (Corbel, 1997). In 2010, *B. melitensis* was reported as absent in goats in the region of Mizque (Zambriski *et al.*, 2010).

The current National Program for the control and eradication of bovine and bubaline brucellosis was established in 2014 (SENASAG, 2014). The purpose of the program is to join efforts between producers, veterinary professionals, health authorities, and government, among others (Agronegocios, 2017). Since 2014, 3-8 months old calves and females older than 8 months are vaccinated with S19 and RB51, respectively. Currently, the serological test used is milk ring test, cELISA, and Buffered Plate Antigen (BPA) (SENASAG, 2012). Positive reactors must be sent for slaughter with no compensation (Agronegocios, 2017).

Official data provided by the veterinary authorities at WAHIS (2018a), reported the presence of *B.abortus* from 2006 to 2018, *B. melitensis* was suspected but not confirmed during the same period of time and *B. suis* was reported in 2006 and 2008 and absent from 2009 to 2018. In wildlife, including alpacas and lamas, brucellosis has been reported since 2005 (Suxo Blanco, 2005). However, no clear epidemiological data is available.

Brucellosis in humans was reported in Cochabamba during 2017. A study of 276 samples from blood donors resulted in 1.1% seropositive using ELISA IgG (Vargas-Chiarella *et al*., 2017). Health authorities reported one human brucellosis case per year since 2018 (WAHIS, 2018b).

Colombia

Colombia started the brucellosis control campaign in 1970. The estimated national individual seroprevalence from 1971-1978 ranged from 0.4% to 11.4%, with an average individual seroprevalence of 4.22%. Vaccination was performed with a complete dose of S19 in females of all ages. The owners were responsible for vaccinating the animals (García Carrillo, 1981). In 1997, the only species reported was *B. abortus* in bovines (Corbel, 1997). Presently the objective of the Program for Prevention, Control, and Eradication of Bovine Brucellosis in Colombia is to reduce the herd prevalence of the disease in bovine, buffalo, ovine, caprine, and swine (IICA/SENACSA, 2017; ICA, 2019).

Cattle and buffalo calves between 3-8 months of age are vaccinated with S19 or RB51. Although vaccination is compulsory, revaccination with RB51 is recommended in females and buffaloes between 13 and 18 months (ICA, 2019). Likewise, revaccination with RB51 is recommended in non-pregnant females (bovines and buffaloes) at the age of 5 years and thereafter every five years. Before mobilization of bovines from free areas, vaccination with RB51 should be performed. In spite of these, there are no reliable epidemiological data reporting the success or failure of this idiosyncratic vaccination strategy. The screening tests include RBT, FPA, and iELISA. The competitive ELISA is used as a confirmatory test for bovine, buffalo, ovine, caprine, and swine. In the case of horses and dogs, the CFT is used for diagnoses. Bacteriological and PCR are currently performed by the Colombian Animal Health Authorities (ICA, 2019).

B. abortus, biovar 1, 2, 4 in bovines, and *B. suis* 1 in swine have been identified in Colombia in domestic animals but not in wildlife (Corbel, 1997; Lucero,

2008). *B. melitensis* has never been reported, and *B. suis* has not been detected in the last 15 years (WAHIS, 2018a). *B. canis* was isolated in dogs in Medellin Colombia (Giraldo Echeverri, 2009), and an individual seroprevalence of 2.76% was reported (Agudelo-Flores *et al.,* 2012). During 2012-2013 an active surveillance of humans with undifferentiated tropical febrile illness (fever without a focus of infection) reported a seroprevalence of 1% of antibodies against smooth *Brucellae* (Mattar *et al*., 2017). According to WAHIS (2018b), three clinical brucellosis human cases were reported in 2008 and 25 in 2015.

Ecuador

In 1979, the Ministry of Agriculture started the National Program of Animal Health, based on the official S19 vaccination of calves and the voluntary elimination of the reactors with no compensation (García Carrillo, 1981; IICA, 1980). Currently, the National Program works on a voluntary basis all over Ecuador and it includes test and slaughter strategy, as well as epidemiological surveillance, certification of brucellosis-free herds, control of the mobilization of animals, and information (SESA, 2008). The vaccination is compulsory throughout the national territory, and it is carried out in female calves (individually tagged) between three and six months of age, using the S19 or RB51 vaccines. Vaccination is a requirement for selling and trading of dairy products and slaughter, exhibitions, selling cattle, commercialization of meat and breeding purposes (SESA, 2008). The diagnostic screening tests currently used are milk ring test and RBT, while confirmatory tests are ELISA. Herds vaccinated with RB51 are considered brucellosis-free after two consecutive tests with a separation of 6 months each, while herds vaccinated with S19, require three negative serological tests with a separation of 6 months each (SESA, 2008). Currently, the control program receives financial support from governmental and private sectors in a proportion of 23% and 77%, respectively. There is no compensation for the owners after the slaughter of positive animals (SESA, 2008).

According to the official data at WAHIS (2018a), *B. abortus* has been reported in bovines from 2006 to 2017, and *B. suis* in swine has been suspected

but not confirmed from 2011 to 2018. There is no information of *B. melitensis* infections nor on brucellosis in wildlife. The brucellosis status of 11000 bovines in the Galapagos Islands is unknown. Based on previous studies on the island, during 1997 and 2014, cattle seemed free of the disease (Gioia *et al.,* 2018). Since 2005, an increasing number of human patients with brucellosis has been reported in Ecuador (figure 16).

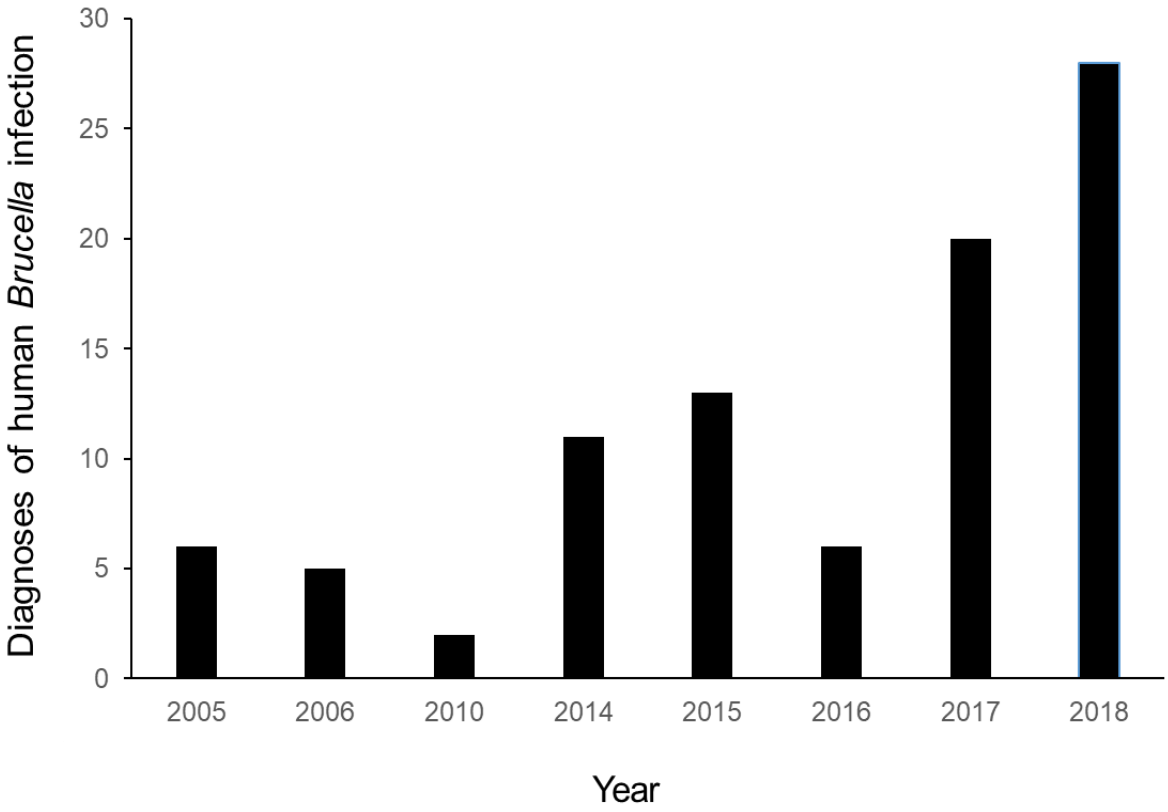

Figure 16. Brucellosis human cases officially reported in Ecuador between years 2005-2018 (WAHIS, 2018b)

Peru

In Peru, the reported individual seroprevalence of brucellosis from 1972 to 1975 ranged between 2% and 4% (García Carrillo, 1981). During 1997, it was reported that *B. abortus* was present in bovines and *B. ovis* in ovines (Corbel, 1997). Currently, the National Service of Animal Health (SENASA) is responsible for the brucellosis control and eradication program. The program gives priority to areas of intensive breeding of dairy cattle or dual-purpose, intending to establishing disease-

free areas. The diagnostic tests are the RBT as a screening test and the CFT and indirect ELISA as the confirmatory tests, while the milk ring test is used for surveillance. Vaccination of calves and ear tagging is compulsory in farms with high seroprevalence of the disease. Positive animals are sent to slaughterhouses with no compensation. Replacements and animals participating in fairs and exhibitions must originate from brucellosis-free herds. Every six months, SENASA publishes a list of herds free of bovine brucellosis, and it also includes those that have lost their status. In both cases, the processing plants are informed. Herds free of bovine brucellosis, enjoy a bonus corresponding to 1% of the base price per kilogram of milk. The owners enrolled in the program are entitled to the benefits that are granted for trading bovine replacements. Trading raw milk is only allowed from brucellosis-free herds.

According to WAHIS (2018a)*, B. abortus* had been reported from 2008 to 2018 and was absent in wildlife, while *B. melitensis* was present in caprine herds from 2007 to 2017. *B. suis* was reported in swine in 2007. Brucellosis has been detected in camelids with 20% individual prevalence in some regions of Peru (Murray & Fow*,* 1998). In Lima, in 2016, the individual seroprevalence in dogs using double immunodiffusion in agar gel was 21.3% (Zavala *et al*., 2016). There is no information regarding brucellosis in wildlife.

Despite the efforts mentioned above to control brucellosis, Peru is the second country with more human brucellosis cases reported in the Americas, with a total of 2868 patients since 2005 (figure 17) (WAHIS, 2018b). Most of the human cases are caused by *B. melitensis (*Lucero *et al*., 2008). Two humans infected with *B. ceti* ST 27 have been reported (Sohn *et al*., 2003).

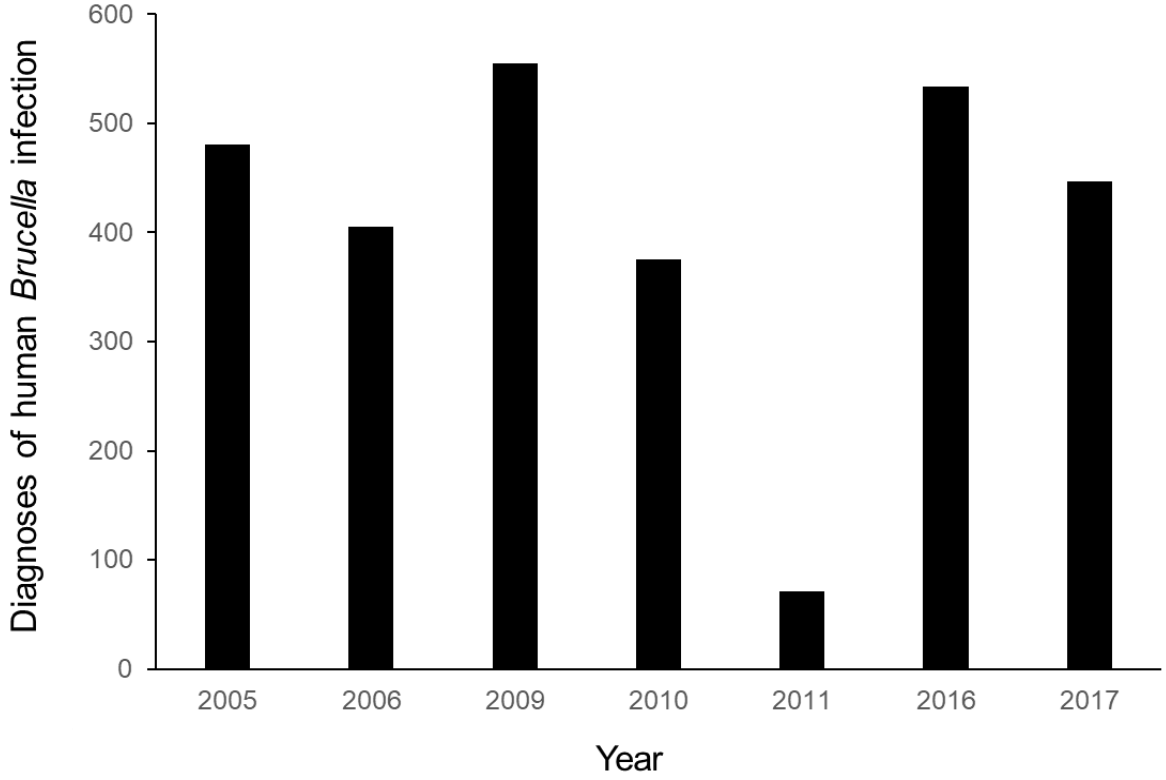

Figure 17. Brucellosis human cases officially reported in Peru between years 2005-2017 (WAHIS, 2018b)

Venezuela

The first official brucellosis control program in Venezuela started in 1968, with S19 vaccination, test and slaughter with no compensation. In 1975, with a cattle population of 9 million bovines, the vaccination coverage was close to 35 %, with a herd seroprevalence from 16% to 33.4% or individual seroprevalence from 1% to 3.4% depending on the area (García Carrillo, 1981; FAO, 2017). In 1999, the government approved the use of strain RB51 together with S19 for vaccination of 3-8-month-old female calves and revaccination with RB51 at 10 to 15-month-old as well as adult cows in high herd prevalence areas (Vargas, 2000). In 2002, S19 was replaced by RB51 in some regions, however, S19 is still used in others (Vargas,

2002). Presently, the vaccination strategy has drawbacks such as i) the low availability of the vaccine in market ii) the low quality of the vaccine; iii) absence of notification after vaccination, and; iv) lack of supervision of the vaccination program (González, 1999). Venezuela has established the slaughter of the positive bovines with no compensation.

Since 1999, the Autonomous Service of Animal Health (SASA) established RBT as a screening assay every 6 months in female cattle older than 20 months, and 2- Mercaptoethanol, CFT, and competitive ELISA as confirmatory assays. Herd surveillance has been carried out by milk ring test and ELISA in milk (Vargas, 2000). Herds that give a negative result in two consecutive samplings separated by 6 months receive a "brucellosis-free" status. Only animals from brucellosis-free herds are allowed to move in the territory.

In 2002, the reported herd seroprevalence in cattle was 10.5%. Even higher values were reported in some areas of the country. Official data in WAHIS (2018a), reported *B. abortus* from 2006 to 2018 and it was suspected but not confirmed in wildlife from 2009 to 2018. *B. suis* was suspected in domestic animals from 2009 to 2017 and in wildlife in 2011, and from 2015 to 2017 (WAHIS, 2018a). The presence of *B. abortus* biovar 1 and 4 in bovines, *B. suis* in swine, and *B. canis* in dogs have been reported in Venezuela (Corbel 1997; Lucero 2008; Contreras, 2000). Serological tests and isolation of *B. abortus* have been demonstrated in water buffaloes, while *B. suis* was recovered from capibaras (*Hydrochaeris hydrochaeris*), feral pigs and peccaries (*Tayassu tajacu*) (Lord *et al.,* 1983; Lord *et al.,* 1991).

B. melitensis was never reported in domestic animals or wildlife, according to the official data submitted to WAHIS (2018a), from 2006 to 2018. However, positive serological reactions have been detected in sheep and goats (Vargas, 2002). *B. abortus*, *B. melitensis* and *B. suis* have been detected in humans in Venezuela, with the former bacteria being the most commonly isolated (figure 18) (Vargas, 2002; Lord *et al*., 1998).

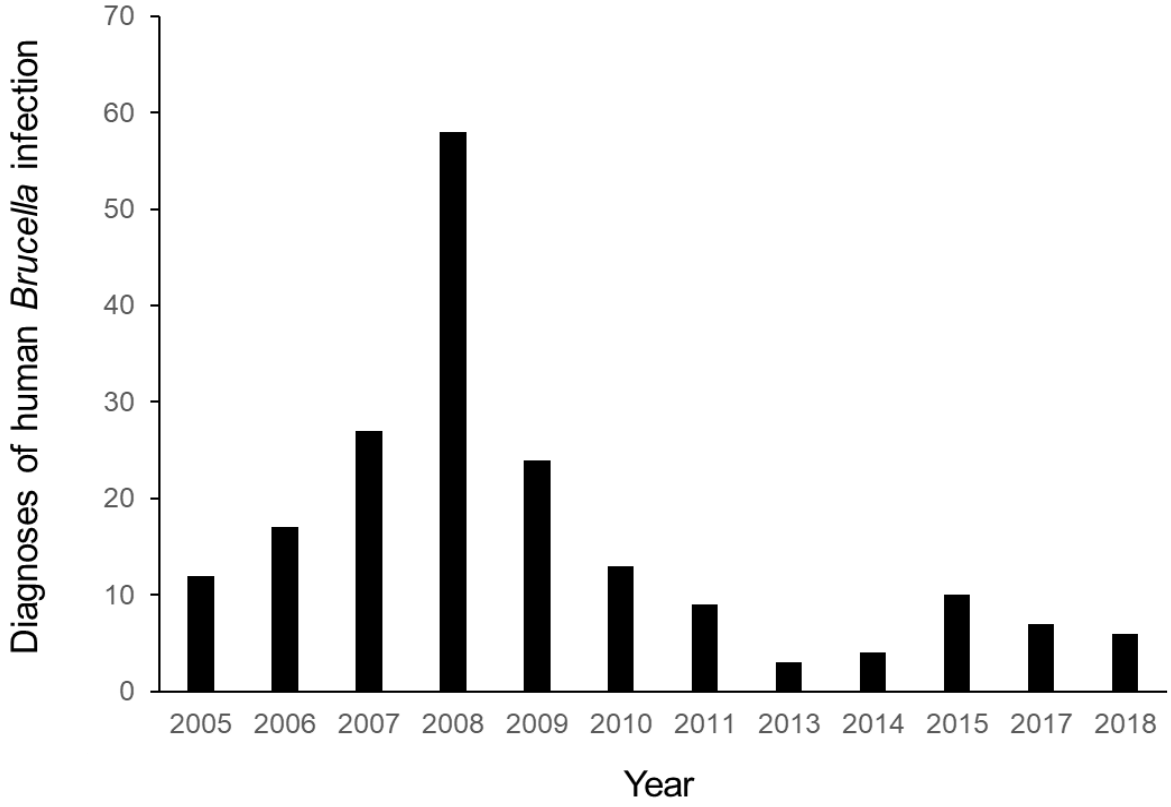

Figure 18. Brucellosis human cases officially reported in Venezuela between years 2005-2018 (WAHIS, 2018b)

Guyana

The population of bovines, swine, and goats in Guayana is 100249, 12600, and 82606, respectively. There is no epidemiological data certifying the absence or presence of brucellosis in Guayana. During 2006 to 2018, *B. melitensis* and *B abortus* were suspected but not confirmed. *B. suis* was reported as absent. There is no information regarding brucellosis in wildlife (WAHIS, 2018a). In spite of this, human cases have been reported with *B. abortus, B. melitensis,* and *B. suis* (Berger, 2018), indicating the presence of *Brucella* organisms in this region.

Suriname

The population of bovine, swine, and goats in Surinam is 33857, 34465, and 3852, respectively (FAO, 2017). An individual brucellosis seroprevalence of 6.4% was reported in a total population of 40200 bovines in 1970, (Kooy,1970; FAO, 2017). In subsequent years, the animal health authorities reported no brucellosis cases in 25000 bovine heads (WAHIS, 2018a; Berger, 2018). However, no epidemiological data regarding the current status of brucellosis is available, and the risk with neighboring countries with brucellosis exists.

French Guiana

The population of bovine, swine, and goats in French Guiana is 18592, 4772, and 1640, respectively (FAO, 2017). In 1981, the national authorities reported no brucellosis testing, and in recent years, the human health authorities have not reported any cases (García Carrillo, 1981; WAHIS, 2018b). There is no information regarding the presence of *B. melitensis, B. abortus,* and *B. suis* from 2006 to 2018 in domestic animals or wildlife (WAHIS, 2018a) nor any epidemiological studies. Still, it is a susceptible area, since it borders with countries with reported cases of animal and human brucellosis.

Brucellosis in the Southern Area countries (Argentina, Brazil, Chile, Paraguay and Uruguay)

Argentina

The herd prevalence of brucellosis in cattle during 1980 ranges from 10.75% and 13.86% (García Carrillo, 1981; FAO, 2017). Vaccination with S19 produced in Argentina since 1980 (García Carrillo, 1981) has been compulsory nationwide in calves of 3-8 months of age. In 1982, the Agriculture Department integrated a commission for the control of the disease that included governmental institutions, federal agriculture offices, private veterinarians, and producers. This program included mandatory as well as voluntary strategies, involving vaccination and test and slaughter, with no economic compensation. The assays used included plate and

tube agglutination test, 2 mercaptoethanol, and complement fixation. In dairy farms, the ring milk test was used for surveillance. By 1985, the estimated national herd seroprevalence decreased to 10% and individual seroprevalence above 5% (Samartino, 2002).

In 1989, the vaccination coverage with a reduce dose of S19 administrated subcutaneously increased from 33.7% calves (in 1980) to 70-74% until the beginning of the 1990's. In 1993, the vaccination increased to more than 90% (Samartino, 2002). The vaccination program included animals from 3 to 10 months of age and adults. In 1998, the milk prices from brucellosis-free herds received better payment, promoting the dairy market. During these years, the National Veterinary Services (SENASA) performed protection experiments with RB51 vaccine; however, the comparative results with S19 revealed that RB51 had low protection and reduced herd immunity. Following this, RB51 vaccine was banned from Argentina (SENASA-Argentina, 2002). In 2004, the national individual seroprevalence of bovine brucellosis was estimated to be 2.10% (12.4% in beef cattle farms), while in 2008, the herd seroprevalence ranged from 10% and 13% with an individual seroprevalence of 4-5% (Aznar *et al*., 2012; Lucero, 2008). Among the factors that promoted the increase in prevalence was the absence of compensation for slaughtering the reactors. This contributed to the hiding and selling of positive animals and spread of the infection (Aznar *et al*., 2012).

The *Brucellae* species isolated in Argentina included *B. abortus* biovar 1, *B. abortus* biovar 2, and *B. abortus* biovar 4, *B. melitensis* biovar 1, *B. suis* biovar 1, *B. suis* biovar 1a, *B. ovis* and *B. canis* (Lucero, 2008). *B. melitensis* bv.1 has been isolated in goats and sheep, and the herd seroprevalence was estimated at 3.6%, 12%, and 36% in the eastern, central, and western regions of Formosa province (border with Paraguay), respectively (Russo *et al*., 2016). The persistence of *B. melitensis* follows the distribution of goats in the central, western, and northern regions of the country, whereas *B. suis* and *B. abortus* had a higher prevalence in the Humid Pampa region where exploitation of cattle and pigs predominates

(Ministerio de Salud Argentina, 2013). *B. ovis* is present in rams all over the regions with herd seroprevalence ranging from 3% to 50% (SENASA, 2015).

In 2014, *B. abortus bv* 1 and 2 were reported as the most frequent biovars isolated in the cattle of the country (Aznar *et al.,* 2012). Antibodies in wildlife have been found in up to 11.8% of the animals tested in the Pampas in 2009. In 2012, *B. suis* biovar 1 was isolated in armadillos (*Chaetophractus villosus)* from La Pampa and European hare (*Lepus auropaeus)* from Buenos Aires province (Kin *et al*., 2014; Fort *et al*., 2012). From 1994 to 2006, *B. suis* was described as the agent responsible for 41% of the reported human cases, while *B. melitensis*, *B. abortus,* and *B. canis,* were responsible for the 38%, 20% and 1% zoonotic cases, respectively (Lucero, 2008). According to WAHIS (2018b), there were 2885 human brucellosis patients in Argentina from 2005 to 2018 (figure 19). Cases of *B. canis* infecting humans have been described in the provinces of Neuquen, Corrientes, and Tierra del Fuego (Lucero, 2008).

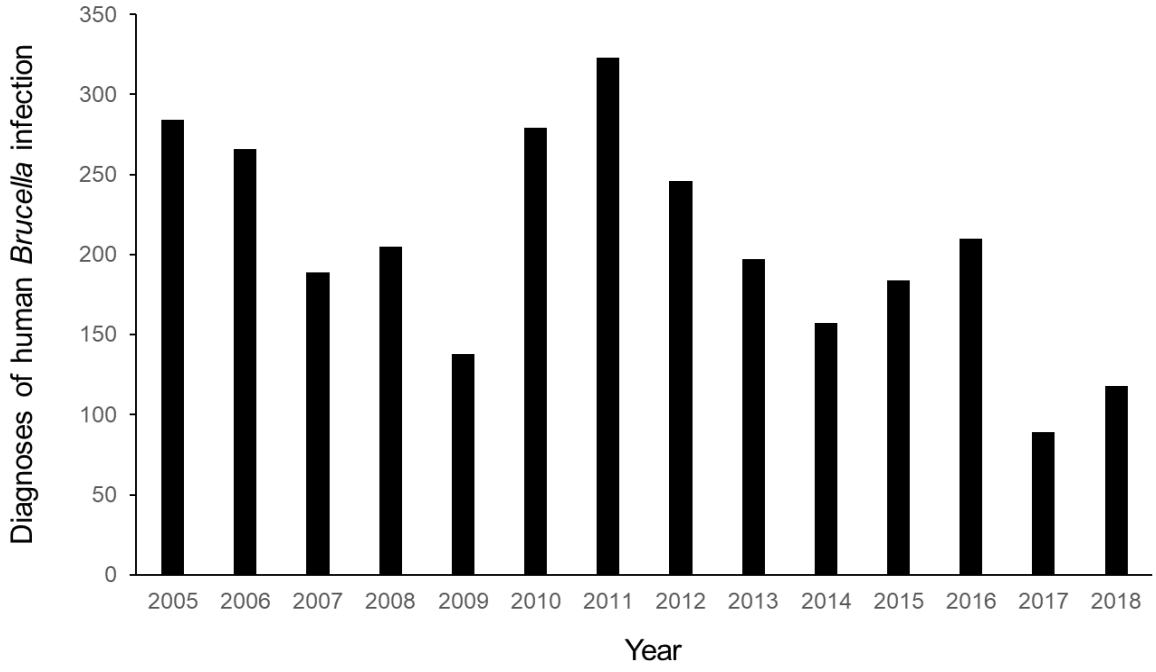

Figure 19. Brucellosis human cases officially reported in Argentina between years 2005-2018 (WAHIS, 2018b)

Brazil

During 1972 and 1974, the individual and herd seroprevalences of brucellosis in cattle from Sao Paulo and other regions ranged fro 10% and 90%, respectively (Correa *et al*., 1972; Costa *et al*., 1974). Some states such as Rio Grande do Sul started an aggressive vaccination program to achieve 80% coverage of eligible heifers. Following this, a significant decrease in brucellosis seroprevalence was observed in this region (García Carrillo, 1981). In 1975, a national survey determined a herd seroprevalence of 13.2% with a variation between 0.4% in Santa Catarina to 17.7% and 32.0% in Minas Gerais and Goias, respectively (García Carrillo, 1981). In 1976, the government proposed a National Program on a voluntary basis, with S19 vaccination of heifers from 3-8 months old and test and slaughter, with no compensation. This program was never fully implemented, and therefore the high prevalence remained active in the country, mainly in those regions with a high number of bovines (Poester *et al*., 2002). In 1994, Minas Gerais achieved a S19 vaccination coverage of 75% of the eligible heifers. However, in the rest of the country, vaccination was erratic, and the prevalence of *B. abortus* remained high. In the nineties, *B. suis* was sporadically reported in pigs (Corbel, 1997).

A new National Control Program was implemented in 2001, that included a compulsory S19 vaccination of calves from 3 to 8-month-old. The S19 vaccine was not free of charge, and the payment was assumed by the farmers. The vaccinated animals were eligible for serological testing just after 24 months of age. The diagnoses were based on RBT as a screening test and 2 mercaptoethanol, complement fixation (CFT), fluorescence polarization assay (FPA), and competitive ELISA as confirmatory assays (Poester *et al*., 2002). Positive animals were slaughtered without compensation. This program included voluntary strategies such as accreditation of brucellosis-free herds, voluntary monitoring of beef herds based on periodic sampling, regular test for breeding stocks before mobilization or livestock exhibitions. In 2002, the Ministry of Agriculture set up a program to access cheap loans for replacing the slaughter of positive animals (Poester *et al.*, 2002).

From 2000 to 2010, an individual and herd brucellosis seroprevalence of 9.88% and 86.3%, respectively, was reported in the Cerrado, Pantanal, and Amazon (Furtado *et al*., 2015). From 2007 to 2009, a herd seroprevalence of 11.4% and individual seroprevalence of 2.5% from Maranhao was reported (Borba *et al.,* 2013). In 2012, an individual seroprevalence in cattle from northern and southern Brazil was described as 10.2% and 0.06% with 41.2% and 0.32% herd seroprevalence, respectively (Aznar *et al.,* 2012). An individual prevalence of 31% was reported in semen samples from bulls from cattle-breeding farm in Minas Gerais, by PCR (Junqueira *et al*., 2013). In 2013, an individual seroprevalence of 4.8% was found in water buffaloes from Para State (da Silva *et al.,* 2014). During 2017, a new brucellosis control regulation was established for bovines and buffaloes with compulsory vaccination for both species at 3-8 months old with either S19 or RB51. The official diagnostic test included RBT as a screening assay and 2-Mercaptoetanol, CFT, and FPA as confirmatory tests (Secretaria de Defensa Agropecuaria, 2017).

In addition to *B. abortus* infections, other *Brucellae* such as *B. melitensis, B. suis, B. ovis, B. canis,* and *B. ceti* have been suspected in Brazil. Still, very few epidemiological or bacteriological studies regarding these other *Brucella* species are available (WAHIS, 2018a). A survey in 2011 detected an individual seroprevalence of 0.7% and 6.1% in goats and sheep from Río de Janeiro, suspecting *B. melitensis* and *B. ovis*, respectively (Martins *et al*., 2012). From 2003 to 2007, *B. canis* was isolated in 20.9% of the kennels in Sao Paulo (Keid, 2017). Also, from 2007 to 2017, *B. canis* was described in 50.7% of female dogs with reproductive problems, in 2.85% free-roaming dogs and 20.9% in kennel dogs in Parana (de Paula Dreer *et al.*, 2013). During 2014, according to the World Animal Health, Brazil had 1142 domestic animal outbreaks **(**Hull and Schumaker, 2018). *B. ceti* was detected in the brain of a stranded dolphin in Brazil littorals (Attademo *et al.,* 2018).

B. abortus, B. melitensis, B. suis, and *B. canis* human infections have been reported (Oliveira *et al*., 2012; Berger, 2018). In 2006, 0.66% of the slaughterhouse

workers in Northern Para were reported to have antibodies against smooth *Brucellae* (Gonçalves *et al.,* 2006). An increasing number of human patients have been reported by the health authorities. From 2009 to 2018 a number close to 1000 human brucellosis cases was reported (figure 20) (WAHIS, 2018b).

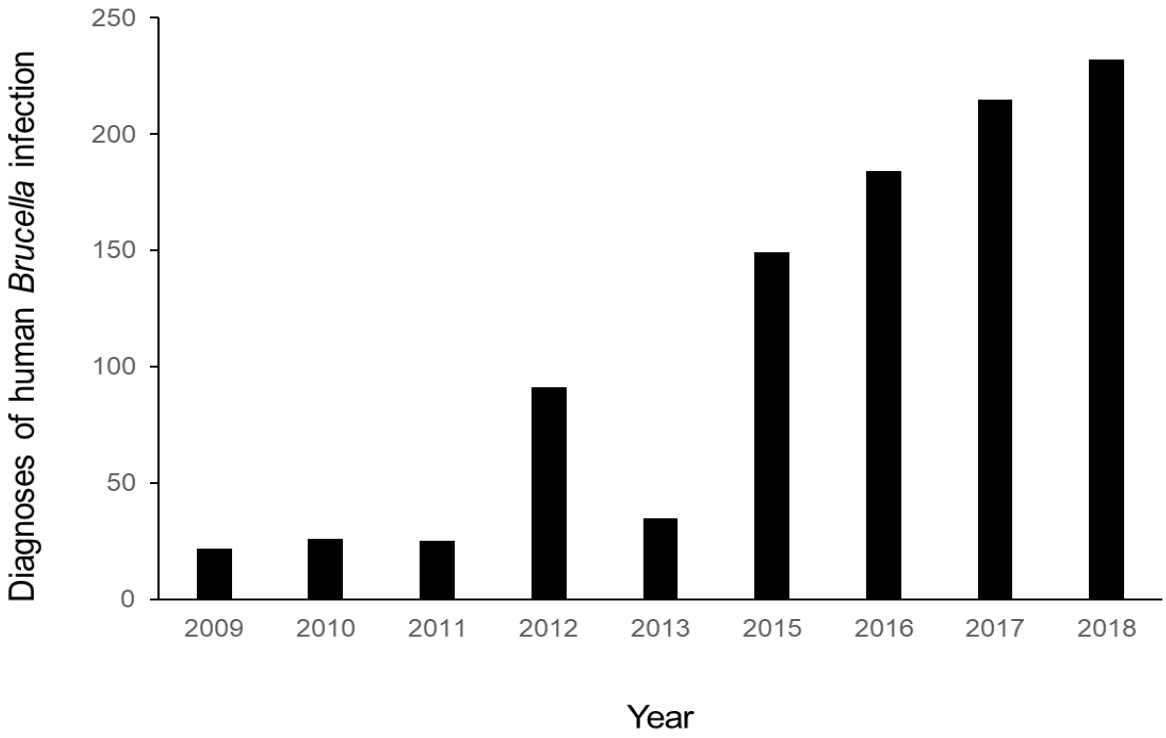

Figure 20. Brucellosis human cases officially reported in Brazil between years 2005-2018 (WAHIS, 2018b)

Chile

During 1974, the herd seroprevalence of brucellosis was 5%, 15%, and 3% in the Northern, Central, and Southern regions of Chile respectively, within a total bovine population of 3.4 million animals (García Carrillo,1981; FAO, 2017). In 1976, the Chilean Agriculture and Livestock Service, the Interamerican Development Bank, and the farmers set up a joint project. This project included mass vaccination with S19 of calves between 3 to 8 months in the Central-Southern region, where 92% of the herds were located. The project considered eradication of the disease in a period of five years using test and slaughter in the rest of the country with no compensation (García Carrillo, 1981).

In 1982, the estimated national herd seroprevalence of brucellosis was 2.9%, and in 1991 lowered to 0.4%. In 1992, the national brucellosis seroprevalence was described as steady, with strict test and slaughter strategy. Therefore, in 1997, with a population of 4.0 million bovines, the government changed the vaccine from S19 to RB51, with *B. abortus* infections still present in the country. In 2012, a national survey indicated a herd seroprevalence of brucellosis in cattle of 0.2% within a population of 3.7 million animals (Aznar *et al.,* 2012; FAO, 2017).

According to WAHIS (2018a), during 2006-2018, the only *Brucella* species reported in Chile has been *B. abortus*. Despite *B. melitensis* and *B. suis* claims to be absent since its eradication in the eighties in domestic animals, though still present in humans. There are no reported cases in wildlife either (WAHIS, 2018a). Other species of *Brucella*, such as *B. ovis,* were reported as sporadic (FAO, 2017; Lopetegui, 1999; Corbel, 1997). *B. canis* is expected to be present in cities like Temuco, where 1% of the free-roaming dogs had positive serology (Tuemmers *et al*., 2013). The Chilean National Reference Laboratory for brucellosis in Chile, reported human infections from 2001 to 2010. From this, 1% were due to *B. suis*, 4% to *B. melitensis*, 16% to *B. abortus,* and in 77% of the patients, the *Brucella* species was not identified. The average incidence rate was 5.5/1000000 inhabitants (Martínez, 2013), and according to FAO, a total of 68 human brucellosis patients were diagnosed from 2005 to 2018 (figure 21) (WAHIS, 2018b).

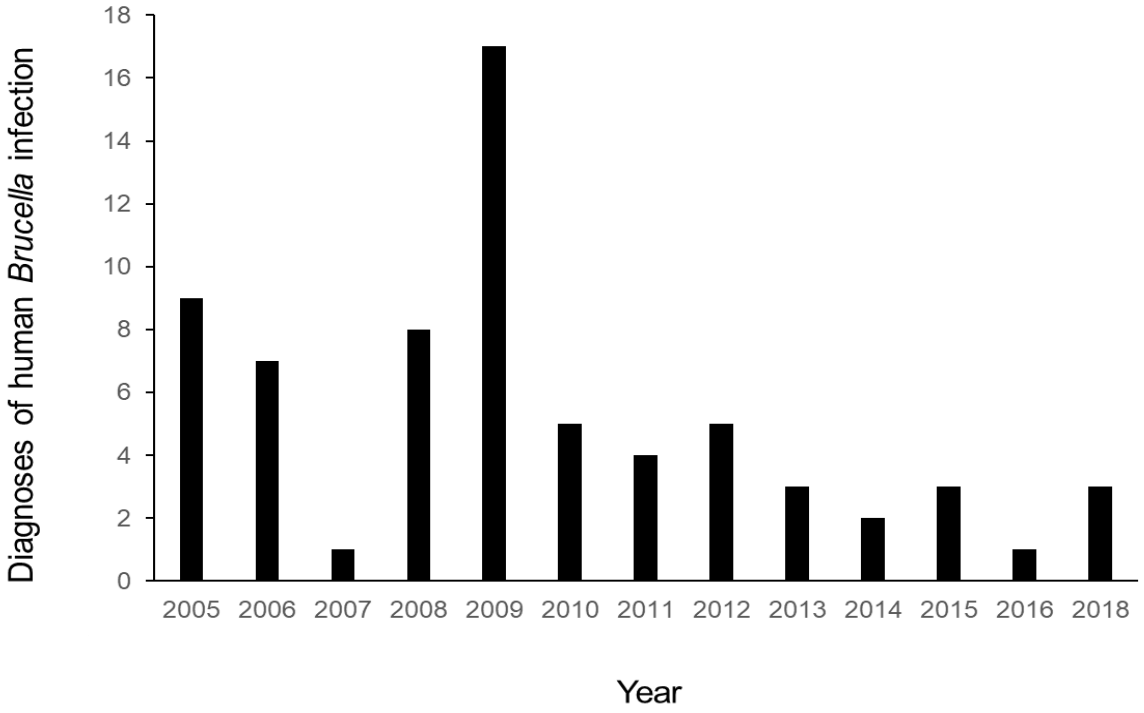

Figure 21. Brucellosis human cases officially reported in Chile from 2005-2018 (WAHIS, 2018b)

Paraguay

A survey performed with rose Bengal test in 6360 bovine serum samples was performed in the Eastern area of Paraguay in 1974, describing 25% of the farms positive for brucellosis. In the Western area, the individual seroprevalence ranged between 7.5% to 25% (Ibañez *et al*.,1975; Ibañez *et al.,* 1977; García Carrillo, 1981). With a population of 4.8 million bovines, the country initiated a strategy of mass vaccination with S19 in calves in 1976. The program coordinated by the Ministry of Agriculture and the Pan American Zoonoses Center (PAHO/WHO) (MAG-Paraguay, 1976; García Carrillo, 1981; FAO, 2017) was followed for eight years on a voluntary basis for slaughter of the reactors with no compensation. By 1978, the estimated herd prevalence was 2%; therefore, it was considered to adopt an eradication program (García Carrillo, 1981).

By 1994, *B. abortus*, *B. melitensis,* and *B. ovis* were described as sporadic infections (Corbel, 1997). From 1994 to 2014, a herd seroprevalence of 5.1% to 8.4% was reported in dairy cattle, and 19.8% to 3.9 % in double purpose and beef cattle, respectively (IICA, 2017; Aznar *et al.,* 2012). *B. melitensis* and *B. abortus* have been reported since 2006 to the present. *B. suis* was only reported as present in 2008 (WAHIS, 2018a). In 2017, the individual prevalence of *B. canis* in mixed-breed dogs was 9.6% in Conception City, estimated by immunochromatography test (Colman *et al*., 2017).

In 2018, the National Animal Quality and Health Service (SENACSA) established the compulsory nationwide vaccination of cattle, under the financial responsibility of the farmers. The vaccines included S19 for calf vaccination between 3-8 months old, and RB51 for adult cows and for revaccination (SENACSA, 2018). However, the vaccination coverage was low, achieved in no more than 50% of the cattle. The program did not include restrictions in the mobilization of herds, with the sole exception of animals in fairs or exhibitions (SENACSA, 2017).

Presently, there are no studies regarding the prevalence of brucellosis in Paraguay. According to the analysis based on routine diagnostic data, the estimated seroprevalence of brucellosis in bovines is close to 5% of individuals and 20% of the herds. Following this, the estimated seroprevalence in dairy cattle is 2% of the cows and 8% of the herds. In beef cattle, the estimated seroprevalence is 6% of bovines and 25% of the herds. According to the information of IICA, the herd seroprevalence may fluctuate as high as 20% (IICA, 2017).

From 2010 to 2018, thirty-two human cases were described including, 21 confirmed humans infected by *B. melitensis* out of 78 suspected students and personnel in a Veterinary School in Asuncion in 2017 (figure 22) (Berger, 2018; WAHIS, 2018b).

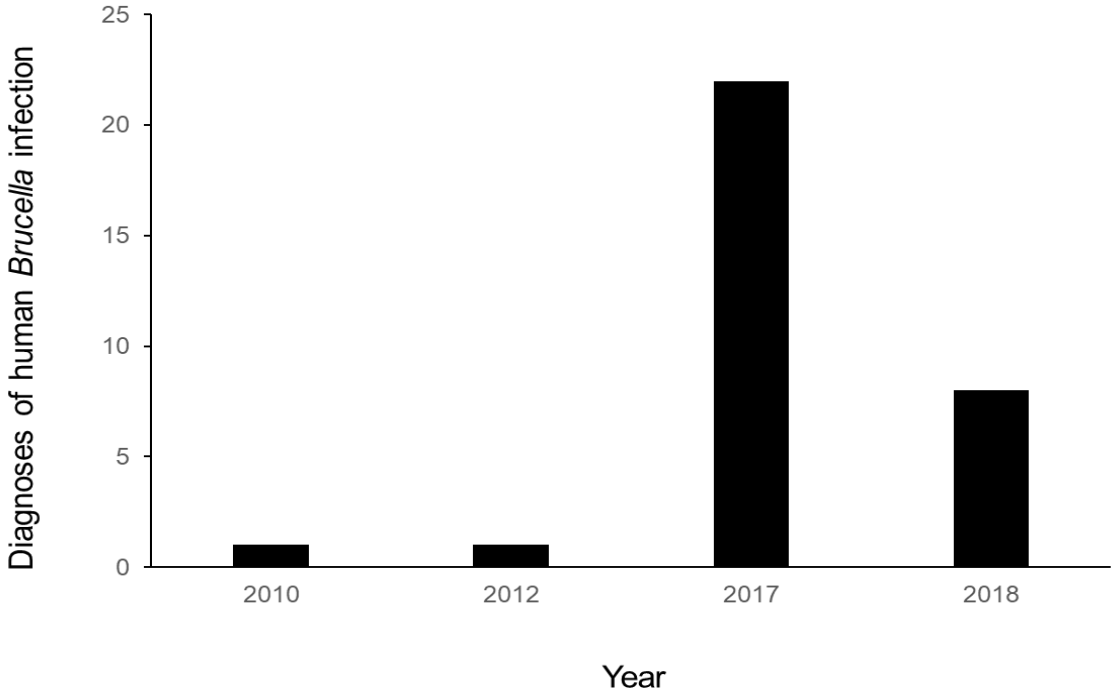

Figure 22. Brucellosis human cases officially reported in Paraguay between years 2010-2018 (WAHIS, 2018b)

Uruguay

In 1964, with a population of 8.7 million bovines, the mass vaccination with S19 of heifers was established as a compulsory strategy by the government (García Carrillo, 1981; Gil, 2009; FAO, 2017). Identification of the vaccinated animals, culling of the brucellosis positive animals, and action for the movement and importation of bovines were established (Garín A, 2011). In 1973, nine years after the compulsory vaccination of more than 80% of the bovines, the estimated herd seroprevalence was 3.3% for beef cattle and 1.4% for dairy cattle (García Carrillo, 1981). During the late seventies, the herd seroprevalence diminished to 1.2%-6.8% for beef cattle and 0.4%-3.2% for dairy cattle (Gil *et al*, 2009). By 1981, the coverage of vaccination with S19 reached 95.5% (García Carrillo, 1981).

In 1998, the Brucellosis Eradication Program was established to achieve the status of country free of brucellosis according to the conditions established by the International Zoosanitary Code of the OIE. The Brucellosis Eradication Program

began, with emphasis on dairy farms on a voluntary basis. The scheme included vaccination with S19, two rounds of serological testing with intervals of 6 to 12 months, and elimination of the seropositive animals. However, this program had low acceptance due to the absence of financial support (Gil *et al*, 2009).

In the early nineties, the national individual seroprevalence was 0.13% in non-random serum sampling and lowered to 0.30% in dairy cattle by random sampling. Therefore, due to the low seroprevalence of the disease, the Veterinary Services banned S19 vaccination in 1996. The program continued with the surveillance using milk ring test and slaughter of seropositive animals. Notification of abortions from farms became compulsory (Gil *et al*., 2009).

Due to a lack of suitable official intervention to the outbreaks in the Brazil border and the southeast region of the country, vaccination with *B.abortus* RB51 started in the risk zones in 2004. At the same time, economic compensation for the slaughter of positive cattle was implemented (Lopetegui, 2004). Still, from 2002 to 2008, the national herd seroprevalence was estimated between 2.04% to 1.30% in beef cattle and less than 0.25% in dairy cattle with an overall herd seroprevalence of 1.70% to 1.10% nationwide (Gil *et al.,* 2009). During 2012, the individual seroprevalence was estimated at 0.04% on 11.4 million bovines (Garín, 2011; Aznar *et al.,* 2012; FAO, 2017). No data are proving that bovine brucellosis has been eradicated yet.

The presence of *B. abortus* is reported mainly in bovine. *B. suis* was reported present from 2006 to 2015, and absent in 2018. *B. melitensis* has never been reported in domestic animals (WAHIS, 2018a). The authorities recognize that the advances that made it possible to reach eradication in cattle have been due to the success of vaccination with S19 back in the decades of sixties to eighties (Gil *et al.,* 2009). There is no information regarding brucellosis in wildlife. From 2005 to 2018, 77 cases of human brucellosis have been reported (figure 23) (WAHIS, 2018b; Pisani *et al*., 2017). The only species reported in humans has been *B. abortus*

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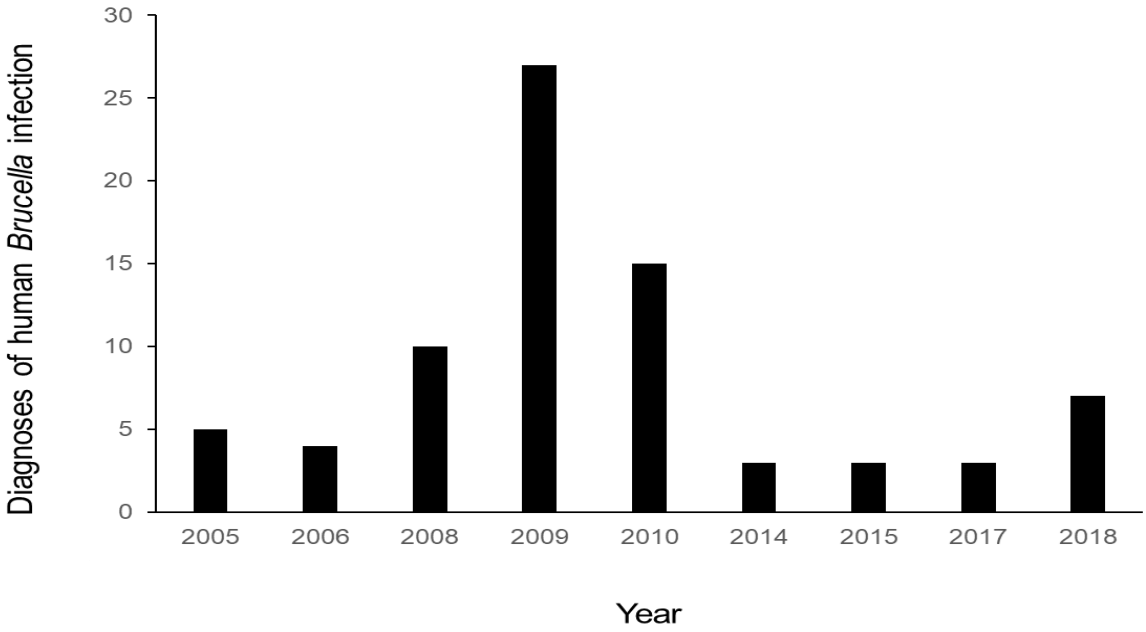

Figure 23. Brucellosis human cases officially reported in Uruguay between years 2005-2018 (WAHIS, 2018b)

Brucellosis in the Caribbean countries

(Antigua and Barbuda, Aruba, Bahamas, Barbados, Bermuda, British Virgin Islands, Cayman Islands, Cuba, Dominica, Dominica Republic, Grenada, Guadeloupe, Haiti, Jamaica, Martinique, Montserrat, Netherlands Antilles, Puerto Rico, St. Kitts and Nevis, St. Lucia St. Pierre and Miquelon, South Georgia and the South Sandwich Islands, Suriname, Trinidad and Tobago, Turks and Caicos Islands, US Virgin Islands, United States Minor Outlying Islands).

According to the WAHIS (2018a) from 2006 to 2018, there are no reports of brucellosis animal cases in the islands of Martinique, Cayman Islands, Dominica, Falkland Islands (Malvinas). However, no epidemiological data or scientific studies determining the presence or absence of *Brucella* organisms in these latitudes are available. In the islands of Anguilla, Aruba, Bermuda, British Virgin Islands, Guadeloupe, Montserrat, Netherlands Antilles, St. Kitts and Nevis, St. Lucia St.

Pierre and Miquelon, South Georgia and the South Sandwich Islands, Turks and Caicos Islands, US Virgin Islands, United States Minor Outlying Islands, no WAHIS (2018a) information was generated. In the case of wildlife of these locations, there is no information available regarding the presentation of brucellosis in these animals.

Antigua and Barbuda

In the islands of Antigua and Barbuda, brucellosis is not considered endemic; however, tests with armed *B. suis* as a biological weapon was linked to this country in 1948 (Willis, 2003). There is no information available on *B. melitensis, B. abortus* or *B. suis* on these islands during 2006 to 2018 (WAHIS, 2018a). However, there are no epidemiological data available nor attempts to isolate the bacteria from either domestic animals or wildlife.

Barbados

In 1975, the program for the eradication of the disease was established in 14000 animals. By 1977 the reported individual seroprevalence varied between 0.1% and 0.9% (García Carrillo, 1981; FAO, 2017). By 1997, neither *B. abortus*, *B. melitensis*, *B. suis,* nor *B. ovis* were reported on the island (Corbel, 1997). Currently, the disease is reported as absent in domestic animals, and there is no information in wildlife or humans (WAHIS, 2018a; WAHIS, 2018b). However, no epidemiological data nor attempts to isolate the bacteria either from domestic or wildlife animals from this island are available.

Bahamas

In this country, *B. suis* was tested as a biological weapon during 1953 and 1954 (Willis, 2003). Currently, there is no official information on *B. melitensis, B. abortus* or *B. suis* in this island from 2006 to 2018 reported in WAHIS (2018a).

Cuba

Brucellosis was first confirmed by the isolation of *B. abortus* in 1937 from a bovine placenta (Pelaiz, 1950). From 1963 to 1973, with a population of 6 million bovines and using tube agglutination test, an individual seroprevalence of brucellosis diminished from 4.33% to 0.3%. After this, a brucellosis control campaign based on test and slaughter, regulation of movement of bovines, and quarantines of imported bovines was established. During 1973-1976, with a cattle population of 5.3 to 5.6 million animals, an individual prevalence from 0.3% to 0.4% was achieved (García Carrillo, 1981).

From 2006 to 2018, the presence of *B. abortus* was reported, while *B. suis* was first reported in 1997 (Corbel, 1997). From 2006 to 2012, *B. suis* was still present, but reported absent in the following years. *B. melitensis* has never been reported in domestic animals or wildlife on the island (WAHIS, 2018a). In humans *Brucella* spp. have been described causing endocarditis (García *et al*., 2012), and a total of 345 cases were reported by the authorities between 2005 to 2018 (figure 24) (WAHIS, 2018b). Although *Brucella* organisms have been reported absent in wildlife, there are no published data on serological or bacteriological studies.

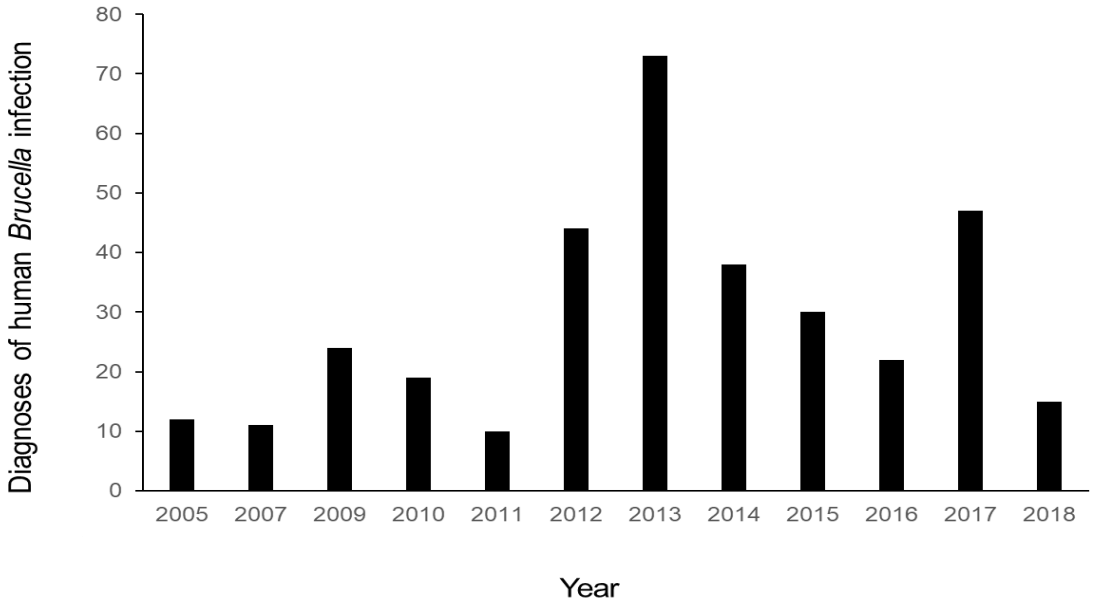

Figure 24. Brucellosis human cases officially reported in Cuba between years 2005-2018 (WAHIS, 2018b)

Dominican Republic

Serological studies in about 100000 animals were performed from 1966 to 1971 from a total population close to 1.3 million heads (García Carrillo, 1981; FAO, 2017). The individual seroprevalence ranged between 4.1% and 12.2%, with an average of 10% (García Carrillo, 1981). In 1972 with a bovine population of 1.4 million, a program to control and eradicate brucellosis was established using S19 vaccination, following, test, and slaughter in farms where it was financially possible (García Carrillo, 1981; FAO, 2017). There are no recent studies for the estimation of the bovine brucellosis prevalence in the Dominican Republic.

Both S19 and RB51 vaccines are used. While S19 is given free by the government, RB51 vaccine has to be purchased by the farmers. Calf and adult vaccination with full dose are allowed. During 2018, an estimated vaccination coverage reached 36% on an estimated population of 230695 bovines. RBT and FPA, as well as RMT, are used as diagnostic tests. Positive animals are marked and slaughtered with no compensation. Quarantine and restriction of mobilization of the positive herds are mandatory (Duran, U, 2019, per commun). In 2014 and 2015, 40 human brucellosis patients were reported in Dominican Republic (WAHIS, 2018b).

B. melitensis has been suspected but not confirmed in domestic animals (WAHIS, 2018a). *B. abortus* was reported as present from 2006 to 2018. *B. suis* seems to be absent; however, isolation or identification of the strains has not been attempted on a regular basis. There is no information regarding *Brucella* infections in wildlife (WAHIS, 2018a). No suitable epidemiological data are available from this country.

Grenada

The number of bovines, sheep, goats, and pigs in Grenada is estimated to be 5400, 13000, 7000, and 5400, respectively. Vaccination of bovines for *Brucella* spp. protection is not performed in Grenada. A survey during 2013 reported a bovine herd seroprevalence of 20% and individual seroprevalence of 6% (Chikweto *et al*., 2013a). Antibodies in dogs against smooth *Brucellae* had been recorded with an

individual seroprevalence ranging from 1 to 20% (Chikweto *et al*., 2013b). The status of brucellosis in other animals is unknown, and there is not information about this bacterial disease in wildlife or humans in Grenada.

Haiti

During 1964, the individual seroprevalence of brucellosis in cattle ranged between 3 to 5% (Grosnier, 1964). In 1966, the individual seroprevalence was found to be over 10% (Laroche *et al*., 1966). The estimated population of bovines and pigs on the island during 2017 was approximately 1.5 million and 1.6 million, respectively (FAO, 2017). Sporadic human cases due to *B. abortus* have been recorded, and the Dominican Republic, the neighbor country, has reported human brucellosis cases imported from Haiti. Currently, there is no information regarding the infection in domestic animal nor wildlife *Brucella* infections (WAHIS, 2018a).

Jamaica

From 1971 to 1975, the reported individual seroprevalence of bovine brucellosis ranged between 1.5% to 0.5% (García Carrillo, 1981). In 1978 and 1979, the individual seroprevalence reported was 0.3% and 1.2%, respectively (García Carrillo, 1981). Presently the bovine and swine population in Jamaica is of 265000 and 80000, respectively, with no reported cases of brucellosis (FAO, 2017). The number of sheep and goats in Jamaica is low, with no reported cases of brucellosis.

From 2006 to 2018, there is no information on *B. melitensis, B. abortus,* and *B. suis* in this island either in domestic animals, wildlife or humans (WAHIS, 2018a; WAHIS, 2018b). No suitable epidemiological data are available from this country.

Puerto Rico

Brucellosis did not exist in the island until 1923, after the importation of infected cattle from mainland USA (García Carrillo, 1981). In 1947, the national herd bovine seroprevalence was 13.6%, while the prevalence in human blood donor was 4.7% (Morales Otero, 1949). Vaccination with S19 started in 1942, followed by a test and slaughter strategy and certification of brucellosis-free areas. In 1949, the herd prevalence lowered to 1%. The herd seroprevalence described in 1977, 1978 and

1979 was 0.77%, 0.59% and 0.61%, respectively (García Carrillo,1981). During 2006 to 2018, there are no reports of *B. melitensis*, *B. abortus* or *B. suis* in domestic animals, wildlife or in humans (WAHIS, 2018a; WAHIS, 2018b). Presently it seems that Puerto Rico, as part of the confederation of the United States, is free of brucellosis; however, no published epidemiological studies are available.

St. Vincent and the Grenadines

B. melitensis, *B. abortus, B. suis* have been suspected in domestic ungulates but there is no confirmation from 2006 to 2018. There is no information regarding brucellosis in wildlife (WAHIS, 2018a). No epidemiological data are available from this island.

Trinidad and Tobago

B. abortus was present in bovines, including water buffalo from 2006 to 2014, and it was reported absent from 2015 to 2017 (WAHIS, 2018a). *B. melitensis* and *B. suis* have not been detected in domestic animals or wildlife. However, systematic attempts to isolate the bacterium have not been carried out; no epidemiological studies are available.

Annex 2. Official data of the presentation of brucellosis in domestic animals or wildlife 2006-2018 of the Americas. (WAHIS, 2018a; FAO,2017; García Carrillo, 1981).

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Anguilla	NI	NI	NI	NI	NI	NI	NI	NI
Antigua and Barbuda	5000	NI	NI	NI	NI	NI	NI	NI
Argentina	53353787	1922 human	P 2006-2018	P 2006-2011 L 2012-2018	P 2006-2017 L 2018	NI	L 2012 P 2014- 2017	A 2017-2018
Aruba	NI	NI	NI	NI	NI	NI	NI	NI
Bahamas	740	NI	NR	NR	NR	NR	NR	NR
Barbados	10743	1948	A	A	A	NI	NI	A
Belize	113122	NI	A	NR	NR	A	NR	NR
Bermuda	649	NI	NI	NI	NI	NI	NI	N
Brazil		NI	NR	P 2006-2018	P 2006 A 2007-2018	NR	NI	NI
British Virgin Island	2400	NI	NI	NI	NI	NI	NI	NI

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Canada	11535000	NI	NR	L 2007 SN 2008 A 2006,2009 -2018	A	NR	SN 2009-2011 L 2012-2018	P 2009-2018
Cayman islands	2111	NI	A	A	A	NI	NI	NI
Chile	2890840	NI	A	P 2006-2018	A	A	A	A
Colombia	22461179	1944 bovine placenta	NR	P 2006-2018	A	NR	A	A
Costa Rica	1420979	1914 hemoculture human	NI	P 2006-2018	NI	NI	SN 2009- 2012	NI

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Cuba	3865500	1937 bovine placenta	NR	P 2006-2018	P 2006-2012 A 2013-2018	NR	A 2010-2018	A
Dominica	14076	NI	NR	NR	NR	NI	NI	NI
Dominican Republic	3000000	NI	SN 2017	P 2006-2018	A	NI	NI	NI
Ecuador	4190611	1952 vaginal fluids bovine	NI	P 2006-2018	SN 2011 P 2012-2018	NI	NI	NI
El Salvador	962889	NI	A	P 2006-2017	A	A	NI	NI
Falkland Islands (Malvinas)	4201	NI	NR	NR	NR	NR	NR	NR
French Guiana	18582	1941 human	A	A	NI	NI	NI	NI

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Grenada	4552	NI	A	A	A	NI	NI	NI
Guadeloupe	76975	NI	NR	NI	A	NI	NI	NI
Guatemala	3850206	NI	NR	P 2006-2017	NR	NR	SN 2009- 2017	NR
Guyana	100249	NI	SN 2006	SN 2006, 2010	A	NI	NI	NI
Haiti	1497228	NI	NI	NI	A	NI	NI	N
Honduras	2869201	1977 Bovine	NI	L 2006-2009, 2014-2017 P 2010-2014	A	NI	NI	NI
Jamaica	130668	NI	NR	A	NR	NR	NI	NR
Martinique (France)	13594	NI	A	A	A	A	NI	NI
Mexico	31771736	NI	P 2006-2014 L 2014-2017	P 2006-2010 L 2010-2017	P 2013-2014	SN 2009-2014 A 2016-2017	SN 2009-2014 A 2015-2017	SN 2009-2014

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Monstserrat	10071	NI	NI	NI	NI	NI	NI	NI
Netherlands Antilles	644	NI	NI	NI	NI	NI	NI	NI
Nicaragua	4848341	1977	P 2006	P 2006-2018	P 2006, 2012	NI	NI	NI
Panama	1521500	NI	A	P 2006-2012, 2015-2018 L 2013-2016	A	A	NI	NI
Paraguay	13821526	1976 cow milk	P 2006-2008, 2017,2018	P 2006-2008 L 2008-2018	P 2008	NI	NI	NI
Peru	5535569	NI	L 2007, 2008,2011, 2016,2017	L 2008-2018	P 2007	NI	A 2016-2018	NI

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Puerto Rico & US Virgin Islands	372524	NI	NI	NI	NI	NI	NI	NI
St. Kitts & Nevis	2000	NI	NI	NI	NI	NI	NI	NI
St. Lucia	10981	NI	NI	NI	NI	NI	NI	NI
St. Pierre & Miquelon	38	NI	NI	NI	NI	NI	NI	NI
St. Vincent & the Grenadines	3931	NI	SN 2006-2010	SN 2006-2010 A 2017-2018	NR	NI	NI	A
South Georgia and the South Sandwich Islands	NI	NI	A	NI	NI	A	NI	NI
Suriname	33857	NI	A	A	A	A	A	A

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Trinidad and Tobago	35895	NI	NR	P 2006,2009, 2014 A 2007-2008,2010-2013,2015-2017	NR	NR	NI	NR
Turks & Caicos Islands	NI	NI	NI	NI	NI	NI	NI	NI
US Virgin Islands	8101	NI	NI	NI		NI	NI	NI
United States Minor Outlying Islands	NI	NI	NI	NI	NI	NI	NI	NI
Uruguay	11739000	NI	NR	P 2006-2018	P 2006-2012 L 2015 A 2013,2014, 2016/2018	NR	NI 2009-2014	A

Country	Cattle Population	Year of first report of brucellosis -specie	Domestic animals			Wildlife		
			B. melitensis	***B. abortus***	***B. suis***	***B. melitensis***	***B. abortus***	***B. suis***
Venezuela	16482742	1930 Bovine	NR	P 2006,2018	SN 2009-2017	NR	SN 2009-2018	SN 2011,2015, 2017

*Disease reported as: (P) Present, (L) Limited to one or more zones, (SN) Suspected but Not confirmed, (A)Absent, (NI) No Information, (NR) Never Reported