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Specific Detection Of Brucella Spp.In Cattle Slaughtered In The Province Of Bolivar (Ecuador)

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ABSTRACT

In this research, we set out to study the prevalence and molecular characterization of Brucella isolates obtained from the blood of cattle slaughtered in 2 herds of the province Bolivar, Ecuador.Therefore, 100 samples of bovine blood were selected, 50 from the Guaranda slaughterhouse and 50 from the Echeandía slaughterhouse.All blood samples were analyzed using the Rose Bengal (RB) serological test, as well as the culture on selective brucella Agar + blood "5% sheep blood supplement" (BAS), under microaerobic conditionsat 37 °C for 24-48 h.Similarly, the isolates obtained by culture were characterized by PCR using a pair of primers that amplify a 905-bp fragment. After the RB serological test, 54% of the analyzed samples were positive, while 63% were positive by culture.Regarding the molecular characterization, 42% of the isolates presented the characteristic band, resulting in being positive for the Brucella genus, being the San Pablo sector the one with the highest prevalence 75%, followed by the Salinas sectorwith 50%, San Lorenzo 47,05% and Echeandía with 31,9%.In relation to the prevalence of the pathogen by sex, this was higher in female cattle.In conclusion, the PCR technique can be considered as a useful molecular tool in the diagnosis of Brucella spp.in cattle, especially due to its greater sensitivity.

Keywords: Brucellaspp., Rose Bengal, culture, PCR, prevalence.

INTRODUCTION

Brucellosis is a contagious disease of cattle that has important economic consequences caused by bacteria of the genus Brucella, which are Gram - coccobacilli, measuring 0,6–1,5 µm long by 0,5 – 0,7 µm wide,are immobile and non-sporulated bacteria, they do not form flagella or fimbriae, and they do not have a capsule.Brucella grows at 37 °C in the presence of 5 to 10% CO_2.In addition, they present an oxidative metabolism, due to the fact that they use nitrates as an electron receptor, they do not use citrate as a carbon source and they do not ferment sugars, they produce the enzymes catalase and oxidase, and urease activity is variable.(**Stanchiet al., 2017**). On a world scale, the production of meat and milk is one of the most important items, due to the contribution of proteins and vitamins that it generates in the diet of the world population.However, this source of nutrients can be decimated by a group of pathologies, among which are bovine brucellosis, a disease that can be transmitted to humans (**Khurana et al., 2021**). The dairy farm is one of the most important sources of income to the

family economy of the rural sector in Ecuador, the dairy production chain unfolds an uncountable number of jobs and underemployment(**Jauregui, 2016**). Ignorance due to lack of technical and epidemiological information about bovine brucellosis, can cause the disease to spread silently between properties, which is why it was necessary to implement the study to establish the epidemiological parameters of the disease (**Díaz &Lamiña, 2013**).

Brucellosis is considered the most widely distributed zoonosis in the world;causes great losses in the livestock sector, since annually around 500 thousand new cases in humans are reported worldwide this is a zoonosis with a high occupational risk, especially in the slaughter area(**Paredes, 2012; Díaz &Lamiña, 2013**).

In the countries of Latin America and the Caribbean, brucellosis control and eradication programs have been undertaken by government entities;despite these efforts, there are worrying prevalence data in South America(**Tique et al., 2009**).

Within Ecuador, the disease causes economic losses equivalent to 2 or 3% of livestock production(**Maigua, 2018**).

The National Bovine Brucellosis Control Program in which regulations, standards and activities are compiled that must be carried out to reduce, control and eradicate Bovine Brucellosis in Ecuador.There are no scientific studies published in Ecuador that allow us to know the main risk factors for brucellosis in cattle, nor are there studies carried out with molecular techniques(**Morales et al., 2011**).

Within the Brucella genus, 11 species are distinguished, highlighting the brucellosis of cattle (B.abortus), sheep and goats (B. melitensis) and swine (B. suis) are diseases that appear in the Animal Health CodeTerrestrial of the World Organization for Animal Health (OIE) and must be notified in a mandatory way to the OIE (Terrestrial Animal Health Code)(**Casey & Wilson, 2011; OIE, 2021**). For conventional diagnosis, the RB serological test (Rose Bengal) is widely used, it is an agglutination technique for qualitative and semi-quantitative detection of anti-Brucella antibodies, the bacterial and colored suspension is agglutinated by IgG or IgM antibodies present inserum from the patient animal(**Zambrano Aguayo et al., 2016**).

Regarding the determination and detection and more reliable diagnosis, several authors recommend the application of more sensitive techniques such as PCR (Polymerase Chain Reaction), which allows generating a large number of copies of a DNA fragment(**Guamán et al., 2018**; **Ketchum et al., 2018)**. This being a very important diagnostic tool to advance in the control of bovine brucellosis in the country, avoiding the elimination of valuable animals or leaving false negative animals that continue to spread the disease in the bovine herd.(**Saadat et al., 2017**).

Considering everything previously described, in this study we did not propose as an objective;Detect and isolate Brucella strains spp.from biological samples of slaughtered cattle from two herds of the province Bolivar (Ecuador) through serological, culture and molecular tests.

MATERIALS AND METHODS

Research location.

One hundred samples of bovine blood were collected from 2 herds of the province Bolivar in Ecuador;50 from the city of Guaranda and 50 from the city of Echeandía, the blood samples with caps and vacutainers tubes from the coccygeal and jugular were immediately transferred to the Molecular Biology laboratory of the Research Department of the State University of Bolivar for lateranalysis.The sampled bovines came from 4 sectors as shown in table 1.

Table 1. Sectors of origin of slaughtered cattle

Sector	N
Salinas	32
San Pablo	4
San Lorenzo	17
Echeandía	47
Total	100

Rose Bengal serological test

For this test, 50 µL of the blood sample and 1 drop of each of the (+) (-) controls are deposited on a glass slide;the rose bengal reagent was vigorously homogenized and a drop of 50 µL was placed on the initial homogenate, the mixture was spread over the entire surface in a circle.Finally, the slide was placed on a rotary shaker at 80 rpm for 4 minutes, observing at the same time that each sample was duly identified.After this time, the reading was carried out, where the positive samples presented sero-agglutination in the form of a ring.

Culture and isolation of the microorganism of interest

The 10 mL blood samples obtained were homogenized in a flask, and the addition of 90 mL of buffered peptone water (ATP) (VM666728, Merck KGaA) was made;the flasks were allowed to incubate under microaerophilic culture conditions at 37 °C for a period of 24 hours.After this time, 200 µL of each culture broth was transferred to brucella agar (CM0169, RG24, United Kingdom) + Blood (BAS) “supplemented with 5% sheep blood) plates.The plates were left at rest for 30 minutes, then they were incubated at 37 °C under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2), with the use of an atmospheric generation system Anaerojar TM(OXOID, iAG25, UK), added to an envelope to generate said conditions MikrobiologieAnaerocult (Merck KGaA 64271, Darmstadt, Germany).After incubation, colonies with apparent characteristics of the Brucella genus were selected and analyzed by microscopy (Gram stain).

Molecular analysis

Each of the isolates obtained had their DNA extracted using a DNA extraction kit from the Genejet Genomic DNA purification Kit 50 RX kit.(Thermo Scientific, K0721, USA) following the manufacturer's guidelines.DNA concentration was observed using a micro-spectrophotometer (ThermoScientific™, NanoDrop™).

The amplification reaction was run in a final volume of 25 µL, containing 24 µL of the mixture and 1 µl of template DNA (isolated DNA).The reagents used for the reaction were: DreamTaq Green PCR Master Mix 2X (Thermo scientific, K1081, Lithuania) containing (dNTPs, MgCl2, Taq polymerase), forward and reverse primers, plus Mill-Q water.The product DNA was amplified by PCR (Polymerase chain reaction) using 0,2 nM of each of the primers that amplify a 905-bp fragment of the 16S rRNA gene, these primers were previously described by **Casalinuovo et**

al.(**2016**);whose sequences were;F4: (5'-TCGAGCGCCCGCAAGGGT-GAGCGG-3'), R2: (5'-AACCATAGTGTCTC-CACTAACC-3').The conditions for the PCR were: a cycle of 95 °C for 4 minutes;35 cycles at 94 °C for 60s for denaturation, 54 °C for 60s for primer binding, extension at 72 °C for 60s;finally, a final extension at 72ºC for 7 minutes.

The products generated (6 µL of each) were analyzed by horizontal agarose gel electrophoresis (Agarose®) (ThermoiScientific ™, BP160-100, USA) at 1,4%, prepared in 1X TAE buffer, subjected to 100 volts for45 minutes to estimate the size of the amplified DNA fragments was using a BenchTop 50 bp molecular weight marker (G829B, PROMEGA, USA).After running the gel, it was stained with the Diamond nucleid acid reagent in a ratio of 1:1000 and left to act for 30 minutes. Finally, the fragments were visualized on a transilluminator under ultraviolet (UV) light.

RESULTS AND DISCUSSION

Detection of the Brucella genus in bovine blood

Rose Bengal test

After this analysis, agglutination in blood could be determined in 54 samples, Figures 1.

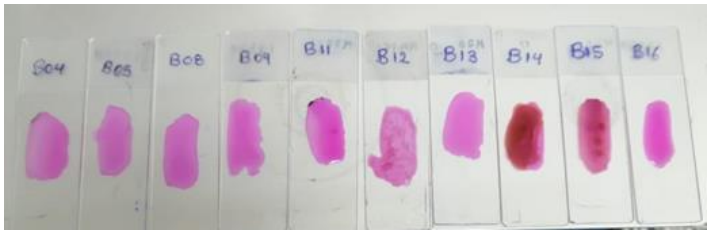

Figure 1. Agglutination test using the rose bengal technique

The results of the serological analysis of the samples taken in the 2 herds of the province Bolivar, detected that the bovines from the Salinas sector presented 21 positive cases, of the bovines from the San Pablo sector presented 2 positive cases, followed by thecattle from the San Lorenzo sector with 18 positive cases, with the highest level of positivity being the cattle from Echeandía with 54 positives.According to the study by **Aucancela (2017),** for the serology test (Rose Bengal) in samples of 200 animals, of which 82 cows were in dairy production, where they obtained a seropositive data of 8% with rose bengal.On the other hand, **Poulsen et al.**(**2014**), of 2.561 cows and 301 goats, after the Rose Bengal card antigen test (RBCT), which yielded an overall apparent prevalence of 5,5%.In the work of **Cevallos et al.**(**2017**), the authors used 40 blood samples from four herds of cattle in central Ecuador, resulting in that 35% of the animals tested positive for the analysis with Rose Bengal.**CevallosFalquez et al (2008),** out of a total of 172 bovine blood samples, 30 were positive with the RB serological test.

Culture detection

After culture, the isolates presented the colonies in smooth and translucent shapes, Figure 2.

Figure 2. Culture and initial bacterial growth.

After microbial culture, 63 samples turned out to be possible positive, for which microscopic observation analysis was carried out in order to confirm that they are Gram -, resulting in all 63 samples being Gram negative.Where, greater positivity by culture was evidenced in the Echeandía sector with 31 of 47 samples, followed by Salinas 19 of 32, San Lorenzo 11 of 17 and finally, San Pablo 2 of 4.

Molecular characterization

The results show data ranging from 1,2 to 54,3 ng / µL, the DNA concentration presents satisfactory results as recommended by **Guamán,** (**2017**).After the analysis, amplification of the DNA obtained and electrophoresis, the fragments were observed at a height of 905-bp Figure 3.Of the 100 isolates of biological samples of cattle obtained by culture, 42 were found to be positive for belonging to the Brucella genus by means of the PCR test, 16 positive out of 32 (50%) came from cattle from the Salinas sector, 15 positive out of 47 (31,9%) from Echeandía, 8 positive out of 17 (47,05%) from the San Lorenzo sector and 3 out of 4 (75%) from San Pablo, the amplification gel can be seen in figure 3.

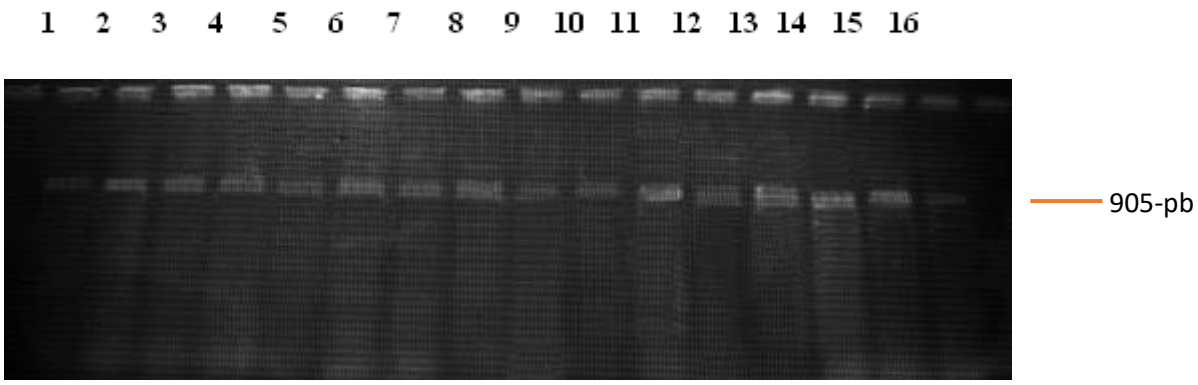

Figure 3. Electrophoresis of the PCR results of isolates of Brucellaspp, Lanes 1-16: isolates of Brucella;pb: Base pairs

Analyzing the results, it is done individually by study sector, it can be said that San Pablo presented a higher point prevalence of contamination with Brucella, this being 75%, followed by the Salinas sector with 50%, San Lorenzo with 47,05% and finallyEcheandía with 31%.It should be noted that the lowest percentage value was obtained in the sector with the highest number of samples analyzed.In general, the work of **Cevallos et al.**(**2017**), showed results similar to those obtained in the present investigation, where the authors used 40 blood samples from four herds of cattle in central Ecuador, resulting in 45% of the animals tested positive by PCR. Another similar result, but mostly milk samples, was reported in the work of **Mosquera et al.(2008)**, where they analyzed 136 animals from three farms located in the

municipality of Durania (Colombia), where they found that 13,2% of the milk samples were positive for PAL, of which 30,3% (10/33) were positive by PCR.These authors also analyzed blood samples only initially positive by PAL animals, the result was that 94,1% (16/17) were positive by PCR.In another work carried out in Manabí, by **López Balladares (2015),** of 9 isolates suspected of being Brucella, they determined by means of PCR that the species B. abortus belongs.Results with values lower than ours were reported by **CevallosFalquez et al (2008),** a total of 172 bovine blood samples, 29 were positive by PCR.Similarly, **Román Cárdenas & Ramón-Contento, (2021)**, obtained a 14,8% positivity for Brucella by PCR in samples of lymph nodes from the cervical, pulmonary, bronchial, retropharyngeal and mediastinal regions of 115 animals slaughtered in Loja, Ecuador. **Aucancela (2017**), in a work also developed in Ecuador, report a positivity of 1,2% by PCR for Brucella but in milk samples.Similarly**, Poulsen et al.**(**2014**), in total, 2.561 cows and 301 goats were tested, by PCR, to analyze goat milk and lymph nodes, resulting in 9% and 8% positivity, respectively.In another work developed by **Román-Cárdenas,** (**2020**), in the province of Loja, 66,5% of abortions in creole goats of the studied areas were determined through the epidemiological survey, the molecular test did not detect thepresence of B. melitensis, which is the one that causes the highest percentage of abortions in this animal species.Finally, and something quite worrying was the study developed by **CevallosFalquez et al.(2010)**, where they determined that of the personnel who work in the slaughterhouses of the cantons, Buena Fé, Quevedo, El Empalme and Pichincha, out of a total of 115 blood samples collected from the personnel who work in the slaughterhouses as slaughterers and operators, 54(47%) and 15 (13%) were positive with RB and PCR respectively.

CONCLUSION

In conclusion, it can be said that in this work the PCR technique is considered a useful molecular tool in the diagnosis of Brucella spp.and its use in the prevention of the disease in bovines, especially due to its greater sensitivity.

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