UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Postgrados

Detecting *Brucella* species in Ecuador

Ligia Elizabeth Luna Jarrín

Gabriel Trueba, PhD., Director de Tesis

Tesis de grado presentada como requisito para la obtención

del título de Magíster en Microbiología

Quito, mayo de 2015

UNIVERSIDAD SAN FRANCISCO DE QUITO

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HOJA DE APROBACIÓN DE TESIS

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Ligia Elizabeth Luna Jarrín

Gabriel Trueba, Ph.D. ----------
Director de la Maestría en Microbiología
y Director de Tesis

Verónica Barragán, M.Sc. ----------
Miembro del Comité de Tesis

Ivette Dueñas DVM. M.Sc. ----------
Miembro del Comité de Tesis

Victor Viteri Breedy, Ph.D. ----------
Decano del Colegio de Postgrados

Quito, mayo de 2015

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Firma:

Nombre: Ligia Elizabeth Luna Jarrín

C. I.: 1711587392

Fecha: Quito, mayo de 2015

RESUMEN

Brucelosis es una enfermedad zoonótica emergente en varios países alrededor del mundo. En Ecuador existen reportes de infecciones por *Brucella abortus* en ganado y humanos, sin embargo otras especies de *Brucella* no han sido identificadas. Este estudio busca identificar especies de *Brucella* que se encuentran circulando en la región sierra del país. Para ello se tomaron 300 muestras de cabras y 1 muestras de feto canino, provenientes de 8 diferentes provincias de la sierra ecuatoriana. Los resultados evidenciaron la circulación de especies *de Brucella melitensis, Brucella suis, Brucella abortus y Brucella canis.*

ABSTRACT

Brucelosis is an emerging zoonotic disease in many countries around the world. There are some reports of *Brucella abortus* infections in cattle and humans in Ecuador, nevertheless, other *Brucella* species have not been identified. This study was designed to identify circulating *Brucella* species in 300 goat samples and one canine fetus from 8 different provinces of the highland Andes of the country. The results showed isolates from *Brucella melitensis, Brucella suis, Brucella abortus y Brucella canis.*

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AIMS AND

GENERAL INTRODUCTION

AIMS

General Aim

- Detecting *Brucella* species in goat samples from different provinces of Ecuador

Specific Aims

- Detecting *B. melitensis, B. abortus* and B. *ovis* in lymph nodes and raw milk of goats
- Standardize PCR technique for the identification of *B. melitensis, B.abortus* and *B. ovis* in the microbiology laboratory

GENERAL INTRODUCTION

Historical Information of Brucellosis

Brucellosis was reported for the first time in the XIX century on the Mediterranean Island of Malta. This disease was responsible for the deaths of soldiers and people of the community as a result of a fever whose cause remained ignored. An investigative commission led by David Bruce, was sent to determinate the cause of the disease. In 1905 Themistocles Zammit found that goats were related to the transmission of brucellosis. Fifty percent of the goats were sick and human disease was directly related to goat milk drinking. David Bruce, Guiseppe Caruana Scicluna and a group of experts examined the content of the spleens of dead people and found evidence of a bacterial infection. The research group isolated and cultured the bacteria, and identified it as the causal agent of the disease. In 1920 Meyer and Shaw unified all the information related with this bacterium and decided to call it with the common term of *Brucella* (1).

Taxonomy of *Brucella* species

Brucella is a small gram negative coccobacillus, nonfermenting, aerobic, nonmotile, nonspore, noncapsules and facultative intracellular bacteria (2). The genus *Brucella* is within the family Brucellaceae of the order Rhizobiales in the class Alphaproteobacteria of the phylum Proteobacteria. They belong to the alpha-2 subdivision of the Proteobacteria along with *Ochrobactrum, Rhizobium, Rhodobacter, Agrobacterium, Bartonella* and *Rickettsia* organisms that are pathogens or symbionts of plants or mammalian (3, 4).

Alphaproteobacteria are an ecologically diverse group of gram negative bacteria, among which several lineages evolved from niches in the environment toward obligate intracellular parasitism of diverse eukaryotic hosts. The adaptation to intracellular life has been strongly related with genome reduction, due to the loss of genes that are not needed in this new environment (5). Among the organisms affecting mammals in the phylum Proteobacteria are the genera *Bartonella* and *Rickettsia,* all of which are spread by vector-based transmission. These organisms have a small genome size which is consistent with obligate intracellular survival (4). *Brucella* differs from most genera within the order Rhizobiales due to their features: 1. *Brucella* can infect mammalian cells and is a facultative intracellular pathogen; 2. The size of its genome is 50 – 100% larger than *Bartonella* genomes; 3. *Brucella* has conserved metabolic functions which are present in plant pathogens; 4. It can persist in soil for up to 10 weeks, which is consistent with their metabolic ability of utilize plant-based molecules (4).

Brucella genome

The genome of *Brucella* consists in two circular chromosomes and has a size average of approximately 3.29 Mb (3). Chromosome I has approximately an average of 2.11 Mb and their G+C content is 57.2%, this chromosome resembles a classic bacterial circular chromosome with the likely origin of replication adjacent to a gene cluster. Chromosome II has an average approximately of 1.18 Mb and their G+C content is 57.3%, this chromosome have a cluster of plasmid like replication genes, similar to plasmid replication genes from *Agrobacterium* and *Rhizobium* (3, 6).

Chromosome I encodes the majority of the core metabolic machinery processes such as transcription, translation, and protein synthesis; 51 of 53 ribosomal proteins and 41 of 55 tRNAs are encode in this chromosome. In addition, chromosome I has high percentage of phage-related proteins, due the presence of inserter phage remnants (6).

Chromosome II encodes genes involved in cellular process such as membrane transport, energy metabolism and regulation; these functions appear to represent alternative pathways for specific substrates usage. Chromosome II also encodes for genes involved in cellular processes and plasmid functions, due to the presence of clusters for flagellar biosynthesis, secretion genes, conjugation-associated and plasmid-like replication genes. This chromosome is not predicted to be dispensable, because it encodes essential genes such as tRNA-Cys and tRNA synthetases (6).

Some studies suggest chromosome II has been derived from a megaplasmid that was captured by an ancestral *Brucella.* The acquisition of this megaplasmid was presumably a very ancient event due to the fact that the G+C percentage is very similar in the two chromosomes (6).

Brucella **Speciation**

Nine *Brucella* species are currently recognized, seven of them affect terrestrial animals: *B. abortus* (mainly in cattle)*, B. melitensis* (mainly in goats and sheeps)*, B. suis* (mainly in pigs)*, B. ovis* (mainly in sheeps)*, B. canis* (in dogs)*, B. neotomae* (desert woodrats) and two species that affect marine mammals, *B. cetaceae* (dolphins) and *B. pinnipedialis* (marine mammals)(3, 7).

The origin of *Brucella* species was related with the apparent adaptation to specific hosts, an obvious starting place is the coevolution of *Brucella* species with their preferred hosts, but these is not consistent with the overall genetic variation between *Brucella* species, due to few genetic polymorphism (4). Genetic analyses have shown close similarity among the species with a mean diversity between genomes of around 0.22% (8), which suggests a recent adaptation. An study that use a molecular clock based on single nucleotide polymorphisms in 13 different *Brucella* genomes, concluded that most *Brucella* species diverged from a common ancestor (similar to *B. ovis*) in the past 86,000 to 296,000 years. However, it precedes livestock domestication in Middle East (in the past 10,000 years), suggesting that this disease was endemic within wildlife populations rather than emerging due to domestication (9).

Studies show that *B. ovis* share the common ancestor with the clade of *B. abortus* and *B. melitensis*; these two species have a close relationship and are distant to *B. suis.* Another clade share *B. suis* and *B. canis*, these two species are highly similar and *B. canis* appears to have arisen directly from *B. suis* ancestor (Figure 1). Transmittal of brucellae from pigs to canids likely stemmed from infection of wolves or other canids feeding on the ancestor of *B. suis* within the past 22,500 years (9). *Brucella species* may infect animals other than their primary host; however such infection appears to be self-limiting (4).

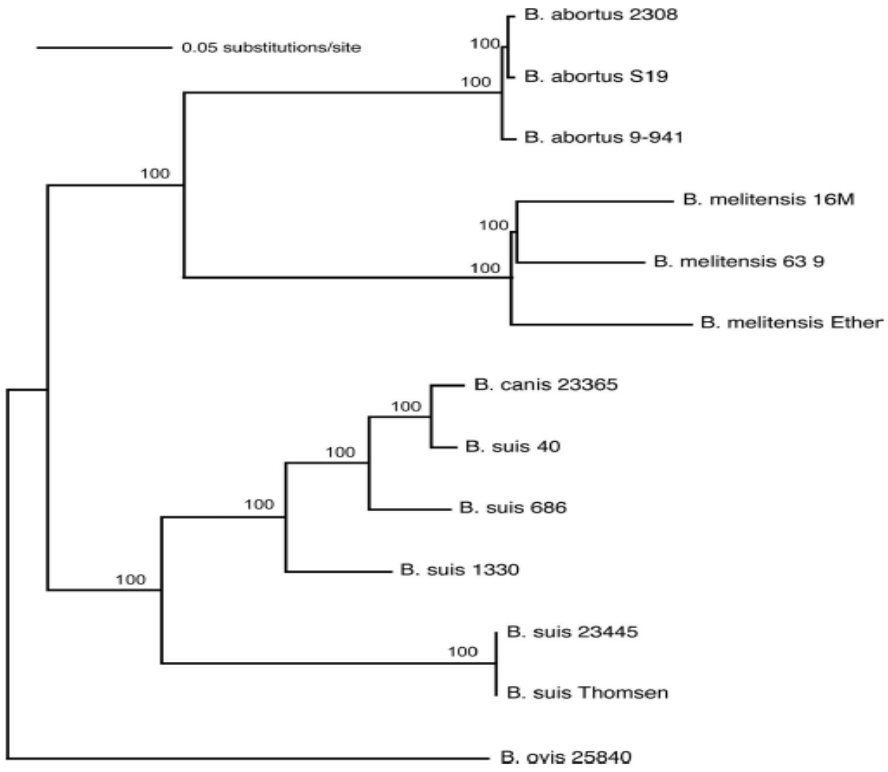

Figure 1. Rooted phylogeny of the genus *Brucella* (9)

Animal Brucellosis

Brucellosis is a worldwide zoonotic disease caused by facultative intracellular bacteria of the genus *Brucella*. Generally, it infects livestock species such as, cattle, sheeps, goats, swine, and camels. Typically the disease is mild, showing the infected animals few signs; they submit the main clinical signs following infection with *Brucella* that is abortion. The principal strains that infect livestock are *Brucella abortus, Brucella suis* and *Brucella melitensis* (3).

Cows are primarily infected by *Brucella abortus* and the main symptoms in pregnant females is abortion, generally in the second half of gestation with retention of placenta and metritis or full term birth of dead or weak calves. Most of

the cows abort only once although, the placenta will be heavily infected at subsequent apparently normal calvings. There is an estimated of 25% reduction in milk production in infected cows. Brucellae is localized in supra-mammary lymph nodes and mammary glands of 80% of the infected animals and thus the bacteria are secreted in milk throughout their lives (3, 10).

In goats the main etiologic agent of brucellosis is *Brucella melitensis* and the disease is characterized by late abortion, stillbirths, decreased fertility and reduction of milk production. Sheep brucellosis is divided into classical brucellosis and ram epididymitis that is a reproductive disease that causes inflammation of the epididymis and can lead to infertility and reduced capacity to produce viable spermatozoa. Ram epididymitis is caused by *Brucella ovis* (non-zoonotic agent) and is generally transmitted venereally, while classical brucellosis is caused by *Brucella melitensis* (3, 11).

Swine infected by *B. suis* develop abortion and may cause orchitis, lameness, hind limb paralysis or spondylitis, metritis or abscesses. Camels can be infected by *B. abortus* and *B. melitensis* when they are kept together with infected sheeps, goats or cattle. The main etiologic agent for brucellosis in dogs is *B. canis*, but can also be infected by *B. abortus, B. suis* and *B. melitensis.* Dogs infected with *B. canis* may develop abortions during the last third of a pregnancy or conception failures (3)

Brucellosis is typically spread during the abortion or during birth, because high levels of bacteria are found in the placenta of infected animals. *Brucella* can survive in the environment for several months, principally in cool moist conditions,

and the bacteria can infect other animals by ingestion and can also colonize the udder (12).

Human Brucellosis

Brucellosis is a zoonosis and nearly every human case has a direct (contact) or indirect origin. The main sources of *Brucella* are infected animals or their fluids or tissues such as: raw milk, urine, blood, carcasses, placenta and miscarriage calf products. Transmission ways are via ingestion or inhalation, or through conjunctiva or skin abrasions. Brucellosis is an occupational disease and the risk groups are veterinarians, slaughterhouse and laboratory workers and livestock caretakers (10, 13).

The incubation period of brucellosis normally is 1-3 weeks, but in some cases can be several months before showing signs of infection. *Brucella melitensis* is associated with acute infection and the other species usually produce subacute and prolonged infections (3).

Symptoms and signs of brucellosis usually referred as fever of unknown origin, which can be confused with other diseases like enteric fever, malaria, rheumatic fever, cholecystitis, fungal infection, autoimmune disease (3)

The most common symptoms of brucellosis include undulant fever in which the temperature can vary from 37°C in the morning to 40°C in the afternoon, night sweats with peculiar odor, chills and weakness. Other symptoms also include malaise, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness and depression (3).

Human brucellosis is also known for complications and involvement of internal organs and its symptoms can be diverse depending on the site of the infection, including encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis and prostatitis. The most severe complication in *Brucella* is endocarditis and it is associated with *Brucella melitensis,* which accounts the 80% of deaths due to brucellosis (3).

Lack of appropriate therapy during the acute phase may result in localization of *Brucella* in various tissues and organs and lead to subacute or chronic disease, which is hard to treat and 2% of *Brucella melitensis* infected patients died (3).

Epidemiology

Worldwide, brucellosis remains a major source of disease in humans and domestic animals. Reported incidence and prevalence of the disease vary widely from country to country. Bovine brucellosis caused mainly by *Brucella abortus* is still the most widespread form. In humans, brucellosis caused by *Brucella melitensis* is the most important clinically apparent disease (14, 15).

The disease is endemic in countries of the Mediterranean basin, Arabian Gulf, Western Asia, parts of Africa and Latin America (16). Human brucellosis is found to have significant presence in rural communities where people live in close association with animals. Worldwide, reported incidence of human brucellosis in endemic disease areas varies widely from <0.01 to >200 per 100 000 inhabitants, this is over half million new cases annually (14, 15, 17).

However, the true incidence of human brucellosis is unknown for most countries. While some areas such as Peru, Kuwait and parts of Saudi Arabia, have a very

high incidence of acute infections, the low incidence reported in other known brucellosis endemic areas may reflect low levels of surveillance and reporting. It has been estimated that the true incidence may be 25 times higher than the reported incidence due to misdiagnosis and underreporting (14, 15).

A study where human benefits through animal interventions for zoonosis control was evaluated, conclude that livestock mass vaccination campaign for small ruminants and cattle could achieve 52% reduction of brucellosis transmission between animals and 51 856 human brucellosis cases could be averted. Costs estimated of the intervention were USD 8.3 million and the overall benefit was USD 26.3 million, suggesting that brucellosis control becomes one of the most cost effective interventions in the public health sector (18)

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PART II

SCIENTIFIC PAPER

Molecular Detection of *Brucella* species in Ecuador

Ligia Luna[a,] Gabriela Chávez [b] Verónica Barragán[a,c], Gabriel Trueba [a,]

Instituto de Microbiología, Universidad San Francisco de Quito, Quito, Ecuador

Colegio de Ciencias Biológicas, Universidad San Francisco de Quito, Ecuador[a]

Hospital Veterinario, Universidad San Francisco de Quito, Quito, Ecuador [b]

MGGEN, Northern Arizona University, Flagstaff, Arizona, USA[c]

Corresponding author:

Gabriel Trueba: gtrueba@usfqw.edu.ec

Abstract

Brucellosis is a zoonosis which causes severe disease in humans and important economic loses in livestock operations. In Ecuador, the only Brucella species reported (in humans and domestic animals) has been *B. abortus.* Nevertheless, other *Brucella* species have been detected in neighboring countries. We used a previously described PCR protocol to reveal the presence of *B. melitensis* and *B. suis* infection in Ecuadorian goats. We also developed a PCR protocol to detect *B. canis* in a dog.

Key Words: Ecuador, Brucellosis, *B. melitensis*, *B. suis, B. abortus, B. canis*

1. Introduction

Brucellosis is a zoonotic disease caused by *Brucella* spp., a Gram negative coccobacillus and facultative intracellular bacterium (Mirnejad, 2012). Different *Brucella* species are associated with a particular animal species: *B. abortus* with cattle, *B. melitensis* with goats and sheep, *B. suis* with pigs, *B. ovis* with sheep, *B. canis* with dogs, etc. (Foster, 2008; Seleem, 2010). Brucellosis in animals is usually investigated using serologic test which don't differentiate *Brucella* species. Detection of *Brucella* species requires bacterial isolation (Gupta, 2006), which is hazardous and difficult (Gupta, 2006; Kang, 2011).

In humans, *Brucell*a spp. causes chronic infection characterized by intermittent fever, arthralgia, and fatigue (Unver, 2006; Nicoletti, 2010). The most severe disease is caused by *B. melitensis* (Ko, 2003; Unver, 2006). Infection occurs mainly by direct contact with tissue (or fluids) from infected animals and by consumption of contaminated dairy products (Paulsen, 2002; Unver, 2006; Wattam, 2014). *Brucella* annually infects more than 500,000 people worldwide and prevalence rates in some countries exceed 10 cases per 100,000 inhabitants (Foster, 2008; Lucero, 2008; Nicoletti, 2010).

In livestock, brucellosis cause an incurable infection characterized by abortion, infertility and decreased milk production (Seleem, 2010). However, the most important problem associated with animal brucellosis is the potential transmission to humans; as a consequence infected animals must be eliminated from herds (Zinsstag, 2007).

Brucellosis is especially prevalent in low income countries where disease control programs and diagnosis are limited (Corbel, 1997; Ron and Benítez, 2005). In Ecuador brucellosis is an underreported disease (Ron, 2014) and recent studies have identified only *B. abortus* associated with disease in human and domestic animals (Ron, 2013; Ron, 2014; Rodríguez, 2015). Nevertheless, other *Brucella* species have been detected in neighboring countries (Corbel, 1997) which leads us to hypothesize that other species of *Brucella* must be present in Ecuador albeit not detected due to low prevalence and methodological difficulties. We use molecular tools to detect for the first time in Ecuador, the presence of *B. melitensis*, *B. suis* and *B. canis*.

Material and methods

Sample collection

Three hundred inguinal lymph from 240 goats were collected at a slaughterhouse in Quito and 60 samples of goat raw milk were purchased in the streets of Quito and Otavalo. Samples were transported in ice and preserved at -20°C until analyzed. Liver and heart samples from a canine fetus were obtained from the Veterinary Hospital at Universidad San Francisco de Quito, were kept at 4°C until cultured and preserved at -20°C for PCR analysis.

DNA extraction

Total DNA was isolated by modified CTAB method. A 2 mm^3 piece of animal tissue was cut with a sterile scalpel, washed twice with 1 ml PBS (pH 7.0) and placed in a sterile tube with 700 µl of CTAB solution. Five hundred micro liters of raw milk samples were suspended in 500 µl of PBS and mixed with 700 µl of CTAB solution (Doyle, 1987). Samples (tissue and milk) were incubated for 2 hours at 65°C. Tubes received 700 µl of chloroform: isoamyl alcohol (24:1). Organic and aqueous phases were separated as previously described (Doyle, 1987), DNA from the aqueous phase was precipitated in sodium acetate 3M and the pellet was washed in ethanol 70%. Finally DNA was suspended in 50 µl of TE buffer and kept at -20°C until used.

PCR assay and sequencing

For the detection of *Brucella* spp., the *bcsp31* gene was amplified as described by Sanjay et al., 2011. To investigate *Brucella* species in goat's in tissue and milk we

used a PCR protocol targeting IS*711,* a trasposable element inserted in distinct chromosomal locations in different *Brucella* species (Bricker and Halling 1994, Ilhan, 2008 and Sanjay, 2011). PCR reactions were performed in a final volume of 25µl, the reaction contained 1.5 mM MgCl2, 0.2mM dNTPs, 2X BSA, 0.5µM of each primer, 1U of GoTaq DNA Polymerase (Promega Corporation, Madison, USA), 50ng of DNA and PCR reaction buffer. The reaction consisted in: an initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 1 min, 70°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min.

Finally, we identified a 210 bp *B. canis* specific region by comparing *B. canis* and *B. suis* genomes using the program gVISTA computational tools for comparative genomics (Couronne, 2003). We designed a pair of primers using Oligos & Peptides design tool of Sigma-Aldrich; bcan 1: 5'GCATTGGCGTCGATCTG3', bcan 2: 5'CGGTCGGATTGACACCAATG3'**. PCR reactions were carried out in a final volume of 25µl; the reaction contained 1.5 mM MgCl2, 0.2mM dNTPs, 2X BSA, 0.4µM of each primer, 0.5U of GoTaq DNA Polymerase (Promega Corporation, Madison, USA), 50ng of DNA and PCR reaction buffer provided by the manufacturer. The reaction conditions consisted of an initial denaturation at 95°C for 3 min; followed by 35 cycles at: 95°C for 1 min, 64°C for 1 min, 72°C for 1 min; a final extension at 72°C for 5 min. All the primers that we used in this investigation and fragments size are listed in Table I.

The amplicons were analyzed by electrophoresis using 1.5% agarose gels. To rule out spurious PCR products, amplicons were sequenced at Functional Biosciences and analyzed using BLAST (http://blast.st-va.ncbi.nlm.nih.gov).

To rule out the presence of inhibitory substances in negative reactions, we amplified the beta-actin gene (Du Breuil, 1993). DNA from *B. melitensis* was donated by Susana Torioni at the National Institute of Agricultural Technology (INTA), Argentina.

***Brucella* culture**

Placental samples from an aborted canine fetus were cultured in chocolate agar with 8µg/ml of nalidixic acid and 8µg/ml of gentamicin. The culture was performed under 5 - 10% CO_2 conditions at 37°C. Colonies were subjected to Gram stain and enzymatic tests (urease, catalase and oxidase). We also used our PCR protocol to confirm these results.

Results

We found that 8.3% of tissue samples from goats were positive for *Brucella sp.* (Table II). Animals arrived from 8 Ecuadorian provinces, but positive samples were from 3 Andean provinces: Cotopaxi 7.4% (2 out of 27 samples), Tungurahua 8.9% (4 out of 45 samples), and Loja 31.7% (19 out of 60 samples), (Table II and Figure I). Samples were positive for *B. abortus* (2.7%), *B. melitensis* (2%), and *B. suis* (0.7%) (Table III). We were unable to identify *Brucella* species in 9 PCR positive samples (3%). Loja was the province with the highest positivity and also the only province where additional *Brucella* species (*B. suis, and B. melitensis)* were found in goats. All 60 raw milk samples from goats collected in two different provinces (Pichincha and Imbabura) were negative.

Additionally, DNA from *B. canis* was detected in a canine fetus in Quito; *B. canis* was also isolated from these fetal samples.

Discussion

The use of molecular tools allow us to detect, for the first time, *B. melitensis (*2% of samples)*, B. suis* (0.7% of samples) in Ecuadorian goats. This finding is relevant because *B. melitensis* is the most pathogenic species and the consumption of raw goat milk is very common in Ecuador.

The percentage of *Brucella* PCR positive samples (8.6%) was similar to that of previous studies, where the overall prevalence estimates based on serology and PCR techniques for dairy herds and goats were 8-9% (Poulsen, 2014).

In this study the southernmost province of Loja had the highest percentage of goats positive for brucellosis. Also, Loja was the only one province where *B. melitensis* and *B. suis* were found. This province has at least 10 times more goats (76.044 goats) than any other province in Ecuador (INEC, 2013; http://www.ecuadorencifras.gob.ec/estadisticas-agropecuarias-2/) and shares a border with Peru a country where B. melitensis is present (Taboada, 2005).

Our results indicate that B. melitensis, B. suis and B. canis are present in domestic animals in Ecuador. These findings should prompt additional studies (especially in Loja province) to determine the possible entry of infected animals from Peru.

Conclusions

In this research we were able to detect for the first time 3 species of *Brucella* in Ecuador. These findings demonstrate that the use of molecular tools in animal tissues obtained from abattoirs may improve the detection and surveillance of *Brucella* species in developing countries.

Acknowledgments: We thank to Metropolitan slaughterhouse of Quito for allow us take, samples of lymph nodes. Susana Torioni at the National Institute of Agricultural Technology (INTA), Argentina for providing the DNA from *Brucella melitensis.* Ivette Dueñas Univerdidad San Francisco de Quito for reviewing and revising the manuscript. The Institute of Microbiology of the Universidad San Francisco de Quito for the financial support for this project.

Authors Disclosure Statement: The authors declare that there are no competing financial interests.

Authors' addresses: Ligia Luna, Gabriel Trueba, Microbiology Institute, Universidad San Francisco de Quito, Quito, Ecuador. Verónica Barragán, MGGEN, Northern Arizona University, Flagstaff, AR.

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Table I. PCR primers used in this study**.**

Primer Names	Sequences	Fragments size (bp)
Brucella genus	**5'**CAATCTCGGAACTGGCCATCTCGAACGGTAT**3'***	208
	5'ATGTTATAGATGAGGTCGTCCGGCTGCTTGG**3'***	
IS711	**5'**TGCCGATCACTTAAGGGCCTTCA**3'***	
Brucella abortus	**5'**GACGAACGGAATTTTTCCAATCCC**3'***	498
Brucella melitensis	**5'**AAATCGCGTCCTTGCTGGTCTGA**3'***	731
Brucella suis	**5'**GCGCGGTTTTCTGAAGGTTCAGG**3'***	285
Brucella ovis	**5'**CGGGTTCTGGCACCATCGTCG**3'***	976
Brucella canis	**5'**GCATTGGCGTCGATCTG**3'****	210
	5'CGGTCGGATTGACACCAATG**3'****	

* Sanjay, K., et al. "Rapid multiplex PCR assay for the simultaneous detection of the Brucella Genus, B. abortus, B. melitensis, and B. suis." Journal of microbiology and biotechnology 21.1 (2011): 89-92.

** Primers developed at the Microbiology Institute, Universidad San Francisco de Quito, Quito, Ecuador

Table II. Detection of *Brucella sp.* on goat samples and canine fetus by province**.**

Province	Sample type	Sample No.	Frequency	Species identified (number of positive samples)
Tungurahua	Lymph nodes	45	0.09	*Brucella abortus* (4)
Cañar	Lymph nodes	20	0	-
Bolívar	Lymph nodes	20	0	-
Imbabura	Lymph nodes	60	0	-
	Raw milk	10	0	-
Pichincha	Raw milk	50	0	-
	Canine fetus	1	1	*Brucella canis*(1)
Cotopaxi	Lymph nodes	27	0.07	*Brucella abortus* (2)
Zamora Chinchipe	Lymph nodes	8	0	-
Loja	Lymph nodes	60	0.32	*Brucella abortus* (6) *Brucella suis* (2) *Brucella melitensis* (6) *Brucella* spp. * (5)
Total		**301**	**0.086** (P=0.086, CI: 0.06-0.13, p-value< 2.2e-16)	

Figure I. Provinces with positive samples to *Brucella sp.*

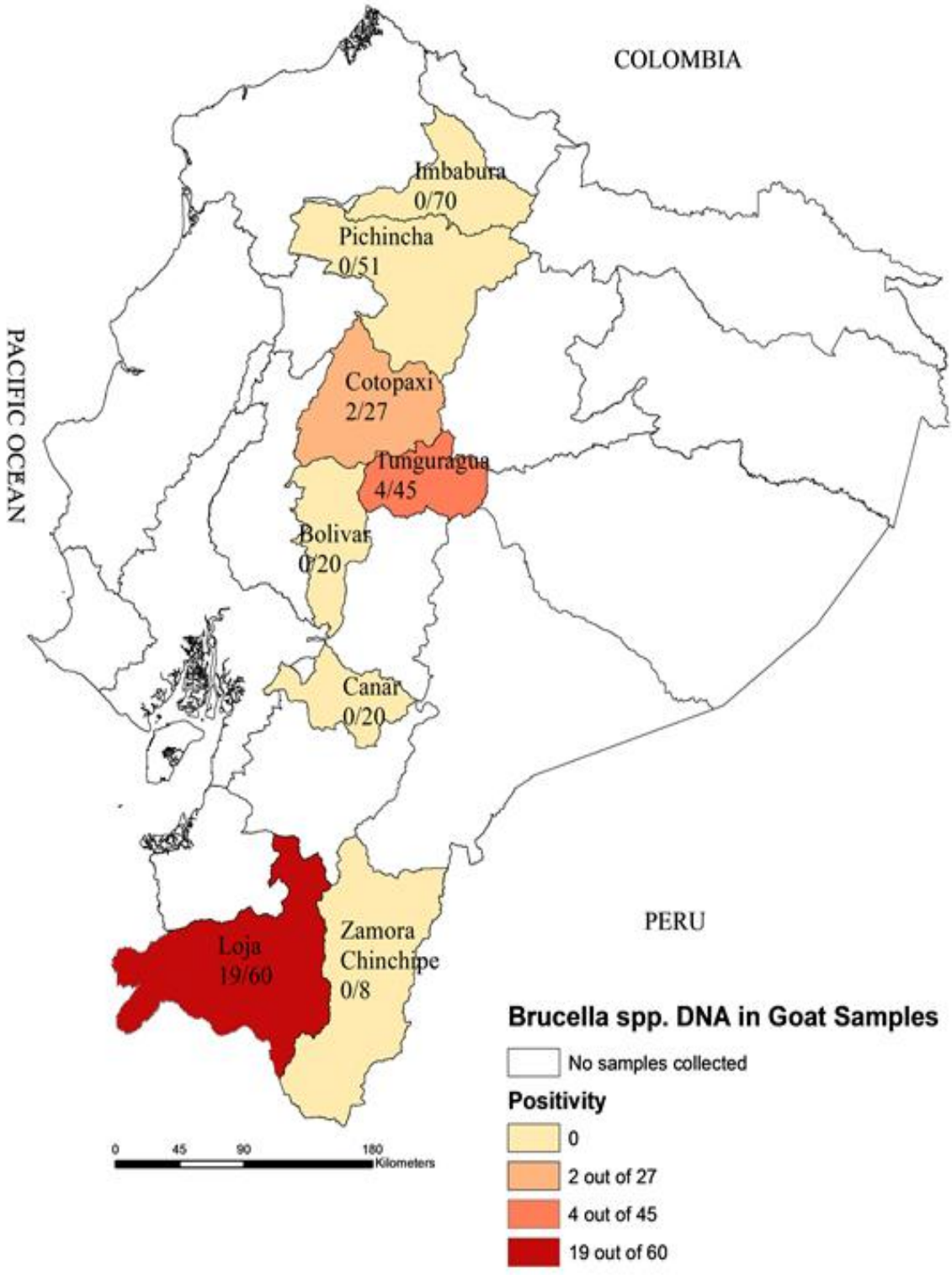

Table III. *Brucella* species identified in goat samples and canine fetus.

Species identified	Positive (%)
Brucella abortus	8 (2.6)
Brucella melitensis	6 (2.0)
Brucella suis	2 (0.7)
Brucella canis	1 (0.3)
Brucella sp.	9 (3.0)
Total	26 (8.6)