Molecular Detection of *Brucella* Species in Ecuador

Ligia Luna[1]

Gabriela Chávez [2]

Lorena Mejía[1]

Veronica Barragán[1,3]

Gabriel Trueba [1]*

[1]Instituto de Microbiología, Universidad San Francisco de Quito, Quito, Ecuador Colegio de Ciencias Biológicas, Universidad San Francisco de Quito, Ecuador

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

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

*CORRESPONDING AUTHOR:

Gabriel Trueba. Instituto de Microbiologia, Universidad San Francisco de Quito. Quito-Ecuador author: Tel.: 59322971836; fax: 59322890070, E-mail: gtrueba@usfq.edu.ec

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ABSTRACT

Brucellosis is a zoonosis which causes severe disease in humans and important economic losses in livestock operations. In Ecuador, the only *Brucella* species reported (in humans and domestic animals) has been *B. abortus*. We used two PCR protocols to investigate the presence of *B. melitensis* and *B. suis* infection in goats. We also report the first isolation and PCR detection of *B. canis* in dogs in Ecuador.

INTRODUCTION

Brucellosis is a zoonotic disease caused by *Brucella* spp., a Gram negative coccobacillus and a facultative intracellular bacterium.[1] Different *Brucella* species preferentially infect a particular animal species: *B. abortus* is more frequent in cattle, *B. melitensis* in goats and sheep, *B. suis* in pigs, etc.[2, 3] Humans get infected by direct contact with tissue (or fluids) from infected animals and by consumption of unpasteurized or raw animal products. [4, 5] Human brucellosis is a chronic infection causing mainly intermittent fever, arthralgia, fatigue and, in fewer cases, a more severe disease.[6] *Brucella* annually infects more than 500,000 people worldwide and prevalence rates in some countries exceed 10 cases per 100,000 inhabitants.[2,6,7] In livestock, brucellosis causes an incurable infection characterized by abortion, infertility and decreased milk production.[3] 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.[8]

Global public health efforts to control major zoonotic diseases require knowledge of the geographic distribution of pathogens such as *B. melitensis* (associated to the most severe brucellosis).[9] Brucellosis in animals is usually investigated using serologic screening tests which don't differentiate *Brucell*a species. Detection of *Brucella* species requires bacterial isolation (which is hazardous and difficult) followed by additional biochemical and serologic

analysis.[10,11] *Brucella* spp. have low genetic diversity and little horizontal gene transfer which reduces de possibilities of using molecular techniques to detect species.[2]

Brucellosis is prevalent especially in low income countries (such as Ecuador) where disease control programs and diagnosis are limited.[12, 13] Previous studies have identified B. abortus as the only Brucella species present in Ecuador.[13, 14, 15] Nevertheless other Brucella species have been detected in neighboring countries[12] which led us to hypothesize that additional species have not been detected in Ecuador due to lower prevalence. We used DNA from animal tissue samples (collected in abattoirs) and PCR to investigate the presence of additional Brucella species in Ecuador. We also isolated B. canis and developed a PCR protocol to detect this bacterium in tissues.

MATERIAL AND METHODS

Sample collection

Three hundred inguinal lymph from 240 goats (from different Ecuadorian provinces) were collected at the municipal slaughterhouse in Quito (from November 2013 to March 2014) and 60 samples of goat´s 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 biopsies from a canine fetus and placental samples were obtained from the Veterinary Hospital at Universidad San Francisco de Quito and kept at 4°C until cultured and preserved at -20°C for PCR analysis.

DNA extraction

Total DNA was isolated by a modified CTAB method.[16] An approximate 2 mm3 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. For milk, 500 µl samples were suspended in 500 µl of PBS, centrifuged and the pellet was mixed with 700 µl of CTAB solution. 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, DNA from the aqueous phase was precipitated in 100% ethanol with sodium acetate 3M and the pellet was washed in 70% ethanol. Finally, DNA was suspended in 50 µl of TE buffer and kept at -20°C until used.

PCR protocol and sequencing

All DNA samples were subjected to Brucella genus specific PCR using primers for the bcsp31 gene. All samples that were positive for *bcsp31* gene were submitted to Brucella species PCR protocol targeting IS711;[16-18] this protocol uses a primer that hybridizes the IS711 element and the other which hybridizes an adjacent region outside the IS711 which is different in each *Brucella* species [16]; amplicons from different species vary in size: *B. abortus* 498 bp, *B. melitensis* 731bp, *B. suis* 285 bp, *B. ovis* 976 bp. We also sequenced (at Functional Biosciences, Madison Wisconsin, USA) the amplicons to rule out spurious PCR products. 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 template and 1X PCR reaction buffer. The reaction program 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* with other genomes (from other species of Brucella) using the program gVISTA computational tools for comparative genomics.[20] We designed a pair of primers using Oligos & Peptides design tool of Sigma-Aldrich; bcan 1: 5'GCATTGGCGTCGATCTG3', bcan 2: 5'CGGTCGGATTGACACCAATG3'. The DNA sequence of this region was submitted to the GenBank (accession number KU671025). This 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 template and 1X 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 and 72°C for 1 min; a final extension at 72°C for 5 min; the expected amplicon size was 210 bp. Positive controls were DNA from *B. melitensis* donated by Susana Torioni at the National Institute of Agricultural Technology (INTA), Argentina, DNA from *B. abortus* strain RB51 and DNA from *B. canis* isolated from a canine fetus.

The amplicons were analyzed by electrophoresis using 1.5% agarose gels. Each nucleotide sequence was aligned independently with reference sequences from GenBank (using MEGA software version 6.0 with ClustalW method). To exclude the presence of inhibitory substances in negative reactions, we amplified the beta-actin gene.[21]

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% CO2 conditions at 37°C. Colonies were subjected to Gram stain and enzymatic tests (urease, catalase and oxidase).

RESULTS

We found that 8.3% of tissue samples from goats (from 8 Andean provinces) were positive for *Brucella* spp, positive samples belonged to 3 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). Samples were PCR positive for *B. abortus* (2.7%), *B. melitensis* (2%), and *B. suis* (0.7%). We were unable to identify Brucella species in 9 PCR positive samples (3%). Loja was the province with the highest positivity and it was also the only province where *B. suis* and *B. melitensis* were detected. We obtained a 24bp *Brucella* DNA sequence from the species specific PCR (3'ATGAAGGCCCTTAAGTGATCGGCA) which was located downstream from the IS711 primer hybridization sequence; larger readable sequences were not obtained maybe because *Brucella* species have 6 to 7 IS711 copies[24] and adjacent sequences of all IS711 locations probably overlapped (unreadable sequences). 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 allowed us to detect, for the first time, evidence of *B. melitensis* (2% of samples) and *B. suis* (0.7% of samples) in Ecuadorian goats. This finding is relevant because not only is *B. melitensis* the most pathogenic *Brucella* species for humans but also the consumption of raw goat milk is very common in Ecuador, there is a common belief that raw milk from goats has medicinal properties. The percentage of *Brucella* PCR positive samples (8.6%) was similar to previous studies which used serologic and molecular protocols.[22]

In this study, the southernmost province of Loja had the highest percentage of goats PCR positive for brucellosis. Also, Loja was the only one province where samples positive to *B. melitensi*s and *B. suis* were found. It is worth noting that this province has at least 10 times more 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.[24]

Our results are evidence that *B. melitensis, B. suis* are present in domestic animals in Ecuador. We also isolated from the first time *B. canis* in Ecuador. These findings suggest that additional studies should be done (especially in Loja province) to determine the possible entry of infected animals from Peru. We also think that molecular tools (along with bacteriological tools) should be used to investigate the presence of *Brucella* species in developing countries in order to establish risks for human infection.

These type studies may help to bring awareness and prompt the implementation of surveillance programs and sanitary measures.[8]

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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