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ÉTUDE DE LA BRUCELLOSE BOVINE DANS LA PARTIE CONTINENTALE DE L'ÉQUATEUR

STUDY OF BOVINE BRUCELLOSIS IN CONTINENTAL ECUADOR

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"I am not accepting the things I cannot change, I am changing the things I cannot accept"

Angela Davis

"For a world where we are socially equal, humanly different and totally free"
Rose Luxemburg

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Abbreviations

°C	Degrees Celsius
µm	Micrometer
16S (rRNA)	16S ribosomal RNA
AGROCALIDAD	Agencia de Regulación y Control Fito y Zoo Sanitario
Bm	Bone marrow
BMA	Bayesian Model Averaging
BPAT	Buffered Plate Agglutination Test
BST	Brucellin Skin Test
CAMEVET	Comité de las Américas de Medicamentos Veterinarios
Cc	Conjugate control
CD4+	Cluster of differentiation 4 - T helper cells
CD8+	Cluster of differentiation 8 - cytotoxic T cells
CFT	Complement Fixation Test
CFU	Colony Forming Unit
CITA	Medium containing Vancomycin, Colistin, Nystatin, Nitrofurantoin, and Amphotericin B
CO_2	Carbon dioxide
Brucella sp.	*Brucella* species
CSMI	Internal Mobilization Sanitary Certificate
CT	Card Test
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
cELISA	Competitive Enzyme-Linked Immunosorbent Assay
iELISA	Indirect Enzyme-Linked Immunosorbent Assay
FM	Farrell medium
FPA	Fluorescence Polarisation Assay
G	grams
GTPases	Guanosine triphosphatase
Ha	Hectares
IATA	International Air Transport Association
IgG	Immunoglobulin G
IgM	Immunoglobulin M
kDa	Kilodalton
LPS	Lipid Polysaccharides
MAG	Ministerio de Agricultura y Ganadería
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time of Flight
Mb	Megabases
Min	Minutes
mL	milliliter
MLSA	Multiple sequence alignment
MLVA	Multi Locus VNTR Analysis
mP	milli-polarisation units
MRT	Milk ring test
Ms	Macrophages

mTMA	modified Thayer-Martin
NGS	Next-Generation Sequencing
NH	Native Hapten test
OD	Optical density
OPS	Organización Panamericana de la Salud
OR	Odds ratio
PANAFTOSA	Panamerican Center for Foot-and-Mouth Disease
PCR	Polymerase Chain Reaction
Pi	Percentage of inhibition
PMNs	Polymorphonuclear cells
PNSA	National Animal Health Program
RB51	Strain RB51 of *Brucella abortus*
RBT	Rose Bengal test
R-LPS	Rough Lipopolysachariden
Rpm	Revolution per minute
S19	Strain 19 of *Brucella abortus*
SAT	Serum Agglutination Test
Se	Sensitivity
SENASA	Servicio Nacional de Sanidad y Calidad Agropecuaria
S-LPS	Smooth Lipopolysachariden
SNP	Single-nucleotide polymorphisms
Sp	Specificity
USD	United States Dollar
VLCFA	Very Long-Chain Fatty Acid
VNTR	Variable copy Numbers of Tandem Repeats
WAHIS	World Animal Health Information System
WOAH	World Organization for Animal Health

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Introduction

La brucellose est une maladie infectieuse zoonotique qui représente un grave problème de santé pour le bétail et entraîne des pertes économiques importantes dans de nombreux pays en développement du monde entier, entravant le commerce et les exportations.

Bien que de nombreux efforts aient été déployés pour contrôler la brucellose, la maladie continue de se propager, en particulier dans les pays où elle est endémique, et où elle représente l'un des risques sanitaires le plus graves pour les animaux et les êtres humains.

En Équateur, *Brucella abortus* est l'espèce la plus couramment impliquée dans la brucellose bovine, qui se caractérise par des avortements spontanés survenant au cours du dernier tiers de la gestation, une rétention des membranes fœtales et une réduction de la production de lait. Les animaux infectés peuvent être porteurs asymptomatiques de la bactérie et servir de source d'infection pour d'autres animaux et pour l'homme.

L'objectif de cette thèse est d'étudier la brucellose bovine en Équateur continental, en accordant une attention particulière à la séroprévalence, aux facteurs de risque, à l'évaluation du contrôle de la qualité des vaccins et de sa mise en place, au développement de méthodologies de diagnostic, à l'isolement et au typage des souches, et de fournir des recommandations pour le programme de contrôle de la brucellose de l'Agence pour la réglementation et le contrôle de la santé animale et végétale (AGROCALIDAD).

Méthodologie

Pour la première étude, un plan d'échantillonnage transversal et stratifié a été mis en œuvre pour étudier la séroprévalence de la brucellose bovine et les facteurs de risque ou de protection associés dans 290 troupeaux issus de 23 provinces de l'Équateur, représentant un total de 3737 vaches prélevées. Un test cELISA a été utilisé pour détecter les anticorps contre *Brucella* et une enquête épidémiologique visant à évaluer les facteurs de risque a été menée dans ces 290 exploitations.

Dans la seconde étude menée en Équateur, le contrôle de qualité des vaccins anti-*Brucella* fournis et enregistrés dans le pays a été évalué. Une analyse du contrôle de qualité des vaccins a été effectuée dans le laboratoire national de référence en tenant compte des points de contrôle de l'Organisation mondiale de la santé animale. En outre, une enquête a été menée auprès de 95 professionnels vétérinaires et de 30 sociétés commerciales afin de déterminer les pratiques de vaccination et de gestion des vaccins en Équateur.

La troisième étude a porté sur l'isolement des souches de *Brucella* à partir de 25 échantillons de ganglions lymphatiques et de 50 échantillons de lait positifs aux tests sérologiques par ELISA et Rose Bengale pour les années 2022, 2023 et 2024 dans le cadre du programme national de lutte contre la

brucellose de l'Équateur. Des cultures bactériennes ont été réalisées sur des milieux spécifiques (CITA et Farrel) et l'identification moléculaire a été effectuée par des techniques PCR et MLVA (Multi-Locus Variable Number Tandem Repeat Analysis).

L'étude d'estimation bayésienne de la prévalence réelle de la brucellose bovine en Équateur a évalué la sensibilité et la spécificité d'un test cELISA et d'un test iELISA basés sur un nouvel antigène synthétique. Un échantillon aléatoire stratifié de 3 299 bovins de plus de 24 mois provenant de 223 fermes a été sélectionné dans la population bovine continentale de l'Équateur. La prévalence réelle et les performances des tests (sensibilité et spécificité) ont été estimées chez tous les animaux (n = 3 299 ; modèle 1) et dans trois sous-groupes : non vaccinés (n = 2 506 ; modèle 2), vaccinés avec la souche S19 (n = 383 ; modèle 3) et vaccinés avec la souche RB51 (n = 392 ; modèle 4).

Résultats

Les résultats de la première étude ont montré que la prévalence apparente au niveau du troupeau était de 21,3 % et de 6,2 %. L'analyse multivariée a révélé que > 70 ha (taille du troupeau) et le nombre de vêlages par animal étaient des facteurs de risque, tandis que la localisation dans la région orientale et l'absence de signes cliniques étaient identifiées comme des facteurs de protection.

Les résultats de la seconde étude ont montré que deux des trois entreprises enregistrées en Équateur ne respectaient pas les paramètres de contrôle de qualité établis par l'Organisation mondiale de la santé animale en termes de numération bactérienne. En outre, les pratiques de vaccination sur le terrain ont été évaluées par le biais d'enquêtes menées auprès de professionnels et de techniciens vétérinaires, ainsi que dans des magasins vétérinaires. Ces enquêtes ont révélé un taux de conformité aux bonnes pratiques de 62,29 % et de 56,86 %, respectivement.

La troisième étude a confirmé la présence des souches de *Brucella abortus* biovars 1, 2 et 4 en Équateur, ce qui représente la première détection du biovar 2 dans le pays. Une identification moléculaire précise est cruciale pour les études épidémiologiques et les stratégies de contrôle.

Dans l'étude 4, la performance de deux tests ELISA (cELISA et iELISA) pour diagnostiquer la brucellose bovine chez les bovins en Équateur a été analysée. Les deux tests ont montré une sensibilité similaire: 94,02 % pour le cELISA et 94,05 % pour l'iELISA. Cependant, la spécificité de l'iELISA était plus élevée (98,10 %) que celle du cELISA (95,85 %). La prévalence réelle de la brucellose était de 1,63 % chez les bovins non vaccinés, de 0,97 % chez ceux vaccinés avec S19 et de 2,75 % chez ceux vaccinés avec RB51. Les résultats indiquent que l'iELISA avec l’antigène synthétique, en raison de sa plus grande spécificité, pourrait être une bonne alternative pour détecter la brucellose bovine en Équateur. Ils soulignent également l'importance d'optimiser le programme de contrôle, en particulier en ce qui concerne l'utilisation adéquate du protocole de vaccination.

Conclusion

L'échec de la lutte contre cette maladie est dû à une combinaison de raisons, telles que l'absence de vaccins hautement efficaces, le faible engagement des agriculteurs à certifier les exploitations exemptes de brucellose, la faible importance de l'investissement gouvernemental et régional dans la lutte, et le principal facteur qui est l'absence de budget pour lutter contre cette maladie. Au niveau national, une approche « One Health » entre les entités compétentes que sont le ministère de l'Agriculture et le ministère de la Santé publique est nécessaire afin de combattre cette maladie et d'améliorer la durabilité du secteur de l'élevage au niveau régional et national.

Les informations engrangées fournissent également une base solide pour l'amélioration du programme national de contrôle de la brucellose bovine en Équateur, notamment en ce qui concerne l'éducation aux bonnes pratiques vétérinaires, le renforcement des contrôles de qualité des vaccins et la gestion des facteurs de risque identifiés.

Summary

Background

Brucellosis is a zoonotic infectious disease that is a serious health problem for livestock and causes high economic losses in many developing countries around the world, hampering trade and exports.

Although many efforts have been made to control brucellosis, the disease continues to spread, especially in endemic countries, and represents one of the most serious health threats to animals and humans.

In Ecuador, *Brucella abortus* is the species most commonly involved in bovine brucellosis, which is characterized by spontaneous abortions in the last third of pregnancy, retained fetal membranes and reduced milk production. Infected animals can carry the bacteria asymptomatically and serve as a source of infection for other animals and humans.

The objective of this thesis was to review the bovine brucellosis status in continental Ecuador, with special attention to seroprevalence, risk factors, evaluation of vaccine quality control and vaccination procedures, development of diagnostic methods, isolation and typing of strains, and to provide recommendations to the Brucellosis Program of the Animal and Plant Health Regulatory and Control Agency (AGROCALIDAD).

Methodology

In the first study, a stratified cross-sectional sampling plan was used to evaluate the seroprevalence of bovine brucellosis and associated risk or protective factors in 290 herds originating from 23 provinces of continental Ecuador, representing a total of 3737 cows. cELISA was used to detect antibodies against *Brucella* and an Epidemiologic survey was conducted to evaluate risk factors in these 290 farms.

The second study conducted in Ecuador evaluated the quality control of *Brucella* vaccines supplied and registered in the country. A quality control analysis of the vaccines was performed at the national reference laboratory, taking into account the World Organization for Animal Health control guidelines. In addition, a survey of 95 veterinarians and 30 commercial companies was conducted to determine vaccination and vaccine management practices in Ecuador.

The third study focused on the isolation of *Brucella* strains from 25 lymph node samples and 50 milk samples that were positive in serological tests by ELISA and Rose Bengal for the years 2022, 2023 and 2024 within the framework of the National Brucellosis Control Program of Ecuador. Bacterial cultures were performed in specific media (CITA and Farrel) and molecular identification was performed by PCR techniques and MLVA (Multi-Locus Variable Number Tandem Repeat Analysis).

In the study of Bayesian estimation of the true prevalence of bovine brucellosis in Ecuador, the sensitivity and specificity of a cELISA and an iELISA based on a new synthetic antigen were evaluated. A stratified random sample was selected from the continental bovine population of Ecuador, consisting of 3299 cattle over 24 months of age from 223 herds. Estimation of true prevalence and test performance (sensitivity and specificity) was performed in all animals (n=3299; model 1) and in three subgroups: unvaccinated animals (n=2506; model 2), vaccinated with the S19 strain (n=383; model 3) and vaccinated with the RB51 strain (n=392; model 4).

Results

The results of the first study showed that the apparent prevalence was 21.3% at the herd level and 6.2% at the animal level. Multivariate analysis revealed that herd size (>70 ha) and number of calves per animal were risk factors, while location in the eastern region and absence of clinical signs were identified as protective factors.

The results of the second study showed that two of the three companies registered in Ecuador did not meet the quality control parameters established by the World Organization for Animal Health in terms of bacterial count. In addition, vaccination practices in the field were evaluated through surveys of veterinarians and technicians, as well as in veterinary stores, showing 62.29% and 56.86% of compliance, respectively.

The third study confirmed the presence of *Brucella abortus* strains of biovars 1, 2 and 4 in Ecuador, the first detection of biovar 2 in the country. Accurate molecular identification is essential for epidemiologic studies and control strategies.

In study 4, the performance of two ELISA tests (cELISA and iELISA) to diagnose bovine brucellosis in cattle in Ecuador was analyzed. Both tests showed similar sensitivity: 94.02% for cELISA and 94.05% for iELISA. However, the specificity of iELISA was higher (98.10%) compared to cELISA (95.85%). The actual prevalence of brucellosis was 1.63% in unvaccinated cattle, 0.97% in those vaccinated with S19 and 2.75% in those vaccinated with RB51. The findings indicate that iELISA, due to its higher specificity, could be the good alternative to detect bovine brucellosis in Ecuador, underlining the importance of optimizing the control program, particularly on the adequate use of the vaccination protocol.

Conclusion

The failure to control this disease is due to several reasons, such as the lack of highly effective vaccines, the lack of commitment of farmers to certify farms free of brucellosis, the limited importance of government investment at national and regional level, and the main factor was the lack of budget to deal with this disease. A "One Health" approach between the Ministry of Agriculture and the Ministry

Of Public Health is needed at the country level to control this disease and improve the sustainability of the livestock sector at the regional and national levels.

These findings provide a solid basis for improving the national bovine brucellosis control program in Ecuador, including education on good veterinary practices, strengthening vaccine quality control, and management of identified risk factors.

Resumen

Antecedentes

La brucelosis es una enfermedad infecciosa zoonótica que representa un grave problema sanitario para el ganado y causa elevadas pérdidas económicas en muchos países en desarrollo de todo el mundo, impide el comercio y la exportación.

Aunque se han hecho muchos esfuerzos por controlar la brucelosis, la enfermedad sigue propagándose, sobre todo en los países donde es endémica, y representa uno de los peligros sanitarios más graves para animales y seres humanos.

En Ecuador, *Brucella abortus* es la especie más comúnmente implicada en la brucelosis bovina, la cual se caracteriza por abortos espontáneos en el último tercio de la gestación, retención de membranas fetales y reducción en la producción de leche. Los animales infectados pueden portar la bacteria de forma asintomática, sirviendo como fuente de infección para otros animales y humanos.

El objetivo de este estudio es investigar el Estudio de la Brucelosis Bovina en el Ecuador Continental, con especial atención a la seroprevalencia, factores de riesgo, evaluación del control de calidad de vacunas, puesta a punto de metodologías de diagnóstico, aislamiento y tipificación de cepas, y brindar recomendaciones al Programa de Brucelosis de la Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD).

Materiales y métodos

Para el primer estudio se implementó un diseño transversal estratificado para investigar la seroprevalencia de brucelosis bovina y factores de riesgo o protección asociados en 290 hatos de 23 provincias de Ecuador, abarcando 3737 vacas. Se utilizó cELISA para detectar anticuerpos contra *Brucella* y se llevó a cabo una encuesta epidemiológica para evaluar los factores de riesgo a 290 fincas.

En el segundo estudio realizado en Ecuador, se evaluó el control de calidad de las vacunas contra *Brucella* suministradas y registradas en el país. Se desarrolló un análisis de control de calidad de vacunas en el laboratorio nacional de referencia tomando en cuenta los puntos de control de la OMSA. Adicionalmente se realizó una encuesta a 95 profesionales veterinarios y a 30 casas comerciales, con el fin de determinas las prácticas de vacunación y manejo de la vacuna en Ecuador.

El tercer estudio se enfocó en el aislamiento de cepas de *Brucella* a partir de 25 muestras de ganglios linfáticos y 50 muestras de leche que resultaron ser positivos a pruebas serológicas de cELISA y Rosa de Bengala para durante los años 2022, 2023, 2024 en el marco del Programa Nacional de Brucelosis de Ecuador. Se realizaron cultivos bacterianos en medios específicos (CITA y Farrel) y se llevó a cabo la identificación molecular mediante técnicas de PCR y análisis MLVA (Multi-Locus Variable Number Tandem Repeat Analysis).

En el estudio de estimación bayesiana de la prevalencia real de la brucelosis bovina en Ecuador se evaluaron la sensibilidad y especificidad de un cELISA y de un iELISA basado en un nuevo antígeno sintético. Se seleccionó una muestra aleatoria estratificada de la población bovina continental de Ecuador, compuesta por 3.299 bovinos mayores de 24 meses procedentes de 223 explotaciones. Se estimó la prevalencia real y el rendimiento de la prueba (sensibilidad y especificidad) en todos los animales (n=3299; modelo 1) y en tres subgrupos: animales no vacunados (n=2506; modelo 2), vacunados con la cepa S19 (n=383; modelo 3) y vacunados con la cepa RB51 (n=392; modelo 4).

Resultados

Como resultados del primer estudio se obtuvo se identifico que la prevalencia aparente a nivel de hato fue de 21,3%, y a nivel animal fue de 6,2%. El análisis multivariante reveló que el tamaño del hato (>70 ha) y el número de partos por animal eran factores de riesgo, mientras que la ubicación en la región oriental y la ausencia de signos clínicos se identificaron como factores protectores.

Los resultados del segundo estudio revelaron que dos de las tres compañías registradas en Ecuador no cumplían con los parámetros de control de calidad establecidos por la Organización Mundial de Sanidad Animal, en términos de la cantidad bacteriana. Además, se evaluaron las prácticas de vacunación en campo a través de encuestas a profesionales y técnicos veterinarios, así como en tiendas veterinarias, arrojando un cumplimiento del 62,29% y 56,86%, respectivamente.

En el tercer estudio se confirmó la presencia de las cepas de *Brucella abortus* de biovares 1, 2 y 4 en Ecuador, siendo la primera detección de biovar 2 en el país. La identificación molecular precisa es crucial para estudios epidemiológicos y estrategias de control.

En el estudio 4 se analizó el rendimiento de dos pruebas ELISA (cELISA e iELISA) para diagnosticar la brucelosis bovina en bovinos de Ecuador. Ambas pruebas mostraron una sensibilidad similar: 94,02% para cELISA y 94,05% para iELISA. Sin embargo, la especificidad de iELISA fue mayor (98,10%) que la de cELISA (95,85%). La prevalencia real de la brucelosis fue del 1,63% en los bovinos no vacunados, del 0,97% en los vacunados con S19 y del 2,75% en los vacunados con RB51. Los resultados indican que iELISA, por su mayor especificidad, podría ser la mejor alternativa para detectar la brucelosis bovina en Ecuador, subrayando la importancia de optimizar el programa de control, particularmente en el uso adecuado del protocolo de vacunación.

Conclusión

El fracaso del control de esta enfermedad, es un conjunto de razones tales como la ausencia de vacunas altamente efectivas, compromiso de los agricultores para certificar granjas libres de brucelosis, la importancia de la inversión gubernamental y regional principal factor por la falta de presupuesto para hacer frente a esta enfermedad. Se debe trabajar a nivel país con un enfoque “Una sola salud” entre

las entidades competentes Ministerio de Agricultura y Salud con el fin de combatir a esta enfermedad y mejorar la sostenibilidad del sector ganadero a nivel regional y nacional.

Estos hallazgos proporcionan una base sólida para la mejora del programa nacional de control de la brucelosis bovina en Ecuador, incluyendo la educación sobre buenas prácticas veterinarias, el fortalecimiento de los controles de calidad de las vacunas y la gestión de los factores de riesgo identificados.

General preamble

Bovine brucellosis is an endemic infectious disease that poses significant threats to both cattle productivity and welfare, along with potential repercussions for human health. Despite numerous efforts to control and eradicate this disease, it remains one of the most widespread zoonoses in the world. Its prevalence is particularly high in countries or regions with poor sanitary conditions, where animal husbandry systems are traditional and where epidemiological surveillance of the disease is insufficient. The relevance of brucellosis lies in its significant impact on reproductive performance, manifested through abortions, infertility, placental retention, neonatal mortality and weakness of progeny. These complications induce considerable economic losses for producers.

Ecuadorian National Brucellosis Control Program has been in operation since 2008 and is managed by the Agencia de Regulación y Control Fito y Zoosaniatrio (AGROCALIDAD). Despite efforts to prevent and control the disease, the prevalence of brucellosis in animals remains high in Ecuador. It is worth mentioning that vaccination against *Brucella* is not mandatory in Ecuador and is under the responsibility of each producer.

Many animal producers may not participate in the Brucellosis-free farm certification program due to the difficulty of diagnosing and monitoring the disease in their animals. Furthermore, the government offer low compensation rate of only US$0.01 per liter of milk in case of brucellosis free status of the herd. This may not be enough incentive to convince producers to participate. Also, the lack of awareness about the risks associated with the disease adds to the lack of attention paid to it.

Controlling brucellosis effectively requires reliable information about the disease and good diagnostic tools. Although several local studies by province have been carried out in Ecuador, there is a need for a comprehensive study at the national level to update the scarce information. Therefore, it has been proposed to carry out a study to determine the prevalence and possible risk/protective factors associated with the disease. Diagnostic methodologies recognized by the World Organization for Animal Health (WOAH), such as *Brucella* isolation and culture, should be implemented as they are considered the gold standard technique. It is also important to evaluate and control the quality of vaccines and the vaccination procedures for proper animal immunization. Additionally, specific tests used in official laboratories should be compared, and the typing of *Brucella* species and biovars should be done at national scale.

The thesis is divided into several chapters. Chapter one provides a review of the literature on brucellosis, the pathogen that causes it, seroprevalence, risk factors and predisposition to bovine brucellosis, eradication and control programs, and a description of the bovine brucellosis program in Ecuador. The second chapter provides an overview of the research objectives. The general objective is

to study Bovine Brucellosis in Continental Ecuador. The chapter also includes a description of specific objectives that will be developed to precise the research. The first one specific objective was to analyze the seroprevalence and risk/protective factors associated with bovine brucellosis in Continental Ecuador **(Study 1).** The second specific objective was to evaluate vaccination management practices and identify possible factors that influence the prevention of brucellosis in the country **(Study 2).** For the third specific objective, we performed *Brucella* culture and isolation in the central official laboratory in Ecuador and determined the genetic diversity of *Brucella* using the Multiple Locus VNTR Analysis (MLVA) scheme **(Study 3).** For the last specific objective, we performed a comparative evaluation of ELISA diagnostic tests for the determination of *Brucella* sp **(Study 4).**

The Chapter three details the experiments conducted to accomplish the general and specific objectives. In order to carry out a national study on bovine brucellosis in Ecuador, the first step was to determine the prevalence of the disease. This was achieved through a stratified randomized study covering 290 cattle herds distributed in the 23 provinces of continental Ecuador. At the same time, an epidemiological survey was carried out to identify the risk/protective factors that influence the presence of brucellosis in the country **(Study 1)**.

The second objective aimed to assess the management practices of brucellosis and the quality control of vaccines that are registered and used in Ecuador. A survey was conducted among veterinary professionals of AGROCALIDAD to gather information about their knowledge and vaccinations procedures. In addition, a survey was conducted among veterinary stores to gather information on vaccine delivery in the three main agricultural provinces of Ecuador. To determine the quality of the vaccines, a vaccine quality control analysis was carried out on the vaccines that are registered for sale in Ecuador **(Study 2).**

Another study focused on the culture and identification of circulating *Brucella* strains from retromammary lymph node and milk samples collected in 2022, 2023 and 2024 under the National Brucellosis Control Program of Ecuador. Cultures were performed on specific media, followed by molecular identification by PCR and MLVA techniques. MLVA-16 analysis allowed discrimination of strains present in Ecuador, which is crucial for epidemiologic studies, outbreak tracing, and design of control strategies **(Study 3).**

Finally, we performed a Bayesian estimation of the true Ecuadorian prevalence of bovine brucellosis in non-vaccinated and vaccinated cattle sub-population, the sensitivity and specificity of a cELISA and an iELISA based on a new synthetic antigen. The results of this study suggested that the iELISA based on a novel synthetic antigen was more standardizable, and should be

recommended as the bovine brucellosis screening test in Ecuador because it has higher specificity and comparable sensitivity as cELISA. In addition, the proposed method provides relevant information on the effectiveness of the vaccination campaign and makes recommendations for improving the national brucellosis Control Program in Ecuador **(Study 4).**

Chapter 4 presents a general discussion, recommendations and conclusions on the overall contribution of the thesis. All this information is crucial to develop and implement effective measures to prevent and control brucellosis in Ecuador. The activities carried out for the achievement of this thesis are detailed below (Table 1).

Studies of bovine brucellosis in Ecuador have made significant contributions to understanding the prevalence of the disease and improving the strategies of the National Brucellosis Program. The first study, which covered the 23 provinces of continental Ecuador, revealed a prevalence of 21.3% at the herd level and 6.2% at the individual level, highlighting risk factors such as herd size and number of calves per animal, while the Amazon region was shown to be a protective factor. These results are essential to focus control efforts in the most affected areas and to strengthen food security through an integrated vision of animal and human health. Another study assessed the quality of vaccines available in the country and found that two out of three vaccines did not meet international standards. This underscores the need to improve vaccine quality control and launch educational campaigns on good vaccination practices, which will help improve immunization and control of brucellosis in Ecuador.

On the other hand, the detection of *Brucella* strains in several regions of the country allowed a better understanding of the epidemiology of the disease, with the identification of biovars 1, 2 and 4. In addition, a study on the effectiveness of serological tests (cELISA and iELISA with synthetic antigen) concluded that the iELISA, based on a synthetic antigen, has a higher specificity and is more standard for the diagnosis of brucellosis. This study also revealed a low vaccination rate in the Coast and Sierra regions, suggesting the need to improve vaccination campaigns and the protocol for the use of S19 and RB51 strains. Taken together, these studies provide valuable information to refine and strengthen the national brucellosis program, thereby improving disease control and public health protection in Ecuador.

Table 1. Field and laboratory work's activities used in this thesis

FIELDWORK ACTIVITIES	STUDY UNIT	SAMPLES	ANALYSIS	STUDY
I. Cross-sectional survey				
A. Survey	Herds	290	Seroprevalence and risk factors related to bovine brucellosis	Study 1
B. Survey veterinary professionals and technicians	National Survey	95	Assessment of brucellosis vaccination practices and quality control of vaccine	Study 2
C. Survey stores	Stores	30	Assessment of brucellosis vaccination practices and quality control of vaccine	Study 2
D. Vaccine companies	Vaccine companies	3	Assessment of brucellosis vaccination practices and quality control of vaccine	Study 2
II. Animal sampling				
Cattle	Herds	290	Seroprevalence and risk factors related to bovine brucellosis	Study 1
Blood serum of cattle	Animal serum	3737	Seroprevalence and risk factors related to bovine brucellosis	Study 1
Milk	Animal	50	Determination and characterization of (novel) circulating strains of *Brucella* sp	Study 3
Retromammary lymph nodes	Animal	25	Determination and characterization of (novel) circulating strains of *Brucella* sp	Study 3
Cattle	Herds	223	Bayesian estimation of the true Ecuadorian prevalence of bovine brucellosis in non-vaccinated	Study 4
Blood serum of cattle	Animal serum	3299	Bayesian estimation of the true Ecuadorian prevalence of bovine brucellosis in non-vaccinated	Study 4

Chapter 1 - Introduction

1.1 Historical data

The British army surgeon Cleghorn, who lived on the Mediterranean island of Menorca in 1751, describes cases of chronic and recurrent febrile disease. In 1861, Allen Jeffery Marston described his case in detail, calling it “body disease”. David Bruce isolated the causative agent in 1886, from scales of soldiers who had died of the disease and who had consumed raw goat milk, this causative agent he called *Micrococcus melitensis* (Saegerman et al., 2010).

Themistokles Zammit, in 1886 discovered the causative agent, *Micrococcus melitensis*, of brucellosis and identified milk products of goats as the source of infection for military troops on the island of Malta (De Figueiredo et al., 2015).

The Danish veterinarian Bernard Bang isolated a new bacterium in 1895 from cattle aborting repeatedly, which he named *Bacillus abortus* (Maurin, 2005).

In 1911 Ferenbaugh, and Gentry and Ferenbaugh reported 12 cases of brucellosis which they had seen that year in the goat-raising section of southwestern, ten of the patients were goatherders or ranchmens who worked with goats and lived in houses surrounded by them; two were boys who had often played in dusty goat-pens (Evans, 1947).

The “relationship” between *Micrococcus melitensis* and *Bacilus abortus* was only established in 1917 by Alice Evans, an American bacteriologist. She proposes the creation of a new genus Bacterium (Maurin, 2005).

Four more classical species were later characterized: *Brucella suis*, isolated in 1914 by Traum from aborting sows and classified as such fifteen years later by Huddleson; *Brucella canis*, isolated in 1966 by Carmichael from a Beagle bitch after abortion*; Brucella ovis* isolated in 1953 from a ram and *Brucella neotomae*, isolated in 1957 from a Desert Woodrat (*Neotoma lepida*, Thomas 1893) in Utah (USA) (Saegerman et al., 2010).

Novel isolates from human breast implant infection, from baboons that had delivered stillborn offspring, and from foxes have also been described, although the natural reservoir of these isolates remains uncertain. While limited isolates of each new type have been described, they have been formally published as the tenth, eleventh, and twelfth *Brucella* species, *B. inopinata*, *B. papionis*, and B. *vulpis* respectively (Scholz et al., 2010a; Whatmore et al., 2014a; WOAH, 2022).

In the year 2023 it was described *Brucella* nosferati infecting Desmodus rotundus (bat) has emerging potential due to the broad foraging range of its bat host for humans and wild and domestic animals (Hernández-Mora et al., 2023).

Different strains of *Brucella* that were recovered from fish, frogs, foxes, rodents, and reptiles were identified as atypical strains that differed from the species that are now known. They are not yet recognized as distinct *Brucella* species (WOAH, 2022).

1.2 Generalities of the disease and causal agent

Brucella spp. is a small coccobacillus Gram-negative having a length of 0.6-1.5 µm and a diameter of 0.5-0.7 µm. They are facultative intracellular pathogens, are immobile, and do not sporulate (Alton y Forsyth, 1996). They have oxidative metabolism, use nitrates as electron acceptors. *Brucella* spp. catalase and oxidase-positive, do not affect gelatin or modify milk, and in general, do not ferment sugars (Álvarez-Hernández et al., 2015).

The genome of most *Brucellae* has two circular chromosomes, one of 2.1 Mb (chromosome 1) and another of 1.2 Mb (chromosome 2). Together they encode approximately 3500 genes (Michaux et al., 1993).

The bacteriological diagnosis of *Brucella* is confirmed through isolation and identification. The most common method is culturing on solid media, which allows clear identification of colonies and limits the development of non-smooth mutants and contaminants. Subcultures can be performed on non-selective media, but for large samples or enrichment, the use of liquid media is recommended. Full identification of species and biovar is performed using cultural, morphological, biochemical, serological, phage-lysis characteristics and molecular tests.", and some strains grow best in an atmospheric sphere containing 5 to 10% CO_2 (Alton et al., 1988; WOAH, 2022).

Brucella is a member of the family *Brucellaceae,* in the order *Rhizobiales,* now named *Hyphomicrobiales*, based on taxonomic decisions analyzed on the basis of interpretations of highly conserved 16S rRNA sequences and DNA-DNA hybridisations, and belong class Alphaproteobacteria (Volpiano et al., 2021). It shows close genetic relatedness to some plant pathogens and symbionts of the genera *Agrobacterium* and *Rhizobium*, as well as animal pathogens (*Bartonella)* and opportunistic or soil bacteria (e.g. *Ochrobactrum*) (WOAH, 2022).

In a recent publication, bacterial taxonomists proposed including the genus *Ochrobactrum* within the genus *Brucella* (Hördt et al., 2020), which has sparked controversy. *Brucella* is known to be a dangerous intracellular pathogen affecting humans and animals, while *Ochrobactrum* consists of free-

living organisms found in soil and plants. The taxonomists justified this merger based on a two-dimensional genomic analysis, focusing primarily on sequence divergence and using a cladistic rather than a systematic evolutionary approach. However, the cladistic approach diverges from the recommended polyphasic taxonomy, which takes into account both genomic analyses and biologically significant traits. The authors of the proposal attempted to downplay the differences between the two genera, arguing that they are phylogenetically related and that *Ochrobactrum* species have also been isolated from clinical specimens (Moreno et al., 2023).

Brucella is a genus of gram-negative bacteria that causes brucellosis, a zoonotic disease primarily transmitted through unpasteurized dairy products and contact with infected animals. This bacterium is highly pathogenic and possesses the ability to evade the host's immune system, allowing for its survival and replication within host cells. In contrast, *Ochrobactrum* includes species that are primarily saprophytic and are associated with nosocomial infections, lacking the zoonotic specificity and the same pathogenic profile that characterizes *Brucella*. The distinction between *Brucella* and *Ochrobactrum* is justified by their differences in pathogenicity, genetic and phylogenetic characteristics, as well as modes of transmission. While *Brucella* exhibits adaptations that make it an effective zoonotic agent, *Ochrobactrum* does not possess the same virulence mechanisms and is not linked to zoonotic infections. These factors make it inappropriate to classify *Brucella* as *Ochrobactrum*, highlighting the importance of maintaining its distinction as a unique and relevant genus in medical microbiology (Moreno et al., 2023).

Studies based on DNA/DNA hybridization or the sequencing of the gene coding for the 16S RNA ribosomal suggested that the genus *Brucella* is monospecific, with DNA similarity above 97%. Despite this, *Brucella* species can be distinguished by single-nucleotide polymorphism analysis, host preference, and conspicuous differences in virulence. Authors explain this genetic homogeneity and the absence of antigenic diversity by an unusual characteristic, namely the absence of a plasmid in *Brucella* spp. (Foster et al., 2012; Moreno, 1998; Saegerman et al., 2010).

Brucellosis is a sub-acute or chronic disease that may affect many species of animals (Corbel et al., 2006). *Brucella abortus* is a facultative intracellular pathogen that causes persistent infection in animals and has been isolated from several species of livestock. It is mostly associated with cattle, it is a natural or primary host, and *B. melitensis* is associated with sheep, goats, and humans (Godfroid et al., 2013a; Pappas et al., 2005). The bacterium preferentially replicates within phagocytic cells of the reticuloendothelial system, and in the pregnant animal, inside placental trophoblasts (Moreno, 2014).

Brucellosis in animals is known as Bang's disease, contagious abortion, or abortion epizootic (Ron-Román, 2003), while in humans it is known as Bruce's septicemia, Mediterranean fever, Malta fever, undulant fever, Rio Grande fever (Organización Mundial de la Salud, 2021).

1.3 Lipopolysaccharides

*Brucella*e are Gram-negative bacteria and as such their cell envelope is composed of two membranes (Figure 1). The outer membrane plays a crucial role in the infection process, as it is the first point of interaction between the bacterium and the host. The outer layer of the outer membrane is composed of lipopolysaccharides (LPS), which consists of three key components: (i) the lipid A, which forms the hydrophobic anchor of the LPS within the outer membrane, (ii) an inner and outer core composed of sugar molecules, and (iii) the O-antigen, a polymerized sugar chain extending into the extracellular environment (Haag et al., 2010) (**Figure 1**).

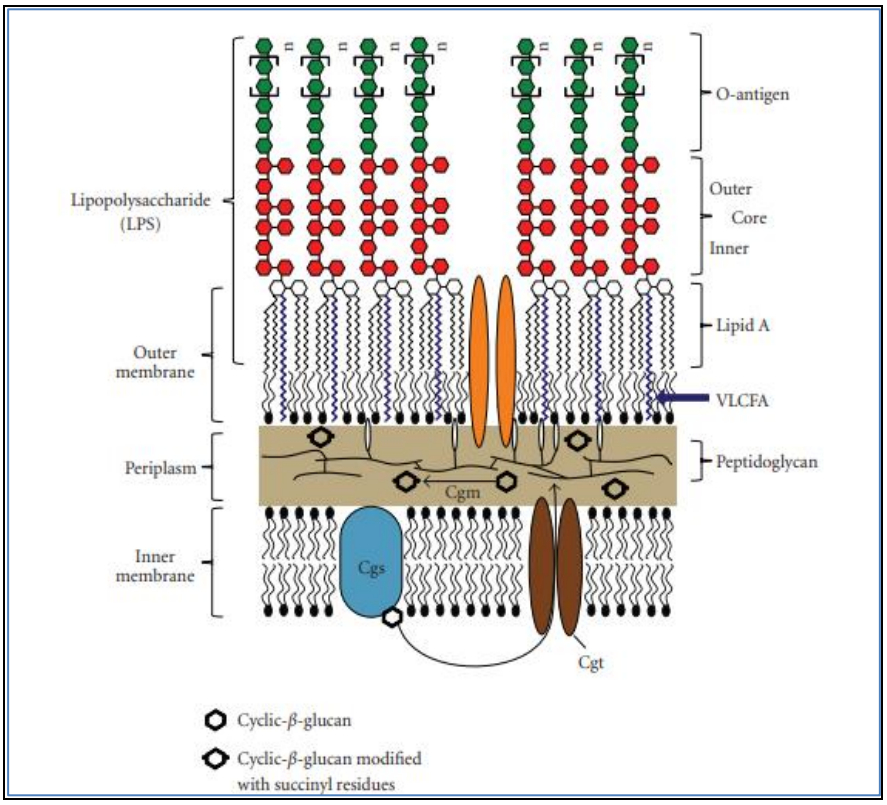

Figure 1- Diagrammatic representation of the *Brucella* cell envelope (Haag et al., 2010)

The presence or absence of the O-chain is responsible for the smooth (S-LPS) or the rough (R-LPS) phenotypes, respectively. The S-LPS contains the O-chain and is found in most wild-type *Brucella* species and biovars. Only *B. canis* and *B. ovis* possess naturally R-LPS (Saegerman et al., 2010). The

ability of *Brucella*e to produce S-LPS with a complete O-antigen is crucial for its virulence in humans (Young, 1995).

This bacterium possesses an unconventional non-endotoxic lipopolysaccharide that confers resistance to anti-microbial attacks and modulates the host immune response. The LPS O-chain it is a key molecule for *Brucella* survival and replication in the host (Cardoso et al., 2006). The cell envelope is comprised of an inner membrane, consisting of a bilayer of phospholipids, an outer membrane with an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS). The LPS consists of three components. The O-antigen faces the extracellular space, and it is the component that is recognized by the adaptive immune response. The O-antigen is connected to a sugar core molecule composed of different sugars. Lipid A forms the hydrophobic anchor of LPS within the membrane and has a backbone of diaminoglucose, which is acylated with saturated and hydroxylated fatty acids. *Brucella* lipid A contains an unusual very long-chain fatty acid (VLCFA). Cyclic β-1,2-glucans are synthesized by the inner membrane protein Cgs and then transported to the periplasm by the predicted ABC-transporter Cgt where they are modified with on average two succinyl residues by a predicted membrane protein Cgm (Haag et al., 2010).

In *Brucella*, lipopolysaccharides (LPS) are crucial to the bacterium's virulence and ability to evade the immune system. The O-specific polysaccharide (O-PS), a component of the LPS, plays a key role in this immune evasion by reducing the detection by the host's innate immune system due to the LPS's low endotoxicity. Mutants lacking the O-PS, often due to spontaneous mutations, produce rough LPS (R-LPS) and are significantly attenuated, illustrating the importance of the O-PS in maintaining *Brucella's* virulence (Fontana et al., 2016).

The presence of chains composed exclusively of l,2-linked residues in the LPS seems to be responsible for the antigenic cross-reactivity with LPS *Yersinia enterocolitica* O:9 (Bundle et al., 1989). The LPS of *Brucella* spp. shares also some antigenic relationship with the LPS of *Escherichia hermann, Escherichia coli* O:157, *Salmonella* serotypes of Kaufmann-White group N, *Francisella tularen*sis, *Xanthomonas maltophilia and Vibrio cholerae* O:1 (Perry and Bundle, 1990; Saegerman et al., 2010).

Brucella in primo-culture usually requires long incubation periods, typically two to three weeks, but sometimes longer (in this case is a mix of liquid and solid of media Castañeda). Growth can be improved using enriched media such as gelose supplemented with serum and glucose. In Petri dishes, colonies of smooth types are translucent, smooth, and convex with distinct contours. This morphology of the colonies is due to the presence of LPS in the external membrane of the bacterium (Saegerman et al., 2010).

1.4 *Brucella* species

The medically important genus *Brucella* comprises a historical clade of six so-called classical *Brucella* species (the type species *Brucella melitensis, B. abortus, B. canis, B. ovis, B. neotomae,* and *B. suis*) plus some recently described species including *B. pinnipedialis, B. ceti, B. microti, B. inopinata, B. papionis and B. vulpis* (Foster et al., 2007; Scholz et al., 2010b; Whatmore et al., 2014). Some further atypical strains have been isolated in the past decade from humans, wildlife mammals, amphibians, and fish (Eisenberg et al., 2020; Tiller et al., 2010). In 2023, a new species was determined, called *Brucella nosferati* sp. nov., which affects *Desmodus rotundus*, vampire bats (Hernández-Mora et al., 2023) (**Table 2**).

Table 2 - *Brucella* species and biovars, preferential hosts and pathogenicity for humans (Saegerman et al., 2010; Godfroid et al., 2010; Whatmore et al., 2014; Hernández-Mora et al., 2023)

Species	biovars	Colony morphology	Preferential host(s)	Pathogenicity in humans
B. melitensis	1-3	smooth	sheep, goat	high
B. abortus	1-6, 9	smooth	cattle	high
B. suis	1, 3	smooth	pig	high
	2	smooth	wild boar, hare	low
	4	smooth	reindeer, caribou	High
	5	smooth	rodent	No
B. neotomae	-	smooth	desert rat	Moderate
B. ovis	-	rough	ram	No
B. canis	-	rough	dog	Moderate
B. pinnipedialis	-	smooth	seal	?
B. ceti	-	smooth	cetacean	?
B. microti	-	smooth	vole, fox	?
B. inopinata	-	smooth	human	?
B. papionis	-	smooth	baboons	?
B. vulpis	-	smooth	red fox	?
Brucella nosferati	-	smooth	bats	?

(?) Although some human cases have been described, the actual pathogenicity remains unknown.

Brucella abortus is a facultative intracellular pathogen that causes persistent infection in animals and has been isolated from several species of livestock. It is mostly associated with cattle, it's a natural or primary host, and *B. melitensis* with sheep, goats, and humans (Godfroid et al., 2013a; Pappas et al., 2005). Bacteria of the genus *Brucella* are highly infectious, and doses as low as 10 to 100 bacteria are thought to be sufficient to cause human disease (Fugier et al., 2007) (**Figure 2**).

The most virulent species with a higher zoonotic spectrum are those from domesticated animals; while those displaying lower pathogenicity and zoonotic potential are those from wildlife animals. One exception is *B. ovis* which is a pathogen for rams and does not infect other hosts (Moreno, 2014). Another exception is the *B. suis* biovar 1 isolated form hares that is highly pathogenic for humans (Saegerman et al., 2010).

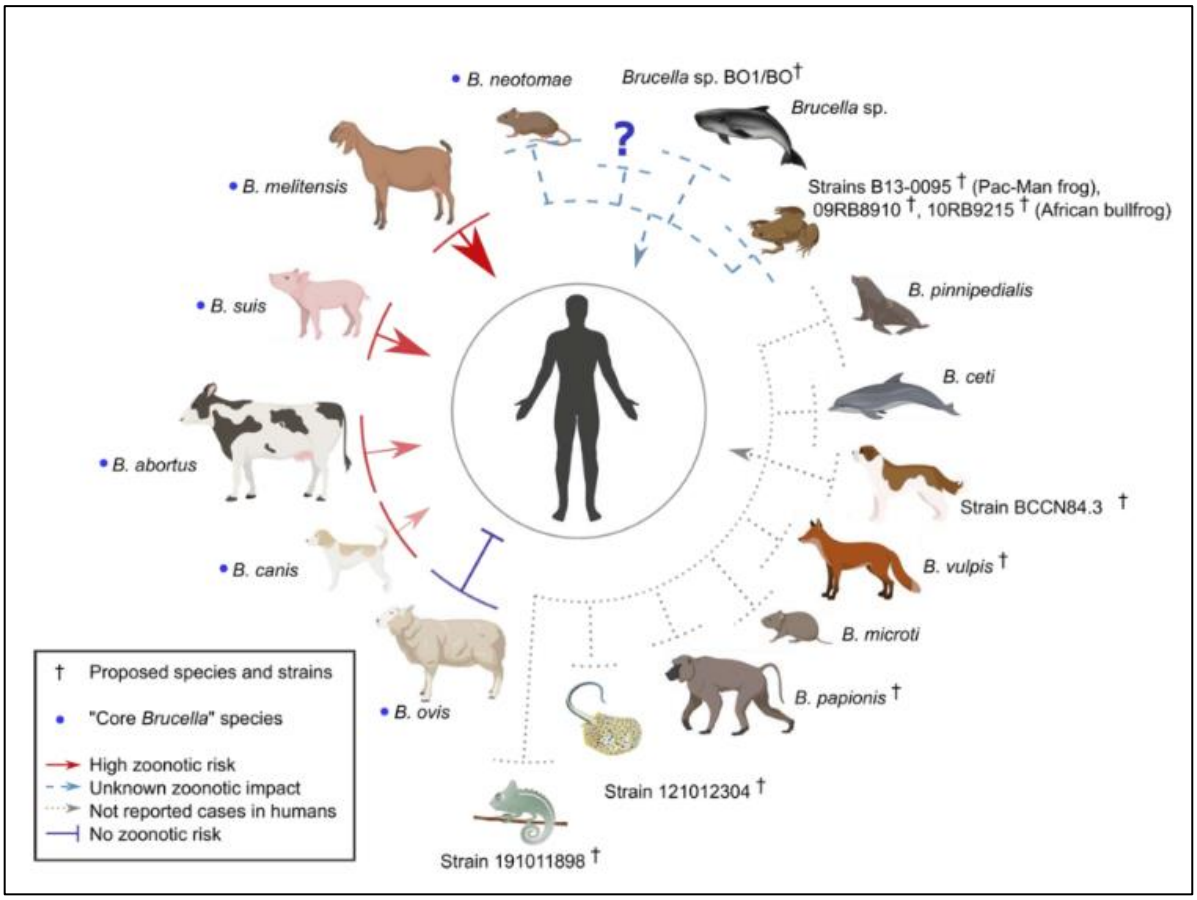

Figure 2 - Zoonotic and non-zoonotic *Brucella* species (Suárez-Esquivel et al., 2020).

Brucella is considered a monotypic genus. However, taxonomy recognizes several species based on host preference, phenotypic and genotypic traits, and biological behavior. Additionally, it is clear that the classic *Brucella* organisms fit the definition of ecotypes from biological, biochemical, genomic, and medical perspectives. It is intriguing how the various species or strains of *Brucella*, which share a very close genetic relationship (~97% similarity), still exhibit different host preferences, virulence, and zoonotic potential (Suárez-Esquivel et al., 2020) **(Figure 2).**

The *Brucella* genus includes a growing number of species, some of which are relevant to public health and economic concerns in various regions of the world. Only a few cases of infection by *B. ceti* sequences have been documented in humans, and therefore the zoonotic potential of marine *Brucella* species remains unclear (Grattarola et al., 2023).

A potential new species of *Brucella*, provisionally named *Brucella amazoniensis sp. nov.*, has been identified in French Guiana. The pathogen was isolated from two patients working in illegal gold mining in the Amazon rainforest, where zoonotic transmission is suspected. Genomic and phenotypic analyses revealed that this bacterium does not belong to any previously known *Brucella* species,

suggesting the need for a new taxonomic classification. This discovery highlights the epidemiological significance of *Brucella* in tropical forest regions (About et al., 2023).

1.5 Routes of infection

Cattle become infected after ingesting contaminated food, milk, forage, and water, or close contact with infected animals (mechanical vectors, including domestic and wild animals, humans) which can spread the disease, or contact with uterine secretions or aborted fetuses, or by vertical and sexual transmission (Saegerman et al., 2010; Godfroid et al., 2013; Ragan et al., 2013). Horizontal transmission occurs through close contact from host to host using secretions, sexual intercourse (mating), and more commonly, through liking of aborted fetuses (Moreno, 2014) (**Figure 3**).

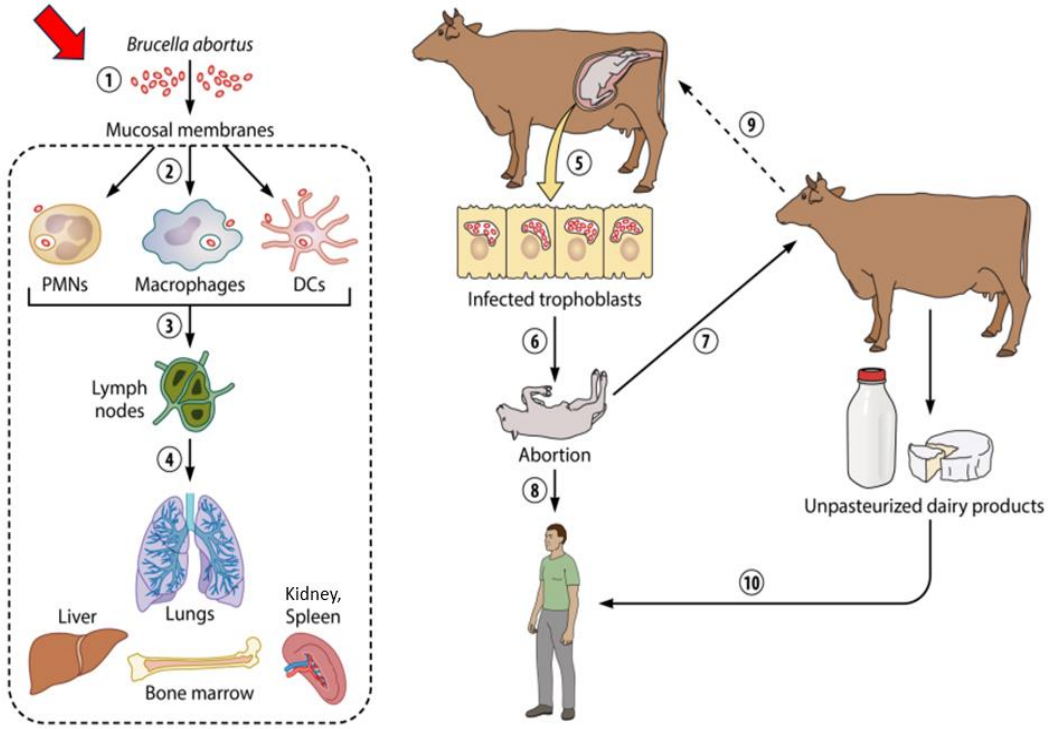

Figure 3 - Cycle of *Brucella abortus* in the bovine host and humans (Moreno and Barquero-Calvo, 2020)

Legend: *Brucella* organisms infect through the mucosal membranes (1); once inside, local professional phagocytes such as Macrophages (Ms), Dendritic Cells (DCs), and Polymorphonuclear cells (typically refers to neutrophils) (PMNs), internalize the invading bacterium (2). From here, the bacterium moves to regional lymph nodes (3), and then it spreads to different organs of the reticuloendothelial system, such as the lungs, spleen, liver, and Bone Marrow (BM) (4). In the pregnant animal, *Brucella* invades the placental trophoblasts, replicating extensively within these cells, causing placentitis (5) and abortion in the last trimester of pregnancy (6). The fetus becomes a source of infection for other animals (7) and humans (8). If the newly infected animal is pregnant, the abortive bacterial cycle continues (9). Humans may become infected by direct contact with the secretions of animals with brucellosis or through ingestion of unpasteurized, contaminated dairy products (10) **(**Moreno and Barquero-Calvo, 2020).

The elimination of bacteria in an infected cow occurs, from day 39 post-infection (Bercovich, 2000a). The abortion is associated with the shedding of 10^{12}-10^{13} bacteria. The quantity of bacteria shed in the course of a brucellosis-induced abortion could theoretically infect 60,000 to 600 000 gestating females. It has been estimated that around 10^{11} microorganisms per gram of tissue are eliminated into the environment after birth or abortion (Saegerman et al., 2010). The excretion of *Brucella* bacteria varies depending on the species, infection status, and stage of the disease. Vaginal fluids from infected animals, especially after reproductive events, contain high concentrations (10^5 to 10^7 bacteria/ml), being a major source of contagion. Urine presents a lower risk, with small amounts in chronic infections. Milk from infected animals is a significant source of human infection, with concentrations of 10^3 to 10^6 bacteria/ml, causing brucellosis mainly through consumption of unpasteurized dairy products (Jansen et al., 2020; Moreno & Moriyón, 2006).

Slaughterhouse workers become inoculated with *Brucella* through aerosolization of fluids, splashing of mucous membranes, and contamination of skin abrasions. Veterinarians are usually infected by inadvertent inoculation of animal vaccines against *B. abortus* and *B. melitensis* (Saegerman et al., 2010). In laboratories, it can infect technicians from specimens of animals (including humans) whose tissues are operated upon or submitted for culture or pathologic analysis (Hayoun et al., 2022).

In cattle the most common route of infection is the gastrointestinal tract (Payne, 1959), *Brucella* replicates intracellularly in phagocytes in the lymph nodes, producing a bacteremia that leads to a systemic infection colonizing the pregnant uterus, the male genital organs, and the mammary gland (Anderson et al., 1986; Ko y Splitter, 2003). In the phagosomes of macrophages, *Brucella* survives and multiplies by inhibiting the fusion of the phagosome-containing bacteria and the lysosome by rapid acidification of the medium (Pizarro-Cerdá et al., 1998; Rivers et al., 2006). Upon entering the cells, *Brucella* follows a multistage intracellular cycle: first, it resides in endosomal vacuoles (eBCV), then transforms into replication-permissive vacuoles derived from the endoplasmic reticulum (rBCV), where the bacteria replicate, and finally into autophagic vacuoles (aBCV), facilitating the release of bacteria for new infections. This cycle depends on the secretion of effector proteins via its type IV secretion system (T4SS), which modulates host functions to promote bacterial survival and replication (Celli, 2019).

It is believed that *B. abortus* exhibits a significant tropism to the uterus in the final trimester of pregnancy because of elevated erythritol and steroid hormone concentrations. *B. abortus* may use Erythritol as a source of carbon and energy during its metabolism, it promotes the survival of bacteria (Samartino and Enright, 1993). The initial source of invasion of embryonic placental tissues is thought to be erythrophagocytic trophoblastic cells, which are situated at the base of ruminant chorionic villi. From there, *B. abortus* spreads to intercotyledonary trophoblasts. The expansion of *Brucella* causes

infiltration of inflammatory cells, trophoblastic necrosis, vasculitis, and ulceration of the allantochorion. Abortion results from impaired fetal-maternal metabolic exchanges as a result (Anderson et al., 1986; Neta et al., 2010).

In contrast to other intracellular pathogens, *Brucella* spp. do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistant forms, or fimbriae and do not show antigenic variation. The key aspect of the virulence of *Brucella* is its ability to proliferate within professional and non-professional phagocytic host cells (Liautard et al., 1996).

Like other pathogenic intracellular bacteria, to be a successful infectious agent, *Brucella* requires four steps: adherence, invasion, establishment, and dissemination within the host (Saegerman et al., 2010).

1.6 Immunity against *Brucella abortus*

Brucella does not possess conventional virulence factors such as toxins, fimbriae, and capsules. This suggests the potential presence of distinctive and nuanced mechanisms for infiltrating host cells, evading host defenses, manipulating intracellular trafficking to avoid degradation in lysosomes, and adjusting the intracellular environment to support prolonged survival and replication (Delrue et al., 2004).

Internalization of *B. abortus* is related to the activation of several small GTPases and the extracellular domain of protein tyrosine kinase in nonprofessional phagocytic cells, tending to localize within the rough endoplasmic reticulum (Corbel, 1997).

The immune system's response to *B. abortus* involves various components, ranging from innate to adaptive immunity. The activation of antigen-presenting cells, natural killer cells, CD4(+) and CD8(+) T cells, and B cells all play a role in triggering this response (Golding et al., 2001).

1.6.1 Innate immunity

The innate immune response is what *Brucella* will encounter after it has entered its host. When *Brucella* infections occur, the primary function of the innate immune system is to lower the quantity of live bacteria that can infect the macrophage, which is the preferred host cell for *Brucella*. The complement system is a first line of defense that attacks gram-negative bacteria directly by destroying their membrane or indirectly by activating them through binding antibodies. It is believed that complement-mediated death of *Brucella* involves both the lectin pathway and the classical pathway,

which is mediated by IgM and low quantities of IgG (Fernandez-Prada et al., 2003; Saegerman et al., 2010).

1.6.2 Adaptive immunity

Adaptive immunity serves as a secondary defense mechanism to innate immunity by activating antigen-presenting cells such as dendritic cells, phagocytes, and cytotoxic lymphocytes, and promoting the production of antibodies (Yang et al., 2024).

The acquired resistance to infection by *B. abortus* is the result of the effects of antibodies and T-effector cells of both CD4 and CD8 phenotypes (Saegerman et al., 2010).

1.6.2.1 Cellular immunity

Cell-mediated immunity is the main defense mechanism, through the activation of macrophages and their ability to eradicate intracellular and its ability to eradicate intracellular bacteria through the action of some cytokines (interferon-γ, tumor necrosis factor α, and interleukin-12) produced by sensitized lymphocytes (Dorneles et al., 2015).

1.6.2.2 Humoral immunity

The LPS is the first antigen against which IgM and IgG antibodies appear after natural infection or vaccination with the S19 (Corbel, 1997). This increase in antibodies is observed between 7 and 14 days after infection. However, after 3 to 8 months post-vaccination, antibodies in prepubertal calves decrease to undetectable levels, suggesting a loss of agglutination activity. Agglutination is an indicator of the immune response, and as antibodies decline, the serum's ability to agglutinate antigens also decreases, which may affect the animal's protection against subsequent infections (Bercovich, 2000b).

Humoral immunity involves the production of antibodies that can opsonize and mark *Brucella* for elimination. Although these antibodies help in the early stages of infection, their ability to neutralize the pathogen is limited because *Brucella* is an intracellular pathogen, meaning it hides within host cells (such as macrophages) to replicate, where antibodies cannot reach it (Skendros & Boura, 2013).

Seroneutralization, which is the ability of antibodies to block a pathogen, plays a limited role in defense against *Brucella*. This is because, once the bacteria invade the cells, antibodies cannot access them. While antibodies can opsonize bacteria outside of the cells and activate the complement system, the complete elimination of *Brucella* depends on cellular immunity, particularly T cells, which activate macrophages to destroy the intracellular bacteria (De Figueiredo et al., 2015).

In the case of vaccination against *Brucella*, seroneutralization plays a limited role because *Brucella* is primarily an intracellular pathogen. While vaccines like *Brucella* strain S19 can stimulate the production of antibodies, these antibodies mainly help by opsonizing the bacteria for destruction before they enter host cells. However, once *Brucella* invades cells, the antibodies cannot neutralize the pathogen. The primary defense against *Brucella* post-vaccination depends on cellular immunity, especially T cells that activate macrophages to eliminate the bacteria within the cells (Skendros & Boura, 2013; Titball, 2008; Yang et al., 2023).

1.7 Survival in the environment

Generally speaking, high temperatures, direct sunlight, and aridity hinder the survivability of *Brucella* in the environment **(Table 3).**

Table 3 - Survival periods of *B. abortus* in various substrates (Corbel et al., 2006)

Medium	Temperature or environment	Survival
Solid surfaces	<31 °C, sunlight	4–5 hours
Tap water	–4 °C	114 days
Lake water	37 °C, pH 7.5	<1 day
Lake water	8 °C, pH 6.5	>57 days
Soil – dried	~20 °C	<4 days
Soil – wet	<10 °C,	66 days
Manure	summer	1 day
Manure	winter	53 days
Farm slurry animal waste	ambient-temperature tank	7 weeks
Farm slurry animal waste	12 °C tank	>8 months

1.8 Clinical Signs

Clinical signs are not pathognomonic. In cattle, ruminants and pigs, the initial phase following infection often is not apparent. In sexually mature animals the infection localizes in the reproductive system and typically produces placentitis followed by abortion in the pregnant female, usually during the last third of pregnancy, and epididymitis and orchitis in the male (Corbel et al., 2006). The majority of animal hosts of brucellosis exhibit characteristic, albeit non-specific, symptoms such as retained placenta and abortion or premature birth, and produce weak newborn calves, and infertility in cows and bulls (Enright et al., 1984).

Hygromas and abscesses are sometimes the main clinical symptoms in bovine herds that are semi-nomadic or nomadic and have *B. abortus* biovar 3 infection. Premature deliveries cause a reduction in milk production, an increase in the number of somatic cells in the milk, and impaired reproductive

efficiency (Carpenter and Boak, 1928; Corbel et al., 2006). Fertility disruption is often transient; most of afflicted animals will only miscarry once, while some will remain unaffected. It is common for the udder to become persistently infected, especially in goats and cows (Corbel et al., 2006).

Calves that acquire the infection vertically or by ingesting contaminated milk may remain serologically negative and not show any sign of the disease (Saegerman et al., 2010). However, heifers with latent asymptomatic infection may abort or give birth to infected calves, which are central to maintaining the disease in a herd (Neta et al., 2010; Nicoletti, 1980; Wilesmith, 1978).

Venereal transmission is not a major route of infection under natural conditions, but artificial insemination with contaminated semen is a potential source of infection (Neta et al., 2010; Rankin, 1965).

1.9 Diagnosis

The diagnosis of brucellosis can be made directly through bacterial isolation and species – biovar identification on molecular tests, and indirectly through the evaluation of the clinical history and signs of the disease, and serological tests (Khurana et al., 2021).

The choice of a particular testing strategy depends on the prevailing epidemiological situation of brucellosis in susceptible animals (livestock and wildlife) in a country or a region (Saegerman et al., 2010; Godfroid et al., 2010). Isolation of *Brucella* allows certainty of diagnosis and biotyping furnishes precious epidemiological information that allows tracing of infection sources, particular in countries where several biotypes are co-circulating (Saegerman et al., 2010).

Species and biovars can be identified by phage lysis, and by cultural, biochemical, and serological tests. PCR can provide the basis for complementary identification and typing methods based on specific genomic sequences. The recommended methods for observing colonial morphology are Henry's method by obliquely reflected light, the acriflavine test described by Braun & Bonestell, or White & Wilson's crystal violet method of staining colonies (Alton et al., 1976; WOAH, 2022).

Identification of *Brucella* organisms to species and biovar level can be carried out by a combination of the following tests: organism morphology after Gram or Stamp's staining, direct observation of colonial morphology, growth characteristics, urease, and oxidase tests, and the slide agglutination test with a polyclonal anti-*Brucella* serum. Species and biovar identification require elaborate tests (such as phage lysis and agglutination with anti-A, -M, or -R monospecific sera) (WOAH, 2022).

MALDI-TOF (matrix-assisted laser desorption ionization time of flight) is increasingly used in diagnostic microbiology and has been applied to the identification of *Brucella.* However, some limitations exist in commercial database coverage, have not yet enabled robust and unambiguous discrimination of strains of *Brucella* sp, in addition to *Brucella* strict genetic relationship (WOAH, 2022).

1.9.1 Culture

In the case of clinical brucellosis, valid samples include aborted fetuses (stomach, spleen, and lung), fetal membranes, vaginal secretions, colostrum, milk, sperm, and fluid collected from arthritis or hygroma (Saegerman et al., 2010). At slaughter, to confirm suspected cases of acute or chronic brucellosis, the preferred tissues are the genital and oropharyngeal lymph nodes, the spleen, and the mammary gland and associated lymph nodes (Godfroid et al., 2010).

A useful tool to consider is the medium CITA, which containing Vancomycin, Colistin, Nystatin, Nitrofurantoin, and Amphotericin B, which isolates the main *Brucella* species including B. *suis*. However, the simultaneous use of CITA plus Farrell (FM) or modified Thayer-Martin (mTMA) media, results in the best diagnostic performance for isolating B. *melitensis* and B. *suis* or B. *ovis*. (Miguel et al., 2011).

1.9.2 Molecular detection

Several PCR-based methods have been developed. The best-validated methods are based on the detection of specific sequences of *Brucella* spp., such as the 16S-23S genes, the IS711 insertion sequence, or the bcsp31 gene encoding a 31-kDa protein (Godfroid et al., 2010).

Nevertheless, as a general rule, brucellosis PCR techniques show a lower diagnostic sensitivity than culture methods, although their specificity is close to 100%. The best results have so far been obtained by combining culture and PCR detection on clinical samples (Godfroid et al., 2010; Hinić et al., 2009; Leyla et al., 2003).

The first technique created to identify and distinguish between all known species of *Brucella* and vaccine strains in a single test is the multiplex "Bruce ladder" PCR. The specificity of the Bruce-ladder PCR has been tested previously, using as targets DNA from 30 strains phylogenetically or serologically related to *Brucella* (Godfroid et al., 2010). The IS711-based real-time PCR assay is specific and highly sensitive and appears as an efficient and reproducible method for the rapid and safe detection of the genus *Brucella* (Bounaadja et al., 2009).

Numerous novel fingerprinting methods exhibit potential in distinguishing isolates within the same biovar of a particular species: single-nucleotide polymorphisms (SNP), which identify single-nucleotide variations in a species' members' DNA sequences; multiple sequence alignment (MLSA), which identifies variations in a set of housekeeping genes' DNA sequences and characterizes strains based on their distinct allelic profiles; and multiple loci containing repetitive sequences (MLVA) (Saegerman et al., 2010).

MLVA is one of the most widely used molecular techniques in the molecular typing of microorganisms. It is based on naturally occurring variations in the number of consecutive repeats of DNA sequences in different regions of the genome. The term MLVA is the acronym for multiple-loci VNTR analysis. In bacterial populations this characteristic can be associated with speciation and even the geographic distribution and dispersion of disease outbreaks, contributing to their epidemiological tracing (Le Flèche et al., 2006).

Next-generation sequencing (NGS) is an emerging sequence-based pathogenic microorganism identification technique and is increasingly used to diagnose the infectious disease (Yu et al., 2023).

1.9.3 Indirect methods

The purpose is to detect an immune response to *Brucella* antigens, and reveal the titers of antibodies present in the patient's blood serum (Ron-Román, 2003). These tests present variable sensitivity and specificity in the function of numerous variables, such as the acute or chronic stage of the disease, sensitization by cross-reacting bacteria (i.e. bacteria presenting antigenic similarities), the vaccination status, and the kinetics of the induced immune responses (Saegerman et al., 2004).

The use of indirect diagnostic tests is the core of the control or eradication strategy and the tests used have an impact on the success or the failure of the strategy (Renukaradhya et al., 2002a).

Numerous immunological diagnostic tests exist, including milk ring test (MRT), buffered *Brucella* agglutination tests (i.e., Rose Bengal test (RBT); Card Test (CT), and buffered plate agglutination test (BPAT), complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and fluorescence polarisation assay (FPA) (Saavedra et al., 2019).

1.9.4 Milk ring test (MRT)

In lactating cattle, the MRT can be used for screening herds for brucellosis. In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable (WOAH, 2022). The test consists of mixing colored *Brucella* haematoxylin-staining whole-cell antigen with fresh bulk/tank milk. In the

presence of anti-*Brucella* antibodies, antigen-antibody complexes form and migrate to the cream layer, forming a dark blue ring on the surface, the presence of IgA and IgM induces the formation of a colored ring (blue) after incubation (Bercovich, 2000b; Saavedra et al., 2019).

1.9.5 Rose Bengal Agglutination Test (RBT)

The RBT uses whole cells of *B. abortus* S99 or S1119.3, stained with Rose Bengal. The RBT is performed with a stained *B. abortus* suspension at a pH of 3.6-3.7. The RBT consists of a simple spot agglutination test where drops of rose Bengal-stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction (WOAH, 2022). The use of low pH, prevents some IgM agglutination and stimulates IgG1 agglutination, hence reducing non-specific interactions (Corbel, 1972).

1.9.6 Buffered Plate Agglutination Test (BPAT)

The BPAT is also a spot agglutination test where a stained *B. abortus* antigen is used. Two staining solutions are required: brilliant green (2 g/100 mL) and crystal violet (1 g/100 mL) mixed in equal volumes to prepare a stained-cell suspension with a blue–green color. This antigen is mixed on a plate with serum and any resulting agglutination signifies a positive reaction (Saavedra et al., 2019; WOAH, 2022).

1.9.7 Slow Agglutination Test or Slow Agglutination of Wright (SAT or SAW)

The principle of this antigen test is to detect agglutinin antibodies mainly of the IgM isotype directed against *Brucella* spp, to a lesser extent IgG. At an optimum concentration of antigens and antibodies, large antigen-antibody complexes form and precipitate at the bottom of the test tube. This reaction is slow because, in contrast to the rapid agglutination tests, it requires an overnight incubation at 37°C. The specificity of the test is enhanced by treating the serum with a chelating agent such as EDTA, which reduces cross-reactivity due to IgM. (Saegerman et al., 2010; Godfroid et al., 2010).

1.9.8 ELISA

ELISAs are mainly divided into two categories, indirect ELISAs (iELISA) and competitive ELISAs (cELISA). Most of the iELISAs use purified S-LPS as an antigen. Their main quality is their high sensitivity, but they are also more vulnerable to aspecific reactions notably to those due to infection by *Yersinia enterocolitica* serotype O:9. The specific cross-reaction of indirect ELISA with the LPS of *Yersinia enterocolitica* O:9 has led to the development of cELISA (Saegerman et al., 2010).

The iELISAs that use S-LPS or OPS as antigens are highly sensitive for the detection of anti-*Brucella* antibodies in cattle, small ruminants, and pigs, but are not capable of fully resolving the problem of differentiating between antibodies resulting from *B. abortus* S19 and *B. melitensis* Rev.1 vaccination. The *B. abortus* RB51 vaccine may also interfere in S-LPS-based iELISAs (WOAH, 2022).

The O-chain of the smooth LPS of *Brucella* contains specific epitopes that are not shared with the LPS of *Yersinia enterocolitica* O:9. Therefore, by using monoclonal antibodies directed against specific epitopes of the *Brucella* LPS, the development of more specific cELISA has been possible. These tests present a better specificity than the iELISA tests but their sensitivity is less (Saegerman et al., 2010). In the cELISA the main rationale for these assays was that vaccines induced antibodies of lower affinity due to the shorter exposure to antigen due to immune elimination compared to field infection in which antigen persisted, resulting in increased antibody affinity. This type of assay utilized a competing antibody of higher affinity than cross-reacting or vaccinal antibody thereby eliminating some antibodies that confuse diagnosis, unlike other serological tests such as indirect enzyme immunoassay and the classical tests, all of which provide global antibody measurements. This increase in specificity was obtained with a minimal loss of sensitivity (Nielsen et al., 2007; Samartino et al., 1999).

Recent discovery of synthetic oligosaccharide antigens representing the capping M epitope elements of *Brucella* O-polysaccharides offers the opportunity to standardize more easily the quality of the antigen used and to increase the specificity of the brucellosis serodiagnosis. Antigen enzyme-linked immunosorbent assay (ELISA) is a technique used to detect and quantify the presence of specific antibodies in biological samples, such as sera. The method is based on the binding of a synthetic antigen, designed to mimic the epitope of the pathogen of interest, to a microtiter plate. Samples are then added, allowing the antibodies present to bind to the antigen. A secondary antibody labeled with an enzyme that binds to the specific antibodies is then added. The addition of a substrate for the enzyme produces a colorimetric reaction, the intensity of which is measured spectrophotometrically and correlates with the concentration of antibody in the sample. This approach provides high sensitivity and specificity and is widely used in clinical diagnostics and epidemiologic studies (McGiven et al., 2015; David et al., 2017).

1.9.9 Complement Fixation Test (CFT)

The complement fixation test (CFT) allows the detection of anti-*Brucella* antibodies that can activate complement. Cattle IgG1, IgG2, and the IgM activate bovine complement. This test is not highly sensitive but shows excellent specificity and is used as a confirmatory test or in enzootic conditions to detect chronic infection. This test being difficult to standardize and is progressively being replaced by the ELISA tests (Godfroid et al., 2010; WOAH, 2022).

1.9.10 Fluorescence polarization assay (FPA)

Is based on the rotational differences between a small soluble antigen molecule in solution (labeled with a fluorochrome) and the antigen molecule complexed with its antibody. A small molecule will rotate randomly at a rapid rate, resulting in high depolarization rate of light, while a larger complex molecule will rotate slower and depolarize light at a reduced rate. The rate change in depolarization can be measured (Nielsen and Gall, 2001).

The molecules of small size spin faster and depolarize a polarized light beam more, while bigger molecules spin more slowly and, consequently, depolarize light less. The degree of depolarization is the measure of the test and it is expressed in milli-polarization units (mP) (Saegerman et al., 2010).

For diagnosis of brucellosis, a fluorescence polarization analyzer is used to obtain a background measurement of the fluorescence of diluted serum. Antigen consisting of an OPS fragment, approximately 22 kDa in size, labeled with fluorescein isothiocyanate is added and incubated for 2 min, followed by a final reading in the analyzer which automatically subtracts the background reading (Nielsen et al., 2007).

1.9.11 Tests for the detection of cellular immunity (Brucellosis skin test)

The Brucellin Skin Test (BST) is a cell-mediated immune assay that is based on the use of purified and standardized protein allergen, almost completely devoid of LPS. The BST relies on the delayed-type hypersensitivity reaction whose results are interpreted 48 or 72 hours post brucellin injection. The BST has a very high specificity, such that serologically negative unvaccinated cattle that are positive reactors to the Brucellin test should be regarded as infected animals (Pouillot et al., 1997).

1.9.12 Native hapten test (NH)

Native hapten test, have been effectively utilized in conjunction with the RBT as a screening test in cattle, is highly specific in *B. abortus* S19 vaccination situations. Immunoprecipitation tests with NH or polysaccharide B are sensitive and specific in discriminating infected animals from cattle vaccinated with *B. abortus* S19 (Muñoz et al., 2005).

Table 4 - Test methods available for the diagnosis of infection with *Brucella abortus* (WOAH, 2022)

	Purpose					
Method	**Population freedom from infection**	**Individual animal freedom from infection[a]**	**Contribute to eradication policies[b]**	**Confirmation of suspect or clinical cases[c]**	**Herd/flock prevalence of infection – surveillance**	**Immune status in individual animals or populations postvaccination**
Detection of the agent						
Staining methods	-	-	-	+	-	-
Culture	-	-	-	+++	-	-
PCR[d]	-	-	-	+/++	-	-
Detection of immune response						
BBAT (RBT or BPAT)	+++	++	+++	+	+++	-
FPA	++	++	+	++	++	-
CFT	++	++	+++	++	+++	-
iELISA	+++	++	+++	++	+++	-
cELISA	++	+	+	+	++	-
BST	++	-	+	+++	++	-
SAT	++	+	+	-	+	-
NH and cytosol protein-based tests[e]	-	-	+	++	-	-
Bulk milk tests[f] Milk I-ELISA or Milk ring-test	+++	-	+++	+	+++	-

Legend: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction; BBAT = buffered *Brucella* antigen tests (i.e. RBT [rose bengal test] and BPAT [buffered plate agglutination test]); FPA = fluorescence polarisation assay; CFT = complement fixation test; i- or cELISA = indirect/competitive enzyme-linked immunosorbent assay; BST = brucellin skin test; SAT = serum agglutination test; NH = native hapten (a) This applies only to herds/flocks, countries or zones free from infection with *Brucella*. (b) To increase the efficiency of eradication policies in infected herds/flocks, it is recommended to associate tests in parallel to increase the sensitivity of the diagnosis, i.e. two serological tests at least, e.g. BBAT or FPA and CFT or iELISA. The sensitivity is further increased by parallel testing by both serology and BST. (c) In low-prevalence or almost-free zones, the predictive value of positive results to serological tests may be very low. In such situations, agent identification is usually needed to confirm clinical cases. In infected herds/flocks, a positive result to any serological test may be considered as confirmation of a clinical case. Any reactor in any serological test should be considered to be infected even in the absence of clinical signs. In low-prevalence or almost-free zones, singleton serological reactors may be confirmed by culture (or PCR) or BST. In free countries or zones, suspect animals are those positive for both a screening and a confirmatory serological test (tests in series) and may be confirmed by culture (or PCR) and/or BST. (d) False-positive results may occur. (e) In zones where subcutaneous S19 or Rev.1 vaccination is practiced, this test may help in differentiating antibodies due to vaccination from those due to infection. (f) Dairy cattle only

1.10 Vaccines for control bovine brucellosis

Vaccination has been well-established to be a crucial element in the control and prevention of animal brucellosis (Schurig et al., 2002).

1.10.1 B. abortus strain 19 biovar 1

The vaccine that has been most widely used to prevent bovine brucellosis is *B. abortus* strain 19 (S19), which remains the reference vaccine with which any other vaccines must be compared, because the *B. abortus* strain 19 (S19) vaccine is the most widely used to prevent bovine brucellosis and serves as the reference vaccine for several reasons (WOAH, 2022).

- Efficacy: Proven effectiveness in reducing brucellosis incidence in cattle.
- Safety Profile: A well-established safety record with minimal adverse reactions.
- Regulatory Approval: Extensive approval across countries, setting standards for new vaccines.
- Immune Response: Strong immunogenicity, producing crucial IgM and IgG antibodies.
- Practical Application: Easy to administer in field conditions.
- Historical Data: Extensive research and data supporting its use.

Live vaccines have proved to be superior to inactivated products. They are effective, and inexpensive, and immunity is more persistent. The disadvantages of post-vaccinal antibodies can be minimized by the reduction of previously recommended doses and through the use of supplemental diagnostic tests (Nicoletti, 1990). Strain 19 is an attenuated organism of smooth morphology normally unable to grow in the presence of erythritol (Jones et al., 1965).

The S19 strain is based on a live attenuated variant of *Brucella abortus*, which has been modified to reduce its pathogenicity while retaining its ability to induce a protective immune response. This strain belongs to *Brucella abortus* biovar 1, one of the most common biovars and a frequent cause of brucellosis in cattle. Biovars, or biotypes, are sub-classifications within a bacterial species that differ in specific biochemical and serological characteristics (Moreno & Moriyón, 2006; WOAH, 2022).

B. abortus 19 (*B. abortus* S19) can be distinguished from typical field isolates of *B. abortus* biovar 1 by its inhibition on media containing thionin (2 µg/ml) and penicillin (5 IU/ml). Moreover, unlike typical primary isolates of biovar 1, this vaccine strain does not require CO_2 for growth. It is also inhibited by i-erythritol (1 mg/ml) due to a 702-bp deletion in the ery region leading to a metabolic deficiency that likely results in the toxic accumulation of intermediate catabolites (Sangari et al., 1994).

It is used as a live vaccine and is normally given to female calves aged between 3 and 6 months as a single subcutaneous dose of 5–8 × 10^{10} viable organisms. A reduced dose of from 3 × 10^{8} to 5 × 10^{9} organisms can be administered subcutaneously to adult cattle, but some animals can develop persistent antibody titers and may abort and excrete the vaccine strain in the milk. Alternatively, the vaccine can be administered to cattle of any age as either one or two doses of 5 × 10^{9} viable organisms, given by the conjunctival route (Nicoletti, 1990; WOAH, 2022) in bovines.

1.10.2 B. abortus RB51 vaccine

Brucella abortus strain RB5l is a naturally occurring, rugged, attenuated, stable mutant of strain 2308 (Hahn et al., 2015) in the presence of rifampin and penicillin (Schurig et al., 1991). Selection was conducted using the crystal violet and acriflavine assays (Colonies and Dissociation), and significant effort has been devoted to the genetic characterization of RB51. The RB51 strain possesses an IS711 spontaneously inserted in the wboA gene, which is undoubtedly linked to its phenotype and attenuation (Moreno & Moriyón, 2006; Vemulapalli et al., 2000).

Calves are vaccinated subcutaneously between the ages of 4 and 12 months with 1–3.4 × 10^{10} viable organisms. Vaccination of cattle over 12 months of age is carried out only under authorization from the State or Federal Animal Health Officials, and the recommended dose is 1–3 × 10^{9} viable organisms (WOAH, 2022).

Due to its minimal production of O-chain, it usually does not induce antibodies specific to the O-chain in quantities measurable by classical serological tests, this regardless of age, dose, or frequency of injections. However, more recently, it has been reported that the strain induces the production of low levels of anti-O-chain antibodies (Saegerman et al., 2010).

According to the study conducted by Blasco; et al (2023) on controlled studies analyzed in the last 35 years, it has been shown that RB51 is an abortive vaccine, is excreted in milk and presents resistance to rifampicin, which complicates diagnosis and safety in humans. It also mentions that its protection is inferior to that of S19, and there is no evidence that revaccination with RB51 improves immunity, nor that it has the ability to differentiate infected from vaccinated animals (DIVA) in diagnostic tests (Blasco et al., 2023).

1.10.3 Quality control of vaccines in final product

The definition of the vaccine doses, as well as the quality control measures necessary to ensure their safety and efficacy, is outlined by the World Organisation for Animal Health (WOAH). According to WOAH (2022), quality control is essential in the production, packaging, and distribution of *Brucella*

vaccines. For lyophilized vaccine, the control tests should be performed on the reconstituted vaccine. Several parameters must be evaluated such as the purity to determine if the biological materials are pure or contaminated, tests for identity, potency including lot potency and innocuousness. Practically, the potency, smoothness, residual virulence and immunogenicity, as well as live bacterial count are tested (WOAH, 2022).

Appropriate CFU counts are as follows:

S19:

a) 0.5-0.8 × 10^{11} CFU (standard dose; subcutaneous route);

b) 0.3-5 × 10^{9} CFU (reduced dose; subcutaneous route);

c) 5 × 10^{9} CFU (reduced dose; conjunctival route).

RB51:

a) 1-3.4 × 10^{10} CFU (standard dose; subcutaneous route).

To approve the vaccine for registration purposes, all relevant data related to vaccine manufacturing and quality control tests must be sent to the authorities.

In countries with national regulatory programs that include re-analysis for control by official authorities (verification or confirmatory testing), samples of each batch or series are also sent for analysis by the competent authorities in official laboratories prior to release for sale. If the manufacturer or the competent authorities obtain unsatisfactory test results, the batch or series may not be released (WOAH, 2023).

1.10.4 Brucellosis situation in the world

While brucellosis and its transmission routes have been discovered for more than 100 years, it is still a global concern, especially in low-income countries. The highest prevalence of brucellosis has been reported in countries, such as West Asia, India, the Middle East, Southern Europe, and Latin America (Khoshnood et al., 2022; Mantur et al., 2007).

Brucella infection in cattle is distributed worldwide, but in several northern and central European countries as well as Oceania *B. abortus* is considered absent (SENASICA, 2024).

The highest incidence is observed in the Middle East, the Mediterranean region, sub-Saharan Africa, China, India, Peru, and Mexico. Currently, countries in central and southwest Asia are seeing the greatest increase in cases (WOAH, 2024). In the case of Latin America, animal brucellosis causes losses of over 600 million dollars a year (McDermott et al., 2013).

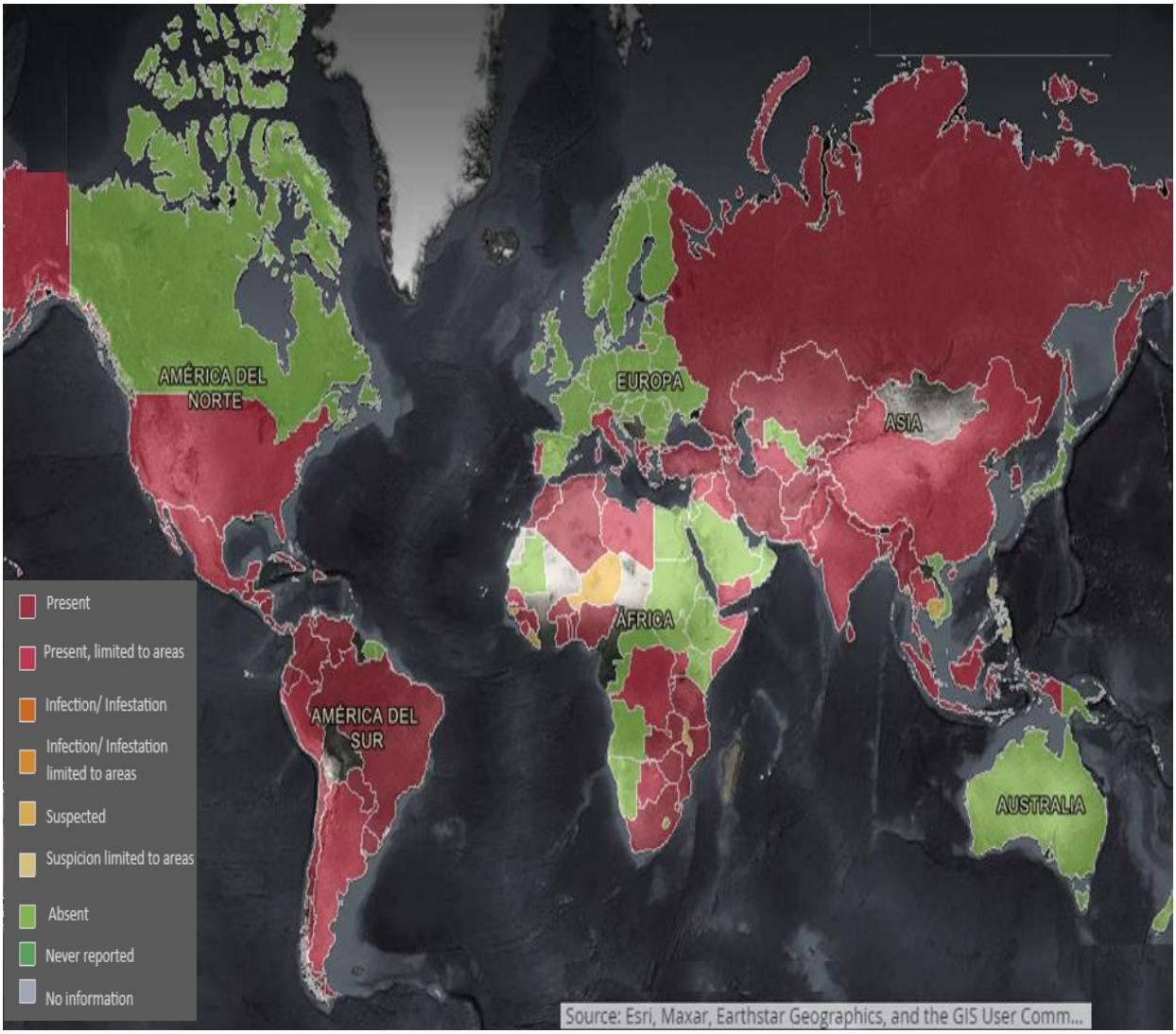

Figure 4 - Brucellosis Status in the World (SENASICA, 2024)

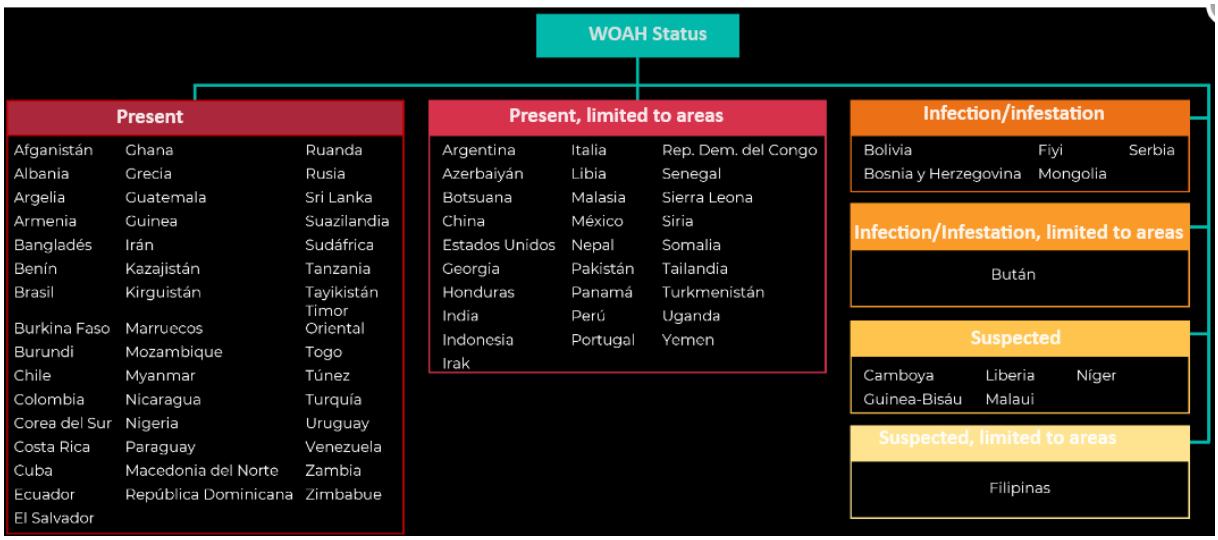

Figure 5 - WOAH Status (SENASICA, 2024; WAHIS, 2023)

Legend: Brucellosis Status in the World: 46 countries with whole presence, 28 countries with presence but limited to some zones, 5 countries with infection/ infestation, 1 country with infection/ infestation limited to zones, 5 countries with suspicion, 1 country with suspicion limited to zones.

1.11 Risk and predisposing factors for bovine brucellosis

Social and political instability, as well as improper diagnosis, reporting, and use of control measures, are common risk factors associated with the challenge of controlling bovine brucellosis. The use of unpasteurized dairy products as a customary cultural practice, livestock husbandry practices, and their contacts with other animals and wildlife all contributed significantly to the spread of brucellosis (Dawood et al., 2023) **(Table 5).**

Table 5 - The risk factors of bovine brucellosis (Dawood et al., 2023)

Risk factors		Association with brucellosis	References
Animal Factors	Species	Cattle exhibit a higher likelihood of infection compared to buffalos	Kumar, 2016
	Age of animals	Sero-positive cases are more prevalent in older animals compared to calves	Lindahl et al., 2014
	Sex	Female and dairy animals are more susceptible to infection than males	Ul-Islam et al., 2013
	Breed	Purebred animals are more prone to seropositivity than native breeds	Shome, 2014
	History of abortion	Cases with a history of abortion are associated with seropositivity	Ndazigaruye et al., 2018

	Status of pregnancy	Late stages of pregnancy are more likely to be seropositive	Khan y Zahoor, 2018
	History of retained placenta	Cases with a history of retained placenta are linked to seropositivity	Mubanga et al., 2021
Pathogen factors		Intracellular replication and evasion of host bactericidal activity: *Brucella* possesses the ability to survive and multiply within host phagocytic cells, primarily macrophages. This bacterium modifies the phagosome to create a favorable replicative niche, avoiding fusion with lysosomes and subsequent degradation. This mechanism allows it to evade the innate immune response and establish a persistent infection. Inhibition of phagocytosis and modulation of immune response: *Brucella* have developed strategies to interfere with the phagocytosis process and modulate the host immune response. This includes: a) Modification of lipopolysaccharide (LPS) structure to reduce its recognition by pattern recognition receptors. b) Secretion of factors that interfere with intracellular signaling in phagocytic cells. c) Negative regulation of antigen presentation and T cell activation.	Avila-Calderón et al., 2020; Celli, 2019; López-Santiago et al., 2019
Occupational factors		Laboratory workers, veterinarians, farmers, butchers, and abattoir workers are at risk	Dadar et al., 2020
Management factors	Herd size	Larger herds show a positive association with seropositivity	de Alencar Mota et al., 2016
	Mixed herd	Cattle housed with goats and/or sheep are more likely to be seropositive	de Alencar Mota et al., 2016
	Breeding method	Artificial insemination is positively correlated with seropositivity	de Alencar Mota et al., 2016; Makita et al., 2011
	Distance between herds	Proximity between herds is positively associated with seropositivity	de Alencar Mota et al., 2016
	Introduction of a new animal from an unknown source	The introduction of animals from unknown sources is linked to seropositivity	Elderbrook et al., 2019
	Clean drinking water	Lack of clean drinking water for animals is positively associated with seropositivity	Coelho et al., 2007

	Clean and hygiene	Insufficient manure removal and dirtiness in farms are positively associated with seropositivity	Coelho et al., 2007
	Routine control milk diagnosis	Herds not routinely tested for *Brucella* infection show a positive correlation with seropositivity	Welburn et al., 2015
Human Factors	Age of owner	Cattle and buffalo owned by individuals above 40 years exhibit a positive association with seropositivity	Pathak et al., 2016
	Awareness	Cattle and buffalo owned by farmers with knowledge and awareness about diseases, particularly brucellosis, show a negative correlation with seropositivity	Pathak et al., 2016
Agro-ecological factors	Origin	Sero-positivity differs in different parts of a country: Seropositivity to *Brucella* varies significantly influenced by factors such as agricultural practices, land management, and the diversity of animal species present in the area. For instance, areas with extensive livestock farming have been associated with increased seropositivity rates.	Alhaji et al., 2016
	The presence of dogs, cats, and wildlife	Their presence is suspected as a source of infection: The presence of dogs, cats, and wildlife in rural areas can be a significant risk factor for *Brucella* transmission. These animals are considered potential vectors for the infection, as they may come into contact with infected livestock, thereby facilitating the spread of the bacteria. Studies have shown that regions with a high density of these species exhibit higher rates of seropositivity in both humans and livestock.	Cárdenas, Awada, et al., 2019

1.12 Bovine brucellosis control and eradication programs

In brucellosis control programs, key actors include veterinary authorities, public health officials, farmers, international organizations such as WOAH and Food and Agriculture Organization (FAO), and researchers. These stakeholders collaborate to implement vaccination, surveillance, and disease management strategies. Beyond laboratories, other critical domains involved are agricultural ministries, public health departments, and education campaigns to raise awareness about the disease. In the context of the **One Health** approach, the control of brucellosis requires interdisciplinary collaboration across veterinary, medical, and environmental sectors. Additional criteria include coordinated surveillance

systems, shared data between animal and human health sectors, and holistic risk assessments that consider ecological, socio-economic, and cultural factors (WOAH, 2022).

Epidemiological contexts including management and trade systems along with well-defined agroecological zones should be evaluated in brucellosis endemic countries to improve milk production and to enhance the sustainability of the livestock sector at both national and regional levels (Dadar et al., 2021).

The sanitation, test, and removal approaches and/or vaccination of cattle between 4 and 12 months of age as well as cattle over the ages of 12 months is the most economic measure for bovine brucellosis control. However, vaccination alone is not advisable for the elimination of brucellosis in any host species (Dadar et al., 2021; Olsen y Stoffregen, 2005).

Many aspects of the planned control program must be assessed, including knowledge of local and regional variations in brucellosis animal epidemiological patterns, cross-sectoral epidemiological coordination and surveillance, husbandry practices, infrastructure support, community awareness, and social customs (Seimenis et al., 2019).

The overall strategy consists of the following steps: (i) generalized compulsory vaccination of the entire susceptible population (medical prophylaxis); (ii) slaughter of known infected animals (test and slaughter) combined with a selective vaccination program including either only the young animals or all animals in a limited area depending on the prevalence (medico-sanitary prophylaxis) and (iii) sanitary slaughter of animals known to be infected or exposed (sanitary prophylaxis). An adequate compensation of the animals slaughtered is an unavoidable prerequisite for the success of the program (Saegerman et al., 2010).

There have also been reports of other preventive measures being successful in controlling brucellosis, such as vaccination of female cows and certification of brucellosis-free herds (Herrera et al., 2008). The control of brucellosis transmission to non-infected animal herds as well as the elimination of the animal carriers of the bacteria such as dogs, cat, and mice in the herd to eradicate the sources of infection (Dadar et al., 2021; Gwida et al., 2010).

The cooperation and support of farmers are crucial for implementing long-term eradication and control programs. Therefore, veterinary organizations should increase farmers' awareness regarding preventive strategies and transmission routes through continuous education and training programs. The accessibility to necessary resources needed for prevention and appropriate veterinary services are also important requirements (Dadar et al., 2021).

An example of a country declared free of *Brucella abortus* is New Zealand, declared free from *Brucella abortus* in 1996 and reaffirmed this status in 2021. The eradication process involved:

- **Initial Measures**: Brucellosis was first detected in 1893, and by 1966, 15% of cattle were infected. Mandatory vaccination of female calves with the S19 strain began, along with compulsory culling of infected animals.
- **Surveillance**: The last case was in 1988, and by 1989, all quarantined farms were cleared. Surveillance continued from 1989 to 1994, confirming no new cases.
- **Ongoing Control**: New Zealand has strict systems for early detection and import controls. From 2010 to 2020, 8,585 tests for *Brucella abortus* were conducted, all negative.
- **Final Declaration**: In 2021, New Zealand reaffirmed over 25 years of brucellosis-free status, with no evidence of the disease in wildlife or domestic animals.

This successful eradication combined vaccination (S19 strain), strict culling, and ongoing surveillance (WOAH, 2021).

1.13 National brucellosis program in Ecuador

The presence of brucellosis in Ecuadorian cattle was evidenced by Salvestroni, who in 1926 made the first notification of a case (AGROCALIDAD, 2016).

In Ecuador, AGROCALIDAD is the institution in charge of the national bovine brucellosis control program, which started activities in 2008, establishes the procedures to be followed in the event of the presence of bovines suspected or confirmed positive for this disease, executing activities by the prevention and control guidelines established by the program, which is described below (AGROCALIDAD, 2016)

Epidemiological surveillance is carried out to collect essential information to identify diseases. There are two main approaches: passive surveillance and active surveillance. In passive surveillance, the national population immediately notifies the official veterinary service surveillance system when brucellosis is suspected. A suspect case is considered to be any farm in which one or more animals show clinical signs compatible with the disease. On the other hand, a confirmed case is defined as a farm in which one or more animals are positive in the confirmatory ELISA test. In contrast, active surveillance, directed by AGROCALIDAD, is carried out according to specific priorities and resources. In the context of bovine brucellosis, active surveillance implies prior planning, especially in cases of monitoring plans aimed at disease control and prevention (AGROCALIDAD, 2016).

In Ecuador, the vaccination of female cattle is carried out using the S19 and RB51 strains of *Brucella abortus,* aimed at preventing the transmission of brucellosis and maintaining herd health. It is important to mention that vaccination is not mandatory and is the responsibility of the cattle producer. The official diagnosis is based on serological diagnosis by Rose Bengal (RB), iELISA, and cELISA, the slaughter of positive animals, and certification of farms as free of bovine brucellosis (AGROCALIDAD, 2020).

Concerning the mobilization of animals, bovine animals must be accompanied by the respective Internal Mobilization Sanitary Certificate (CSMI), I adequation of the current mobilization regulations (AGROCALIDAD, 2016).

Farms with certificates receive a bonus of 1 cent USD per liter of milk received by pasteurizers (AGROCALIDAD, 2016).

Any person who is aware of the presence of this disease is obliged to notify it according to Article 2 of DAJ Resolution 2013461-0201.0214 of November 21, 2013 (AGROCALIDAD, 2013).

Until the year 2022, AGROCALIDAD, performed the analysis of quality control of Brucellosis vaccines for registration through documentary review according to the Manual information for the registration of companies and products for veterinary use, which it is mentioned in ANNEX B of the instructions for the registration of biologicals, in point 2. For live or attenuated bacterial vaccines, controls on the finished veterinary biological product, and the methods used in the control must be described in documents or pharmacopoeias, international references, WOAH-CAMEVET, or current legislation of other countries. If self-developed methodologies are used, they must be validated (AGROCALIDAD, 2021).

1.14 Economic losses and the situation in Ecuador

Ecuador has an area of 281,341 km² and is divided into four regions: the Coastal region, Highland region, East (Amazon region), and Galápagos. It comprises 24 provinces (Ministerio de Asuntos Exteriores, Unión Europea y Cooperación, 2021).

In Ecuador, the livestock sector contributes to the gross national product by 8%, with an income value of 325,993,77 USD for the year 2019 (Sanchez et al., 2021). From the national cadaster of AGROCALIDAD for 2020, Ecuador counts 4,525,183 heads of cattle in the total entries obtained.

The cattle sector in Ecuador is characterized by a diversity of breeds, including both dairy and beef varieties. Among the most common dairy breeds are Holstein, Jersey, Brown Swiss**,** and Guernsey,

while beef breeds include Brahman, Hereford, Angus, and Charolais. Ecuador has an estimated population of around 4 million head of cattle, distributed across different management systems, ranging from extensive to intensive. Generally, management practices tend to be traditional, although more modern methods are gradually being adopted to improve productivity and animal health. Ecuador primarily imports specialized breeds from the United States and Brazil (Ministerio de Agricultura y Ganadería, 2023).

According to estimates made by AGROCALIDAD (2016), the discarding of brucellosis-positive animals would generate an approximate loss of USD 2217. Economic losses in the livestock farms of San Pedro de Suma in the province of Manabí, in the Coastal area, would be between USD 1922 - 3843 (Paredes, 2021).

In the study published in the year 2023, about financial losses associated with bovine brucellosis *(Brucella abortus*) in Carchi-Ecuador, losses of USD 262384 were estimated. This is due to the estimated loss of calves as a consequence of abortions and neonatal death for USD. 79170; losses due to reduced milk production of aborted and non-aborted seropositive cows were estimated at USD 158114, the loss due to the death of 4 cows as a consequence of metritis was estimated at USD 5000 and the cost of examination and treatment of aborted cows amounted to USD 20100 (Ibarra et al., 2023).

1.15 Seroprevalence and associated risk factors to bovine brucellosis

In Ecuador, several studies have been carried out to determine the prevalence at the farm and animal levels, as well as to identify risk factors associated with the disease.

The first prevalence study in Ecuador was carried out in 1979, where a serological survey was made in 15,393 cattle, using the rapid plate agglutination test, carried out by the National Animal Health Program (PNSA), obtaining a seroprevalence of 6% (95% CI: 1.3-10) for Ecuador, and in the provinces of the Northern Highlands from 1.97% to 10.62%. In this region the predominant characteristics were the entrepreneurial systems of dairy production. In the provinces of the Coast, a prevalence of 4.12% to 10.62% was found, where the predominant characteristics were extensive livestock production with low technological development, and in the provinces of the Southern Highlands, a prevalence of 1.3% to 2.6% was found, made up of peasant systems with mostly small production units (PNSA - MAG. Programa Nacional de Sanidad Animal del Ministerio de Agricultura y Ganadería, 1979).

Carbonero et al (2018), conducted a cross-sectional studying the provinces of Azuay, Chimborazo, Cotopaxi, Manabí, Pichincha, Santo Domingo, Tungurahua, and Zamora Chinchipe. The seroprevalence of the farm level was 45.1 % (174/386) and 16.7% (445/2666) at the animal level. The

study also estimated the actual prevalence of 17.0% (95% CI: 15.6-18.4%) at the animal level. The associated risk factors were age, gender, animal health and nutritional management, type of farms, and level of biosecurity on the farms (Carbonero et al., 2018)

In a recent nationwide study conducted on small (less than 20 animals) and medium (20 to 70 animals) farms, the seroprevalence of the farm was estimated as 7.9% (95% CI: 6.79-9.03) and 2.2% (95% CI: 1.82-2.67) at the animal level. The study estimated true prevalence as 12.2% (95% CI: 7.8-17.9) at the farm level and 1.6% (95% CI: 1.0-2) at the animal level, associated with risk factors such as farm size, production types (milk, beef or mixed), vaccination against brucellosis and presence of abortions on the farm (Paucar et al., 2021).

In Ecuador, bovine brucellosis is endemic, the apparent prevalence of bovine brucellosis at the herd level was 21.3% (95% CI: 16.8-26.6) and 6.2% (95% CI: 5.5-7) at the animal level (Garrido-Haro et al., 2023).

In the case of human brucellosis in the northwestern part of the country, the significant risk factors associated with seropositivity were contact with cattle, consumption of the fetus and placenta and the group of occupational risks were risk factors for the transmission of brucellosis. Among individuals, the overall seroprevalence was estimated at 1.88% (95% CI: 1.48-2.38), and the circulating strain was *Brucella abortus* biotype 4 (Ron-Román et al., 2014). A 44-year-old man from northern Ecuador, working on a cattle farm with minimal precautions, experienced sudden asthenia and hypersomnia in September 2009. On November 14, he developed pain and swelling in the right testicle, along with abdominal pain. Investigations confirmed the first case of unilateral orchitis in Ecuador caused by *Brucella abortus* biovar 1 (Ron-Román et al., 2012).

In addition, Poulsen et al. (2014) reported a true prevalence of 7.2% in the epidemiological study conducted on *Brucella* infection in two Ecuadorian provinces in northern Ecuador (Poulsen et al., 2014).

In humans, according to the National Secretariat of Public Health Surveillance of Ecuador (SIVE), there were 20 cases reported in 2021 and 12 cases in 2022, affecting the same age group of 20 to 49 years old. By 2023, 6 confirmed cases had been reported in Ecuador. (Subsecretaria Nacional de Vigilancia de la Salud Pública, 2023).

1.16 Bayesian analysis

Bayesian analysis is a powerful statistical tool used to estimate the "true prevalence" of a disease such as brucellosis, taking into account both prior information and observed data (Dendukuri y Joseph, 2001). Estimating prevalence is difficult when no test is considered gold standard for confirming the

diagnosis (Black y Craig, 2002). Bayesian analysis involves calculating a posterior probability for the parameters of a given model from a prior probability distribution and data likelihood (Gardner, 2002).

Several parameters are estimated based on pivot table (Figure 6):

- **Sensitivity**: The probability that the test will be positive if the individual is actually infected.
  Sensivity= [a/(a+c)] x 100
- **Specificity:** The probability that the test will be negative when the individual is not infected.
  Specificity= [d/(b+d)] x 100
- **Apparent prevalence:** The proportion of individuals within a sample who test positive for a diagnostic test.
- **True prevalence:** The adjusted prevalence obtained by taking into account the sensitivity and specificity of the diagnostic test (Trevethan, 2017).
- **Positive Predictive Value (PPV):** probability that a positive test corresponds to a true infected animal
  PPV= [a/(a+b)] x 100
- **Negative Predictive Value (NPV):** probability that a negative test corresponds to a true healthy animal
  PNV= [c/(c+d)] x 100

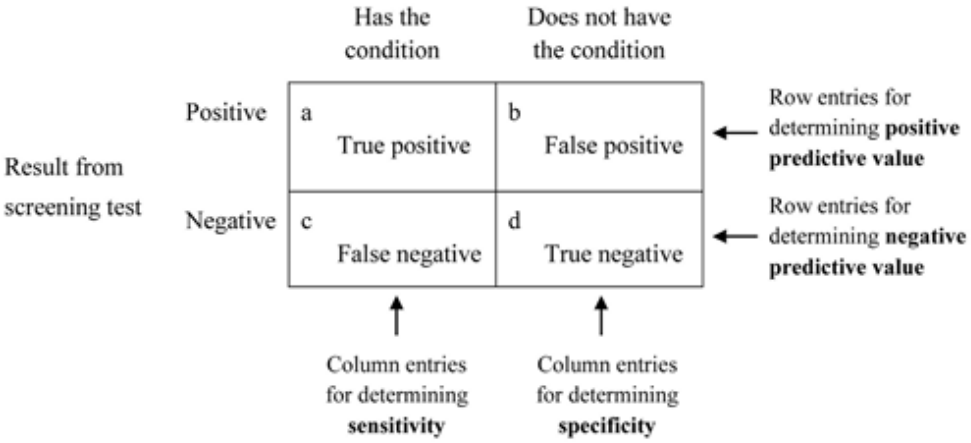

Figure 6. Diagram demonstrating the basis for deriving the sensitivity, the specificity, and positive and negative predictive values (Trevethan, 2017).

The Rogan-Gladen formula is important in studies using imperfect diagnostic tests. It corrects for the bias introduced by the less-than-optimal sensitivity and specificity of the test, providing a more accurate estimate of the true prevalence of the disease in the population (Rogan y Gladen, 1978).

In the context of disease prevalence estimation without a reference standard, Bayesian methods can help to reduce the problem of unidentifiability by incorporating prior information about the joint probability distribution of diagnostic tests, eliminating unlikely solutions based on this information (Black and Craig, 2002).

Bayesian Model Averaging (BMA) is a method that allows the incorporation of multiple models that account for the different forms of conditional dependence between diagnostic tests, thereby improving the robustness of prevalence estimates.

Chapter 2 - Objectives

General objective

The overall objective of this thesis is to contribute to the study and management of bovine brucellosis by developing effective diagnostic tools, identifying and comprehending seroprevalence and risk factors, implementing proper vaccination control practices, and conducting strain typing. By doing so, this research aims to provide valuable information to the authorities and livestock sector, emphasizing the importance of appropriate management of this disease. This thesis emphasizes the importance of managing the disease through a "One Health" approach. The overall data will serve as a guide for authorities regarding the epidemiological situation in continental Ecuador.

Specifics objectives

- In developing countries, brucellosis is a neglected zoonosis and a public health problem that generates large economic losses in livestock farming. Ecuador is considered an endemic country for bovine brucellosis. To prevent the spread of bovine brucellosis in Ecuador, it is crucial to implement multiple measures and carry out studies to know the true situation of the country. The objective of our study was to perform the seroprevalence analysis and risk/protective factors related to bovine brucellosis in continental Ecuador. The seroprevalence of bovine brucellosis in Ecuador was determined at a general level, by region (Coast, Highlands, and East), by farm, and by animal, in the 23 provinces of continental Ecuador, using the cELISA diagnostic test. Additionally, to identify potential risk/protective factors for bovine brucellosis in continental Ecuador, an analysis of the information available from the national serological survey in 2018 was carried out. The findings could serve as a guide for the authorities in managing the identified risk/protective factors, understanding the current epidemiological situation in Ecuador, improving the bovine brucellosis control program and food safety, and focusing on "One Health" **(Study 1).**
- It is important to note that vaccination against bovine brucellosis is not mandatory in Ecuador, and it falls under the responsibility of farm owners. Therefore, our second study focused on evaluating the methodology for managing brucellosis and the quality control of vaccines registered and used in Ecuador. The aim was to assess the vaccination management practices (both the quality of vaccine and the implementation of the vaccination in the field) and identify the possible factors affecting the prevention of brucellosis in the country. The study showed that two vaccines manufactured locally failed to meet the established ranges set by the WOAH. This indicates that the absence of highly effective vaccines, along with improper vaccination practices and a lack of knowledge among vaccinators and vaccine dispensers, could be some of the reasons for the failure of the control program (**Study 2).**

- As a third objective of our work, the technique of culture and isolation of *Brucella* was implemented in central official laboratory in Ecuador, in order to have the Gold standard test determined by the WOAH for the identification of *Brucella* spp. In addition, the isolates were analyzed by PCR for the identification of *Brucella* spp. and subsequently the Bruce-ladder was run, which allows the identification and differentiation of the bacterium by amplification of specific gene sequences by PCR (polymerase chain reaction). Additionally, to determine the genetic diversity of the *Brucella* strains obtained, VNTR Multiple Locus Analysis (MLVA) schemes were used, which made it possible to discriminate between the *Brucella* biovars analyzed and to show the diversity of genotypes of the species circulating in the country. It is important to mention that molecular methodologies were also implemented for the first time in the central Agency's laboratory (Study 3).
- As a fourth objective of our work is to perform a comparative evaluation of ELISA diagnostic tests for the determination of *Brucella* sp. infection (**Study 4 in progress – See appendix 1).**

The objectives of this research provided valuable information for the actors involved in decision-making, in addition to achieving progress towards the control and eradication of the disease in the cattle population.

Chapter 3 - Experimental section

Experimental section

Study 1

Seroprevalence and Risk Factors Related to Bovine Brucellosis in Continental Ecuador

Pathogens 2023;12(9):1134

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Preamble

Brucellosis, caused by *Brucella abortus*, is an important disease of cattle and a zoonosis. Several studies have been carried out in Ecuador to estimate seroprevalence and risk factors. However, it was necessary to have a national study that includes the 23 provinces of continental Ecuador to capture different setting. For this purpose, a stratified randomized study was carried out in 290 cattle herds, for a total of 3,737 cows located in the 23 provinces of continental Ecuador. For the coastal region, samples were taken from seven provinces: Esmeraldas, Manabí, Los Ríos, Guayas, El Oro, Santo Domingo de los Tsáchilas and Santa Elena. In the Sierra, samples were collected from ten provinces: Carchi, Imbabura, Pichincha, Cotopaxi, Tungurahua, Chimborazo, Bolivar, Cañar, Azuay and Loja. In the eastern region (Amazonia), samples were collected in six provinces: Sucumbíos, Napo, Pastaza, Orellana, Morona Santiago and Zamora Chinchipe.

The prevalence of the disease in cattle was 21.3% at the herd level and 6.2% at the individual level. Risk factors, such as herd size and number of calvings per animal were identified, while the region (farms located in the eastern region) and the absence of clinical signs were protective factors. These findings could guide control actions and improve food safety by promoting an integrated animal and human health approach.

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Seroprevalence and Risk Factors Related to Bovine Brucellosis in Continental Ecuador

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Abstract: Bovine brucellosis is a worldwide zoonotic contagious disease. According to World Animal Health Information System reports Ecuador has presented an increasing number of bovine brucellosis outbreaks in the continental territory over the past years (756 in 2018 versus 964 in 2021), generating economic losses for producers and causing a risk to public health. A cross-sectional study was con- ducted to investigate the seroprevalence of bovine brucellosis and associated risk or protective factors between May and June 2018. This stratified random study was implemented in 290 cattle herds located in the 23 provinces of continental Ecuador, which represents a total of 3737 cows aged 24 months or older. A competitive ELISA was used to detect *Brucella* antibodies. Simultaneously, an epidemi- ological survey was implemented to assess the brucellosis risk or protective factors. The apparent prevalence of bovine brucellosis at the herd level was 21.3% (95% CI: 16.8–26.6) and 6.2% (95% CI: 5.5–7) at the animal level. Univariate and multivariate logistic regression analyses were performed to determine the relationship between the potential factors associated with the presence of bovine brucellosis. The risk factors identified after multivariate analysis were a surface in ha per herd > 70 ha (OR = 2.73; 95% CI: 1.18–6.32) and the number of parturitions per animal (two or more with OR ≥ 1.8 and *p*-value ≤ 0.047). On the contrary, the protective factors were the region (farms located in the eastern region) and the absence of reported clinical signs.
In addition, in herds where extensive production predominates, farmers have a low level of knowledge, and the farm biosecurity level is low. These results can guide the authorities in managing the risk factors identified, understanding the current epidemiological situation in Ecuador, improving the bovine brucellosis control program and food safety, as well as increase the one-health approach.

Keywords: *Brucella* spp.; cattle; Ecuador; serological survey; risk factors; competitive ELISA (c-ELISA)

1. Introduction

Brucellosis is a zoonotic infection caused by different bacterial species of the genus *Brucella*, mainly *Brucella abortus*, *B. melitensis*, and *B. suis* [1]. It is a disease related to the evolution of agricultural society, in which animal husbandry is an integral part [2].
Brucella abortus is a facultative intracellular pathogen that causes persistent infection in animals; it has been isolated from several species of livestock. *Brucella abortus* is mostly associated with cattle; it is a natural or primary host, and *B. melitensis* is associated with sheep, goats, and humans [3,4]. Cattle become infected (i) after ingesting contaminated food, milk, forage, or water, (ii) through close contact with infected animals, (iii) contact with uterine secretions or aborted fetuses, (iv) by vertical, and (v) sexual transmission [3,5]. The disease causes substantial economic losses due to abortion in the last trimester of pregnancy, mastitis and reduced milk production in females, and orchitis and epididymitis in males. Infertility can occur in both males and females [6]. In humans, it is considered an occupational disease. Transmission to humans is mainly via close contact with contam- inated placenta, urine, feces, blood, and aborted fetuses. Workers who handle domestic ruminants, such as veterinarians, veterinary assistants, slaughterhouse workers, butchers, as well as laboratory workers, are populations at risk [7,8].

Brucellosis has been reported in Latin America since the first decade of the 20th century and remains up to now a major zoonosis despite control campaigns. Control programs are sometimes ineffective due to the lack of sustainable funding over time [9]. The annual loss caused by bovine brucellosis was estimated at approximately $600 million in Latin America [2]. A 20–30% decrease in milk production has been estimated in brucellosis- affected herds [10,11].

The total area of Ecuador is 281,341 km^2. It is divided into four regions, in which 24 provinces are distributed [12]. In Ecuador, the Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD) is the institution in charge of the national bovine brucel- losis control program. That program started activities in 2008. It relies on the vaccination of females with Buck 19 and/or RB51 strains, serological diagnosis by Rose Bengal (RB), indirect and competitive ELISAs, the slaughter of positive animals, and the certification of herds as free of bovine brucellosis [13]. Certified herds are paid a bonus of USD 0.01 per liter of milk received by pasteurizers [14].
In Ecuador, the agricultural sector contributes to the gross national product by 8% [15], and 5.7 million liters of milk are produced per day at the national level, generating employ- ment for 1,140,000 Ecuadorians [16]. According to the national cadaster of AGROCALIDAD (2020), Ecuador accounts for 4,525,183 cattle heads. Economic losses in the livestock herds of San Pedro de Suma, in the province of Manabí (Coastal area), would reach between US $1922 and 3843 per parish [17].

In Ecuador, several studies have been carried out to determine the brucellosis preva- lence at the herd and animal levels, as well as to identify the risk factors associated with the disease. The first prevalence study in Ecuador was carried out in 1979, where a serological survey was conducted on 15,393 cattle heads, using the rapid plate agglutination test, within the frameworks of the National Animal Health Program (PNSA) [18]; the animal seroprevalence reached 6% (95% CI: 1.3–10) at the national level and from 1.97% to 10.62% in the Northern Highlands provinces, where entrepreneurial systems of dairy production predominate. In the coastal provinces, from 4.12% to 10.62% of animal prevalence was observed; in that area, extensive livestock production with low technological development predominates. Finally, in the Southern Highlands provinces, where most production units are small, the animal prevalence reached from 1.3% to 2.6%.

In 2014, Poulsen et al. [19] reported a 7.2% true animal prevalence (95% CI: 6.0–8.5%) in the epidemiological study conducted on *Brucella* infection in two provinces of Northern Ecuador, using the rose Bengal card antigen test (RBCT) on 2561 dairy animals. Prevalence varied by herd size and was higher in larger commercial herds.

In 2018, Carbonero et al. [20] conducted a cross-sectional study in the provinces of Azuay, Chimborazo, Cotopaxi, Manabí, Pichincha, Santo Domingo, Tungurahua, and Zamora Chinchipe; the seroprevalence at herd level was 45.1% (174/386) and 16.7% (445/2666) at the animal level. The associated risk factors were age, gender, animal health, nutritional management, type of herds, and a poor herd biosecurity level.

In 2021, Paucar et al. [21] conducted a study in small (less than 20 animals) and medium (20 to 70 animals) herds; the herd seroprevalence was 7.9% (95% CI: 6.79–9.03) and 2.2% (95% CI: 1.82–2.67) at the animal level. Their study estimated a true prevalence of 12.2% (95% CI: 7.8–17.9) at the herd level and 1.6% (95% CI: 1.0–2) at the animal level, associated with risk factors such as herd size, production types (milk, beef, or mixed), vaccination against brucellosis and presence of

abortions in the herd. For the diagnosis, they used the Rose Bengal (RB) test and the sero-agglutination test (SAT)-EDTA.

For human brucellosis in the northwestern part of the country, the significant risk factors associated with seropositivity were contact with cattle, consumption of fetus and placenta (traditional Ecuadorian habit), and people with occupational cattle animal contacts. Among individuals, the overall seroprevalence was estimated at 1.88% (95% CI: 1.48–2.38), and the circulating strain was *Brucella abortus* biovar 4 [22]. Ron-Roman et al. (2012) [23] presented the first case of unilateral brucellosis–orchitis in a man from a rural community of Northern Ecuador who provided primary veterinary care in a cattle herd where he performed; the responsible pathogen was *Brucella abortus* biovar 1. According to the Ecuadorian Secretary of Public Health Surveillance (2020) [24], 45 human cases were recorded in 2019, and as of June 2020, two cases were registered; the most affected groups were people aged between 20 and 49 years old.

In Ecuador, the problem of brucellosis, as well as other animal diseases, is mainly related to the lack of microbiological and molecular identification of the causal agent, the lack of control of the antigens used for serological diagnosis, the lack of vaccine quality control, as well as the lack of a compensation system when positive animals are slaughtered [25]. An underlying problem is also the lack of support from authorities and decision-makers, which is reflected by the lack of financial resources for the national disease control program, making studies isolated, repetitive, and without high scientific value and contribution to the program.

A frequent hypothesis circulating among cattle breeders is that due to the existence of cross-reactions with other bacteria and the antibodies generated by the vaccine, many false-positive animals are slaughtered (Ron-Roman J. 2023, personal communication). The situation of bovine brucellosis in continental Ecuador is not completely updated and/or is based on indirect diagnostic tests, which does not allow the implementation of an efficient national control program based on scientific information.

The objectives of the present study are (i) the determination of bovine brucellosis prevalence in continental Ecuador and (ii) the determination of the putative risk/protective factors associated with the disease. The results of this study may be useful in developing and implementing control measures aimed at raising farmers' awareness, making rec- ommendations to strengthen the national bovine brucellosis control program, regulating agricultural management practices, and, ultimately, reducing the prevalence of livestock brucellosis in Ecuador.

2. Materials and Methods

2.1 Study Area

AGROCALIDAD, with the support of the Pan American Center for Foot and Mouth Disease and Veterinary Public Health of the Pan American Health Organization (PANAFTOSA/SVP-PAHO/WHO) in the framework of technical cooperation with Ecuador, conducted between May and June 2018, a serological study on bovine brucellosis in the 23 provinces of continental Ecuador. In the coastal region, samples were collected from seven provinces: Esmeraldas; Manabí; Los Ríos; Guayas; El Oro; Santo Domingo de los Tsáchilas, and Santa Elena. In the Highlands, samples were collected from ten provinces: Carchi; Imbabura; Pichincha; Cotopaxi; Tungurahua; Chimborazo; Bolivar; Cañar; Azuay; and Loja. In the east region, samples were collected from six provinces: Sucumbíos; Napo; Pastaza; Orellana; Morona Santiago; and Zamora Chinchipe.

2.2 Sample Size Calculation

The estimation of the sample size (herds and animals) was based on the number and distribution of cattle in 2017 (4,310,731 cattle heads older than 24 months and distributed in 277,076 herds) and the characteristics of the competitive ELISA (c-ELISA) test used (0.95 sensitivity [Se] and specificity [Sp] were considered). At the herd level, a confidence level of 0.95 was used, as well as an expected design prevalence of 0.15, with 0.05 precision. Indeed, 287 herds were sampled but rounded to 290 units. Herds were sampled based on a stratified random design so that the sample distribution followed the same structure as the animal population, according to the herd size (Table 1).

Table 1. Frequency distribution of herds categorized per size and distribution of cattle per herd category.

Herd Category per N Animals	N Herds per Category	N Herds Tested	Proportion of Herds (%)
≤11	180,162	60	20.7
12–24	52,848	65	22.4
25–44	25,794	58	20.0
45–98	13,893	51	17.6
>98	4379	56	19.3
Total	277,076	290	100.0

N = number.

The number of animals to be sampled in each herd category was estimated considering an expected intra-herd prevalence of 10%, with a 0.05 precision and a 0.95 confidence level (Table 2).

Table 2. Number of females aged 24 months or older to be sampled per category.

Herd Categories per N Animals	N Cattle Heads per Herd Category	N Animals Sampled per Herd Category	Proportion of Animals per Herd Category
≤11	881,675	188	5.0
12–24	885,542	389	10.4
25–44	830,937	620	16.6
45–98	855,267	882	23.6
>98	85,731	1658	44.4
Total	3,539,152	3737	100.0

To minimize the occurrence of false positive c-ELISA results due to the brucellosis vaccination in Ecuador, only 24-month-old female bovines were sampled (N = 3737).

2.3 Estimation of Herd Prevalence and Animal Prevalence

The brucellosis-apparent prevalence was calculated at herd and animal levels for each region. A herd was considered to be positive when there was at least one animal with a positive c-ELISA diagnostic test result. Prevalence was reported as the proportion of positive results (herds or animals) out of the total sample (herds or animals tested). The calculation was performed using the "epiR" and "RSurveillance" packages of the R software version 3.5.1 and 4.2.2 [26].

2.4 Epidemiological Survey

To determine the risk factors, the farmers filled in an epidemiological survey. This survey included 52 questions related to the herd and animal management. The survey collected information on herd identification and location, herd general data, general animal and pasture management, sanitary aspects, reproduction, pathologies, diagnostic tests for brucellosis, and samples collected (Table A1). The information collected was divided into four main categories: (i) herd identification and location (11 variables); (ii) general herd data (10 variables); (iii) general animal and pasture management (7 variables); and (iv) sanitary aspects (24 variables).

2.5 Sampling Method

Blood samples were collected in tubes without anticoagulant through the puncture of the coccygeal vein of each animal. The samples were transported to laboratories of the AGROCALIDAD network, maintaining the cold chain (4 to 8 °C). To extract the blood serum, samples were centrifuged for 5 min at 5000 rpm. The blood serum was stored at 4 to 8 °C until analysis at the AGROCALIDAD serology laboratories located in Tumbaco, province of Pichincha. The blood samples were collected with the prior authorization of the herd owner and did not generate any costs for them.

2.6 Diagnostic Tests Performed and Positivity Criteria

The c-ELISA test was the SVANOVIR® *Brucella*-Ab kit for the detection of antibodies, used according to the manufacturer's specifications. Optical density (OD) values were read at 450 nm

(wavelength) on the ELISA Bio Tek micro-reader (Santa Clara, CA, USA). Positive and negative control sera from the kit were used to validate the ELISA plate result. The limits for validation criteria of the test were OD Conjugate control (Cc) 0.75–2.0, percentage of inhibition (PI) positive control 80–100, PI weak positive control 30–70, and PI negative control <30. The PI for the interpretation was considered negative if <30% and positive if
≥30%. The test Se and Sp were estimated between 0.95 and 1 [27]. PI of each sample was calculated as follows:

2.7 Statistical Analysis

$$PI = 100 - \frac{(OD\ samples\ or\ control \times 100)}{OD\ conjugate\ control} \quad (1)$$

For the analysis of the risk factors, herds were classified according to the surface area in hectares (ha), i.e., four categories: 0–5 ha; 6–30 ha; 31–70 ha; and >70 ha, and according to the number of animals in the herd, i.e., three categories: small (1 to 20 cattle heads); medium (21 to 70 cattle heads); and large (>70 cattle heads).

The cross-sectional study allowed to point out potential risk or protective factors in the presence of positive results for bovine brucellosis by the c-ELISA test [28–30]. Variables considered in this study were divided into two parts, i.e., 27 variables at the herd level and 4 variables at the animal level.

First, a univariate analysis was performed, and OR with 95% confidence intervals (95% CI) was calculated for each variable analyzed (exposure factor). Then, with the variables considered as risk factors (p-value < 0.05), a multivariate logistic regression analysis was performed. The logistic regression model was performed in Stata SE 14.1® (StataCorp LP, College Station, TX, USA). The model was progressively simplified by removing the least significant variable with a p-value > 0.05. The model was consid- ered complete when it could not be further simplified without having a significant dif- ference between the most complex and the simplest model (likelihood ratio test with a p-value < 0.05) [31]. The goodness of fit was assessed using the Hosmer–Lemeshow goodness-of-fit test [32]. The limit of statistical significance of the tests was defined at p-value ≤ 0.05 [33].

In addition, the distribution map of sampled herds with at least one seropositive animal was performed with the QGIS software, version 3.28.1 [34].

3. Results

3.1 Apparent Herd and Animal Prevalence

The overall brucellosis-apparent prevalence at the herd level was above 20%. When considering the regions, a herd prevalence above 20% was reached in the Highlands and in the coastal region, while it was below 10% in the eastern region (Table 3 and Figure 1). The overall apparent prevalence estimated at the animal level was above 6%. The highest animal apparent prevalence, i.e., above 10%, was observed in the Highlands ((Table 3).

Table 3. Distribution of apparent prevalence per region at herd and animal levels.

Region	Herd Prevalence (95% CI)	Animal Prevalence (95% CI)
Coast Region	22.3 (15.8–30.5)	4.4 (3.6–5.5)
Highlands Region	23.7 (16.7–32.5)	10.2 (8.6–11.9)
East Region	8.8 (2.3–24.8)	1.3 (0.5–3.4)
Ecuador	21.3 (16.8–26.6)	6.2 (5.5–7)

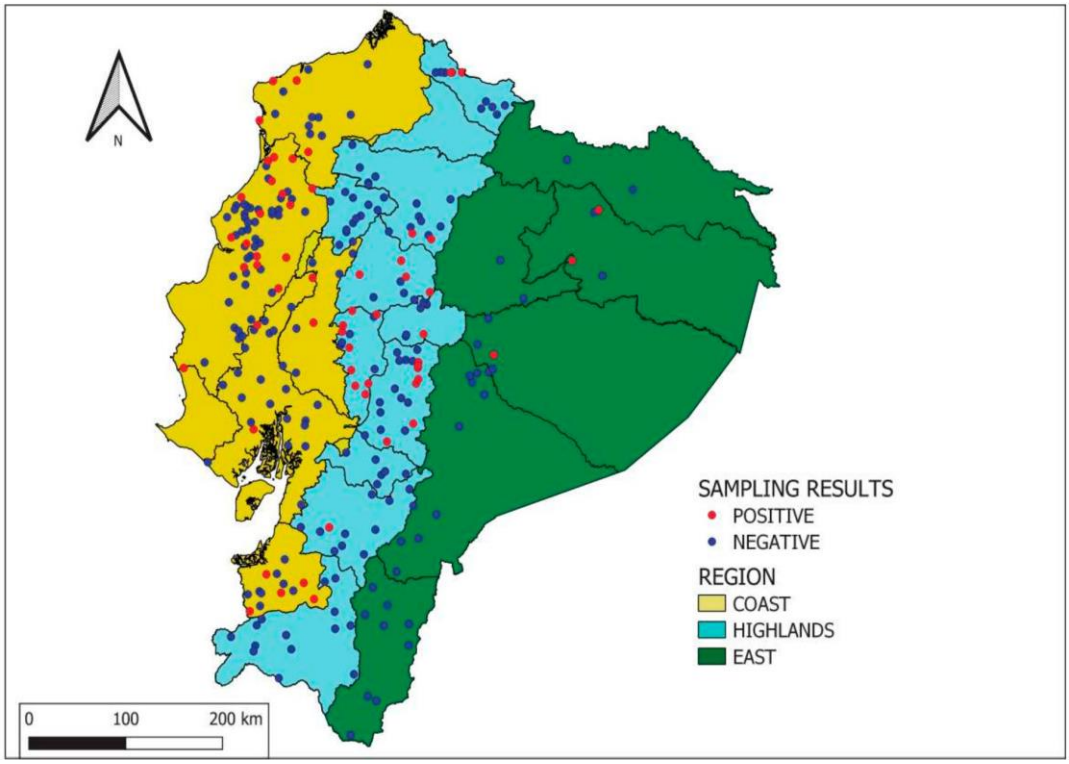

Figure 1. Distribution of sampled herds per region of Ecuador. Legend: positive herd if at least one seropositive animal.

3.2 Descriptive Analysis

In section I of the survey, herd identification and location, 52.4% of cattle holders indicated that they had no knowledge of animal brucellosis. In section II, general herd data, 82.4% of cattle holders implemented an extensive system, and the presence of other animals was reported in 55.5% of the herds; 19.4% of farmers reported the consumption of raw milk. In section III, general animal and pasture management, fencing was reported in 69% of the herds and containment corridors in 41.2% of the herds. Footbaths were present in 3.4% of the herds. The entry of visitors was controlled in 20.4% of herds. Paddocks were shared in 8.8% of herds. In section IV, sanitary variables, vaccination of females against brucellosis as a disease prevention measure was not performed in 89.0% of cattle herds; reproductive problems were reported in 18% of herds, the predominant signs being abortions or retained placenta.

In Ecuador, two types of vaccines are available and used for the prevention of brucellosis in cattle: the nationally-produced Buck 19 strain; and the imported RB51 biological strain. It is worth mentioning that 12.4% of cattle holders mentioned vaccination, and 5.43% of herds were vaccinated with strain 19 and 6.2% with RB51.

Regarding the production characteristics, 87.9% of cattle holders reported a predominant production of meat or milk, while 12.1% of them mentioned a mixed type (meat and milk) (Figure 2). The widest reproduction mode is natural mating; it is practiced in 84.8% of herds. Extensive system is the predominant mode of farming, while intensive farming is performed in only 1.4% of herds. The animals are tied in 16.2% of herds. The presence of other animal species was mentioned for 55.5% of herds. Animal trade is practiced by 80.7% of cattle holders. However, 82.7% of them reported breeding their animals. Diagnostic tests for brucellosis are rarely performed by the farmers. Details on the variables studied by the epidemiological survey are presented in Table **4**

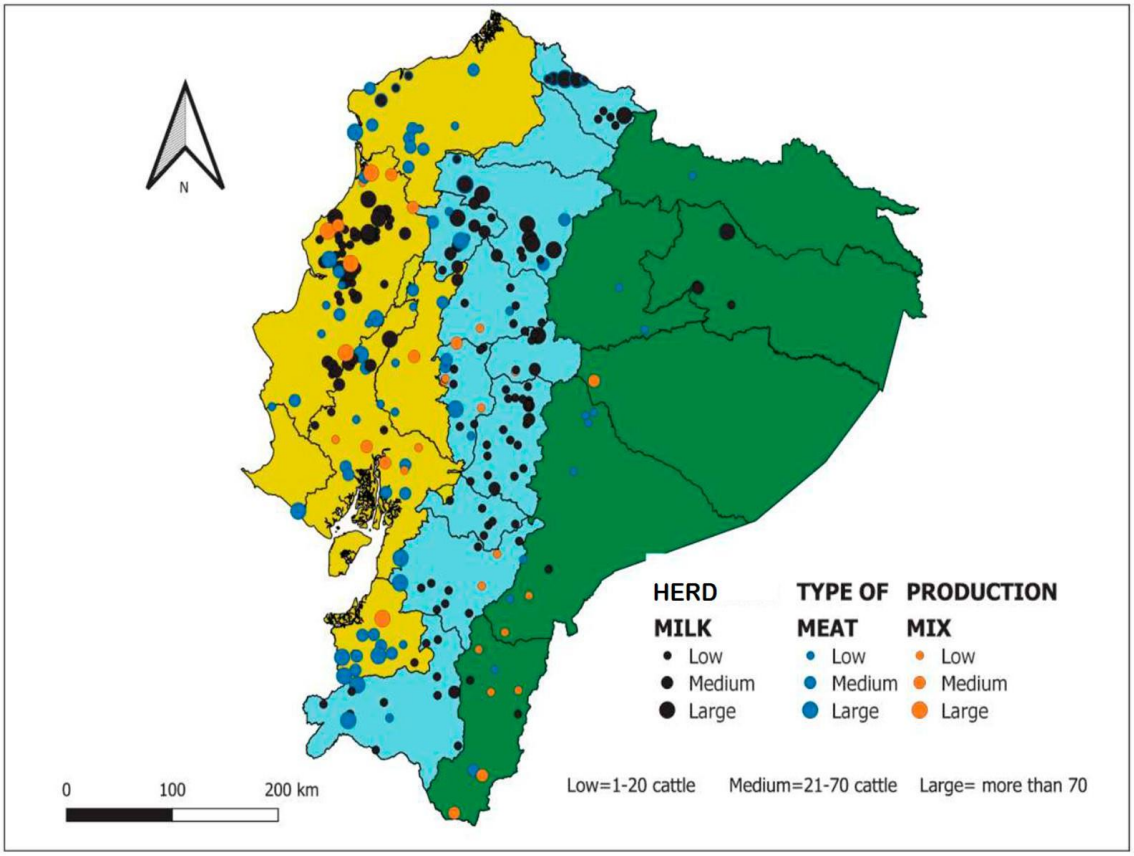

Figure 2. Distribution of herds according to their size and type of production.

The coastal and Highlands regions were represented by milk cattle, with 46.27% and 83.6%, respectively. In the eastern region, 50% of herds sampled were beef holdings. More than half of herds account for 1–20 cattle heads, and most areas cover 0–5 ha.

Table 4. Risk and protective factors at the herd level—univariate logistic regression analysis.

Variables	Variable Type	Modalities	N Herds	N Positive Herds	N Negative Herds	Proportion of Positive Herds (%)	OR (95% CI)	*p*-Value
I. Identification and location of farm								
Region	Categorial	Coastal region	134	30	104	22.3	Ref.	-
		Eastern region	34	3	31	8.8	0.34 (0.10–1.17)	0.09
		Highlands	122	29	93	23.7	1.08 (0.60–1.93)	0.79
Cattle holder's knowledge on bovine brucellosis	Binary	No	152	25	127	16.44	Ref.	-
		Yes	138	37	101	26.81	1.86 (1.05–3.29)	0.03

II. General data on the farm								
Surface area (ha) a	Categorial	First quartile (0–5)	74	12	62	16.21	Ref.	-
		Second quartile (6–30)	77	12	65	15.58	0.95 (0.40–2.28)	0.92
		Third quartile (31–70)	67	15	52	22.3	1.49 (0.64–3.47)	0.35
		Fourth quartile (>70)	70	22	48	45.8	2.37 (1.08–5.39)	0.03
Herd size (N cattle heads)	Categorial	Low (1–20)	167	28	139	16.76	Ref.	-
		Medium (21–70)	80	22	58	25	0.52 (0.24–1.14)	0.1
		Large (>70)	43	12	31	27.9	0.98 (0.43–2.24)	0.96
Type of farming (production) N	Categorial	Milk	173	40	133	23.12	Ref.	-
		Meat	82	17	65	20.7	0.87 (0.46–1.65)	0.67
		Mixed	35	5	30	14.28	0.55 (0.20–1.52)	0.25
Farming system	Categorial	Extensive	239	56	183	23.43	Ref.	-
		Intensive	4	1	3	25	1.09 (0.11–10.68)	0.94
		Tied to the stake	47	5	42	10.6	0.39 (0.15–1.03)	0.06
Breeding system	Categorial	Artificial insemination	26	8	18	30,76	Ref.	-
		Mixed	18	6	12	33.33	1.13 (0.31–4.07)	0.86
		Natural mating	246	48	198	19.5	0.55 (0.22–1.33)	0.18
Level of technification b	Categorial	High	70	17	53	24.28	Ref.	-
		Low	206	43	163	20.87	0.82 (0.43–1.56)	0.55
		Medium	12	2	10	16.6	0.62 (0.12–3.13)	0.57
Other animal species in the herd	Binary	No	129	30	99	23.25	Ref.	-
		Yes	161	32	129	19.8	0.82 (0.47–1.44)	0.49
Number of milking per day c	Categorial	One fold	197	44	153	22.3	Ref.	-
		Two fold	63	14	49	22.2	0.99 (0.50–1.97)	0.99
	Binary	No	233	52	181	22.31	Ref.	-

Consumption of raw milk by the farmer d		Yes	56	20	46	35.71	0.76 (0.36–1.60)	0.47
III. General management of animals and pastures								
Presence of fences in the farm	Binary	No	90	14	76	15.55	Ref.	-
		Yes	200	48	152	24	1.71 (0.88–3.30)	0.11
Presence of footbath	Binary	No	280	62	218	22.14	Ref.	-
		Yes	10	0	10	0	0.17 (0.01–2.88)	0.22
Control of visitors at the entrance	Binary	No	231	50	181	21.64	Ref.	-
		Yes	59	12	47	20.33	0.92 (0.46–1.87)	0.83
Presence of a containment corridor in the farm e	Binary	No	170	28	142	16.47	Ref.	-
		Yes	119	34	85	28.57	2.03 (1.15–3.58)	0.02
Shared pasture f	Binary	No	259	55	204	21.23	Ref.	-
		Yes	25	6	19	24	1.17 (0.45–3.07)	0.75
Mode of animal watering	Categorial	Slope	4	0	4	0	Ref.	-
		Rain	136	30	106	22.05	2.58 (0.13–49.21)	0.53
		River and/or ditch	99	24	75	24.24	2.92 (0.15–56.19)	0.48
		Well and/or drinking water	51	8	43	15.68	1.76 (0.09–35.78)	0.71
IV. Health aspects								
Calving place g	Categorial	Corral	17	4	13	23.52	Ref.	-
		Farrowing pen	11	4	7	36.36	1.86 (0.35–9.79)	0.47
		Paddock	260	53	207	20.38	0.83 (0.26–2.66)	0.76
Disinfection of the calving place	Binary	No	254	55	199	21.65	Ref.	-
		Yes	36	7	29	19.44	0.87 (0.36–2.10)	0.76
Reproductive disorders	Binary	No	238	49	189	25.92	Ref.	-
		Yes	52	13	39	25	1.29 (0.64–2.59)	0.7
Number of clinical sign(s)	Categorial	No	230	49	181	21.3	Ref.	-
		One	35	7	28	20	0.92 (0.38–2.24)	0.86

		Two	9	3	6	33.3	1.85 (0.45–7.65)	0.4
		Three	16	3	13	18.75	0.85 (0.23–3.11)	0.81
Past brucellosis testing in the herd	Binary	No	264	55	209	20.83	Ref.	-
		Yes	26	7	19	26.92	1.40 (0.56–3.50)	0.47
Participate to the trade of animals	Binary	No	56	10	46	17.85	Ref.	-
		Yes	234	52	182	22.22	1.31 (0.62–2.78)	0.48
Origin of purchase h	Categorial	Born in the herd	238	50	188	21	Ref.	-
		Trader	11	2	9	18.18	0.84 (0.17–3.99)	0.82
		Market or exhibition plan	24	7	17	29.16	1.55 (0.61–3.94)	0.36
		Neighbouring area	15	3	12	20	0.94 (0.26–3.46)	0.93
Testing at purchase i	Binary	No	259	56	203	21.6	Ref.	-
		Yes	12	3	9	25	1.21 (0.32–4.61)	0.78
Control of the herd by a veterinarian	Binary	No	181	42	139	23.2	Ref.	-
		Yes	109	20	89	18.34	0.74 (0.41–1.35)	0.33
Use of brucellosis vaccine	Binary	No	258	56	202	21.7	Ref.	-
		Yes	32	6	26	18.75	0.83 (0.33–2.12)	0.7

Legend: OR = odds ratio; N = number; CI = confidence interval; Ref = reference. [a] There is no information on the surface in ha of two herds, while there is information on the number of animals. [b] There is no information available from the two herds on the level of technification. [c] There is no milking information available for 30 herds. [d] There is no information available from 1 herd for consumption of raw milk by the farmer. [e] There is no information available on a 1 herd to have a corridor in the herd. [f] There is no information available from the six herd shared pastures. [g] There is no information available from the two herds for places of calving on the herd. [h]There is no information on the origin of the purchase of the two herds. [i] There is no information on 19 herds for brucellosis testing at the purchase

3.3 Risk and Protective Variables at Herd Level

A significant correlation was found between the results of brucellosis at the herd level and for farms with a farm surface area >70 ha; therefore, the larger the herd is, the greater the risk of brucellosis, and the smaller the area, the less risk of contracting the disease. The lack of knowledge on brucellosis is a risk factor since farmers unaware of the disease are at higher risk of having brucellosis in their herd compared to those who know about brucellosis. The presence of a containment corridor in the herd appeared to be a risk factor in comparison with the herds that do not have such a facility (Table 4).

The multivariate logistic regression analysis (Table 5) confirmed the significant corre- lation between the results of brucellosis at the herd level and a >70 ha-farm surface area. Large herds are at higher risk of seropositivity

compared to smaller ones. In addition, compared to the coastal region as a reference, the apparent prevalence was significantly lower in the eastern region.

Table 5. Risk and protective factors associated with the seroprevalence of bovine brucellosis at herd level—multivariate logistic regression analysis.

Variable	Variable Type	Modality	OR (95% CI)	*p*-Value
Region	Categorial	Coastal region	Ref.	-
		Eastern region	0.22 (0.05–1.00)	0.05
		Highland region	1.37 (0.19–0.43)	0.33
Surface area	Categorial	First quartile (0–5 Ha)	Ref.	-
		Second quartile (6–30 Ha)	1.27 (0.51–3.17)	0.61
		Third quartile (31–70 Ha)	2.38 (0.94–6.02)	0.07
		Fourth quartile (>70 Ha)	2.73 (1.18–6.32)	0.02

Legend: OR = odds ratio; CI = confidence interval; Ref = reference

3.4 Risk and Protective Variables at Animal Level

The univariate model included four variables: vaccine used; birth at the farm; clinical signs; and number of births per animal (Table 6). Registering between two and four births per animal was a risk factor for seropositivity (p-value < 0.05). Knowing the clinical status and the origin of the birth of animals were protective factors (p-value < 0.05). The multi- variate logistic regression analysis at the animal level (Table 7) confirmed the significant correlation between the following factors: births at the farm; the presence of clinical signs; and the number of births per animal. It is important to mention that the animal age was not included as a variable due to the collinearity between the variables "number of births" and the "animal age". Indeed, animals that were born on the farm or not on the farm but for which the origin was known (e.g., born on another known farm) were less likely to contract brucellosis than animals whose origin was unknown or undetermined. The absence of brucellosis clinical signs is a protective factor as well, while animals having calved at least twice were more at risk for brucellosis seropositivity.

Table 6. Risk and protective factors associated with bovine brucellosis seroprevalence at animal level—univariate logistic regression analysis.

Variable	Modality	N Animals	N Positive Animals	N Negative Animals	Proportion of Positive Animals (%)	OR (95% CI)	*p*-Value
Vaccine used	Both	18	0	18	0	Reference	-
	strain 19	390	14	376	3.58	1.43 (0.08–24.83)	0.81
	No	2900	205	2695	7.07	2.82 (0.17–46.00)	0.47
	RB51	429	16	413	3.72	1.48 (0.09–25.58)	0.79
Born on the farm	Not determined	50	8	42	16	Reference	-

	No	333	25	308	7.5	0.43 (0.18–1.01)	0.05
	Yes	3354	202	3152	6.02	0.34 (0.16–0.73)	0.006
Presence of clinical signs compatible with brucellosis	Not determined	73	15	58	20.54	Reference	-
	No	3638	219	3419	6.01	0.25 (0.14–0.44)	<0.001
	Yes	26	1	25	3.8	0.16 (0.019–1.24)	0.08
Number of calving per animal	0	425	16	409	3.76	Reference	-
	1	971	47	924	4.84	1.30 (0.73–2.32)	0.37
	2	818	58	760	7.09	1.95 (1.11–3.44)	0.02
	3	668	51	617	7.63	2.11 (1.19–3.76)	0.01
	4	400	31	369	8.4	2.15 (1.16–3.99)	0.02
	≥5	441	31	410	7.02	1.93 (1.04–3.59)	0.037
	Not determined	14	1	13	7.14	1.97 (0.24–15.97)	0.53

Legend: OR = odds ratio; CI = confidence interval.

Table 7. Risk and protective factors associated with bovine brucellosis seroprevalence at animal level—multivariate logistic regression analysis.

Variable	Modality	OR (95% CI)	*p*-Value
Vaccine used	Both	Reference	-
	Strain 19	1.45 (0.08–25.27)	0.8
	No	2.72 (0.16–45.51)	0.49
	RB51	1.30 (0.074–22.62)	0.86
Born on the farm	Not determined	Reference	-
	No	0.38 (0.16–0.91)	0.03
	Yes	0.31 (0.14–0.66)	0.002
Presence of clinical signs compatible with brucellosis	Not determined	Reference	-
	No	0.22 (0.12–0.39)	< 0.001
	Yes	0.18 (0.032–1.06)	0.058
	0	Reference	-

Number of calving per animal	1	1.19 (0.67–2.13)	0.55
	2	1.81 (1.03–3.19)	0.039
	3	2.03 (1.14–3.60)	0.016
	4	1.99 (1.07–3.69)	0.029
	≥5	1.87 (1.01–3.46)	0.047
	Not determined	2.56 (0.44–14.85)	0.3

Legend: OR = odds ratio; CI = confidence interval.

4. Discussion

The agricultural sector has an important impact on Ecuador's development, as its role is not only limited to sustaining food sovereignty but also to contributing significantly to the economy through taxes [35]. Ecuador produces 5.7 million liters of milk daily on a national scale, generating employment for 1,140,000 Ecuadorians [16].

Ecuador accounts for 4.6 million cattle heads (which represents 68% of animals in the country), distributed in three regions: 41.24% in the coastal region; 49.11% in the Highlands; and 9.65% in the eastern region. There is a total of 280,709 livestock producers nationwide [16,35]. The total income resulting from cattle farming and breeding reached USD 32,599,377 in 2019 [36].

The Ecuadorian livestock sector faces great economic losses due to different factors, among which are infectious and parasitic diseases. The economic cost of discarding each animal due to brucellosis was estimated at USD 2,217 per capita [37].

The Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD) has implemented a national program for the control of bovine brucellosis in the country; it is based on the following pillars: (1) vaccination of susceptible animals; (2) serological diagnosis of animals; and (3) sanitary slaughter of seropositive animals [13]. It is well known that a control program must periodically evaluate its results, with the aim to modify its actions and activities in order to achieve its objectives in an optimal term and with the least investment of resources.

One of the main factors responsible for the presence of bovine brucellosis in Ecuador is the movement of animals with unknown brucellosis status throughout the national territory or that do not comply with established sanitary requirements. Although there is currently control of animal movements within the framework of the foot-and-mouth disease eradication project [38], through the issuance and control of production and mo- bility certificates, to date, no operational strategy has been established for the control of brucellosis-infected animals, in violation of the article 42 of the Agricultural Health Law which focuses on "control of animal movements" [39]. In addition to the lack of control of dairy herds through the use of tests such as the ring-in-milk test (MRT) and the lack of availability of the antigen, there is no operational strategy for the direct control of infected animals in the herds [22].

In such context, this study was developed to determine the prevalence of bovine brucellosis throughout continental Ecuador (23/24 provinces), as well as to identify the possible risk factors associated with this disease. The information generated will be an input of scientific value for decision-makers and health authorities of Ecuador with respect to adjusting the national brucellosis control program.

The results found in the present study show that brucellosis is present in a great proportion of cattle herds at the country level, but especially in the coastal region and in the Highlands. In the eastern region, the prevalence appears to be lower. This updated information is in line with previous studies that highlighted a lower prevalence in the east- ern region [17,18,21,25,40–42]. In addition, the results of herd prevalence in the Highlands and in the coastal region were not significantly different. Such observations may imply the existence of similar risk factors that favor the persistence of the disease in both regions, except climatic factors that differ in both regions, which was demonstrated in the studies of Paucar et al. (2021), Carbonero et al. (2018), and McDermott and Arimi (2002) [18–21,43].

The main difference observed with the results of Paucar et al. (2021) [21] with respect to the herd prevalence for the coastal and eastern regions could be related to the use of different diagnostic tests and possible cross-reactions with other causal agents. Indeed, such theory has already been demonstrated for Gram-negative bacteria closely related to *Brucella*, such as *Yersinia enterocolitica* O:9, *Escherichia coli* 0157:H7, *Xanthomonas maltophilia*, and *Salmonella urbana* [44]. In addition, antibodies are generated by vaccines against bovine brucellosis [45]. The Se and Sp used in the present study differ from the ones used by Paucar et al. (2021) [21]: the Rose Bengal test has 87% Se and 97.8% Sp, and the SAT test has 81.5% Se and 98.9% Sp [46]. In the present study, a c-ELISA was used, and its Sp ranges were between 99.5% and 99.6% [27]. That could minimize cross-reactions with the vaccine

antibodies since it uses the M-84 monoclonal antibody specific for the polysaccharide O [47]. It is important to remember that in the present study, a herd was considered to be positive when there was at least one positive animal.

The results are also in line with reported information on the prevalence of brucellosis in neighboring countries such as Colombia, with a prevalence of 27.5% and 6.6% at the herd and animal levels, respectively [42], as well as Peru, where the prevalence results were found to be higher [1].

The univariate and multivariate analysis applied to the c-ELISA test results, in the light of the epidemiological information collected, allowed the identification of the eastern region as a herd protective factor. This observation could be explained by the climatic conditions of animal management and the apparently few movements of animals in this region [48]. Furthermore, as shown in Figure 1, the concentration of herds is lower in the eastern region, and they are mainly medium and small herds. Other studies mentioned that brucellosis prevalence was influenced by the geographic region in a country [49,50].

Another potential risk factor was a farm extending over a >70 ha-surface area. Studies by Camus (1980) and Sanogo et al. (2012) showed that the incidence of brucellosis varies proportionally with the herd size that is correlated with the farm surface area [51,52]. Ac- cording to Awah-Ndukum and collaborators (2018), large herds face greater difficulties in the management of individual animals; there is often poor sanitary control generally associated with poor herd management [53]. The findings of the present investigation, as well as those presented by McDermott and Arimi (2002) [43], showed that brucellosis prevalence decreases when herd size decreases in pastoral production systems. The re- placement of animals and the performance of the so-called "quarantine" are important aspects to consider in the dynamics of the disease in a herd and area; small herds generally use their own replacement animals and limit the introduction of new and potentially in- fected cattle [54]. Our findings are in line with that statement, as small herds have fewer seropositive animals [43,52].

At the herd level, the presence of a containment corridor was identified as a risk factor in the univariate analysis. In Ecuador, it is well known that large herds have a containment corridor, which facilitates the handling of animals, but, unfortunately, allows the contact of healthy animals with vaginal and fecal secretions of sick animals. It is important to keep in mind that brucellosis is a highly contagious disease [55], especially when considering the multiple routes and intensity of bacterial shedding. Crowded conditions during animal handling make it possible for the pathogen to spread more easily, taking into account that during an abortion, approximately 10^{13} bacteria are shed into the environment. It has been suggested that bacterial shedding at calving could infect between 60,000 and 600,000 females [56].

The univariate analysis showed a relationship between the lack of knowledge of animal brucellosis and the seroprevalence in herds. Although AGROCALIDAD implemented a national control program and brucellosis is one of the major neglected zoonoses worldwide, the Ministry of Public Health minimizes its prevalence and importance in the public health and economic sectors. Therefore, it is necessary to raise awareness of the general public and to provide training for the professionals of the livestock sector on the risks incurred by direct contact with livestock and by the consumption of fetuses and placentas (a traditional habit in Ecuador); the prevention of disease transmission is also important to avoid the spread of the infection [22]. The factors described above increase the risks for an animal to come in contact with the pathogen, especially after abortion storms, which contaminate the environment (pastures and facilities) [21,57,58]. The lack of knowledge on brucellosis has been described in several studies, so it is recommended to educate farmers urgently on the epidemiology, risk factors, and mitigation of the disease [59].

The birth of an animal on the farm itself was identified as a protective factor in the multivariate analysis, as well as the known origin of animals not born on the farm, as opposed to those whose origin is unknown. This can be explained by the fact that the animals born in the herd have an adequate epidemiological follow-up, as well as those that are formally acquired with a known origin. Additionally, this can be explained by the existence of a black market for the sale of animals that do not have the necessary authorization. The introduction of a brucellosis-infected animal in a brucellosis-free herd is a high-risk factor for the spread of the disease [60], in addition to the lack of adequate monitoring for animal movements [48].

The next variable included in the multivariate model was the number of births per animal: it was only included due to the collinearity with ages. Authors have reported the association between the cattle age and *Brucella* infection [53,61]. Age is known to be one of the factors influencing brucellosis seropositivity [62]. Indeed, the older the animal, the greater the probability of previous contact with infected animals. This is due to the lack of adequate follow-up for the elimination of positive animals in the herds [61]. Our study indicates that from the second calving onwards, there is a risk of increased exposure to *Brucella* spp., which, in some cases, may be due to a reduced immune system [63]. In Ecuador, nutritional supplementation of cattle is unusual; it is, thus, common to find cattle with advanced malnutrition; animals with poor nutritional conditions may be more susceptible to infection and a source of disease spread [20]. Other causes could be the low quality of the vaccine, a poor vaccination process, incorrect ages, wrong administration procedures, and vaccinating animals with inappropriate doses [62]. In Ecuador, two types of vaccines are available and used in cattle for the prevention of brucellosis: the nationally produced Buck 19 strain; and the imported biological RB51. Vaccinated animals had a lower risk of seropositivity than unvaccinated animals, although close to 90%

of Ecuadorian farmers do not vaccinate. It is important to mention that within the framework of the national brucellosis program, vaccination is not mandatory; it is the responsibility of cattle holders to implement it [14]. That explains the poor vaccination in the herds involved in this study. Governmental agencies should take into account that incorrect vaccination and inadequate handling directly affect milk quality, as highlighted by Pacheco and collaborators (2012) [64], who determined the excretion of the B19 vaccine strain during a reproductive cycle in dairy cows [62].

One should consider that in farms where cattle holders do not perform diagnosis and elimination of brucellosis-positive animals, the risk of infection increases progressively in the herd as animals get older (permanence of animals in the herd); it was demonstrated by Ramirez et al. (2020) [42] in a study conducted in the Ecuadorian province of Manabí.

The multivariate analysis highlighted the absence of clinical signs compatible with brucellosis as a protective factor. It is important to keep in mind that, in cattle, no pathog- nomonic sign of the disease has been reported; the signs described vary a lot, and the disease is usually asymptomatic in young animals and non-pregnant females [1]. Given the high reproductive problem of brucellosis reported in Ecuadorian cattle [25], a study is needed to determine the causal agent of abortions because, as has been described, the high prevalence of brucellosis is related to a high incidence of abortions [50,61,65].

As for the animal management system, dairy production would favor the multipli- cation and spread of the bacteria within the herd [66]. In beef cattle, although animal management practices would decrease the transmission of the disease, animal holders are not very inclined to implement biosecurity measures due to the lack of perceived real benefits, which, in turn, is the case for free-ranging dairy cattle [13,67].

In the present study, regarding the farming system, herds were characterized as follows: 59.7% were dairy herds; 28.3% were meat herds; and 12.1% were a mix of both. Extensive farming was practiced by 82.4% of cattle holders. A total of 91.2% of herds shared the paddock with other animal types. It is important to point out that the predominant type of reproductive management was through natural mating, which allows the spread of brucellosis by infected males, as highlighted in previous studies [68,69].

Common other factors observed in most herds were the lack of technification, poor veterinary control, the lack of brucellosis diagnosis, and consumption of raw milk by the farmers. All these factors have also been reported in similar studies [11,21,22,25,54,57,58].

5. Conclusions

In Ecuador, the herd prevalence of brucellosis is high, especially in the Highlands and coastal regions (no significant difference between these two regions). That observation suggests the existence of similar risk factors, with the exception of climate, that favor the persistence of the disease. Considering the high prevalence of brucellosis in dairy herds combined with the consumption of raw milk, it is necessary to make cattle farmers, as well as the public, aware of the brucellosis transmission routes and prophylaxis measures, especially in the rural sector. Due to overcrowding and animal handling conditions, it is possible for the disease to spread more easily among animals. In Ecuador, herd vaccination coverage is low, so there is a need to raise awareness among farmers about the benefits of the proper use of vaccines in livestock, especially in high-prevalence geographic areas, to decrease disease prevalence and improve animal welfare and the quality of locally produced meat and milk. It is also recommended to carry out an adequate follow-up of animal movements, with a focus on brucellosis in accordance with the Organic Law of Animal Health. Actions to protect animal and human health should be coordinated with the Ministry of Public Health under a "One Health" strategy.

The main limitation of this study is the use of only one serological test for the diagnosis of brucellosis; applying several tests would increase the sensitivity level and reduce the proportion of false negative results [1,70,71].

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Institutional Review Board Statement: The serological sampling to determine the prevalence of bovine brucellosis at the national level was approved by Official Register No. 248 on Thursday, 24 May 2018, of the Ministry of Agriculture and Livestock. The Authorization for the use of data from the 2018 national sampling was issued through Memorandum No. AGR-AGROCALIDAD/CSA- 2021-000980-M, in which it is mentioned that

the Institutional authorship of AGROCALIDAD must be included under the technical cooperation PANAFTOSA.

Informed Consent Statement: The sampling was free of charge for the farmers, and the animal owners agreed with the sampling.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest

Appendix A

Table A1. Sampling Form and Description of Variables

MINISTERIO DE AGRICULTURA Y GANADERÍA

AGROCALIDAD
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SAMPLING FORM TO ESTIMATE THE PREVALENCE OF BOVINE BRUCELLOSIS IN CONTINENTAL ECUADOR, 2018								
I. IDENTIFICATION AND LOCATION OF THE FARM								
1.Date (day/month/year):				2.Name of respondent:				
3. Owner's name:				4.Cadastral code:				
5.Province:			6.Canton:			7. Parish:		
8.Spindle or zone:		9. X :		10. Y :				
11. Do you know or have you ever heard of brucellosis:								
II. GENERAL DATA ON THE FARM								
12. Surface area of the farm:		ha						
13. Meat cattle – number of heads				14. Dairy cattle – number of heads				
15.Type of production:				16. Number of milking per day.				
17.Technification level:				18.Consumption of raw milk:				
19. Destination of the milk/cheese:				20. Number of cows:				
21. Presence of animals of other species on the farm:								
III. GENERAL MANAGEMENT OF ANIMALS AND PASTURES								
22. Does it have enclosures?				23. There are footbaths on the farm:				
24.Control of the entry of people:				25. Does it have a sleeve, funnel or similar other:?:				
26. Origin of the animals:				27. Do other animals occupy their paddocks?:				
28. Origin of drinking water for animals:								
IV. HEALTH ASPECTS								
29. Has veterinary assistance:				30. Frequency of veterinary visits:				
31. Market your animals:				32.What category does it market:				
IV.I VACCINATION								
33.Vaccination against brucellosis?				34. What vaccine applied?				
35. Who performs the vaccination?:								

IV.II REPRODUCTION								
36. Reproduction system:					37. Do you use a specific place for births?:			
38. Do you disinfect these places?:								
IV.III PATHOLOGIES (occurring in the last year) BRUCELLOSIS								
39. Has there been reproductive problems in the last 2 years?:								
			40	41	42			
40.Symptomology presented (3 options)?:								
43.Destination of aborted material								
IV.IV BRUCELLOSIS DIAGNOSTIC TESTS								
44. Admission with laboratory diagnosis:								
45. Have you carried out brucellosis tests in your herd?:								
46. Name of surveying veterinarian(s):								
V. SAMPLES COLLECTED								
No.	**# EARRING**	**AGE (Months)**	**VACCINE**	**BORN ON THE FARM**	**CALVING (Number)**	**CLINICAL SIGN**		
47.								

- **Location and general data:** From column A to column K, you will find the data described below:
  - NUMBER: default code for each sampled herd;
  - DATE of sampling;
  - RESPONDENT: name and surname of the person who was present during sampling and provided the information;
  - OWNER: name and surname of the farm owner;
  - CADASTRAL CODE: number assigned according to the cadastre during the FMD vaccination (2017 cadastre);
  - Province, canton, parish: spatial location of the farm, according to the geographical distribution of the country;
  - Herd GPS data: georeferencing of farm location on a spatial plane, including (axis, X, Y).
- **The data from columns:** L to BI correspond to the questions of the epidemiological survey carried out during the on-site sampling:
  - Do you know or have you ever heard of brucellosis: it indicates that it is a reproductive disease whose important sign to detect is abortion, so select affirmatively or negatively according to the respondent's answer;
  - Area of the farm: the area corresponding to the premises and pastures, in hectares, determined in the survey, must be specified;
  - Meat-reared animals: indicates the number of animals that are farmed for fattening;
  - Dairy animals: the number of animals that are farmed for milk production (it does not matter if the animal, after its productive life, is for meat consumption destined for the slaughterhouse);
  - Meat and milk percentage: based on the parameters of animals intended for meat and milk, this parameter is considered;
  - Production: select the option according to the following conditions:
    - Extensive: grazing is the only type of feeding;
    - Intensive: balanced feed of vegetable origin and pastures in stables are the main types of feeding in the herd;
    - Hogueo: cattle tied to graze in a specific place, preferably under a tree;
  - Type of production: refers to the type of farming:
    - Meat: animals are farmed for fattening and selling;
    - Milk: animals farmed for the production of milk and by-products;
    - Mixed: animals farmed for both meat and milk production;
- **Number of milkings per day:** enter the number of milkings per cow and per day (maximum);
- **Technification level:** select the option according to the following conditions, depending on the level of technification:
  - Low: manual milking;
  - Medium: it is carried out in hygienic conditions from the udder to a cooling tank;
  - High: use of milking machines;
    - Consumes raw milk: it must be selected if the milk is consumed raw by farmer and relatives, with nopasteurization;
    - Destination of the milk/cheese: select the option according to the following conditions, depending on the final destination of milk and by-products collected on the herd:
      - Collection centres: they gather the milk production or by-products of small holdings so that

they can compete in quantity and quality in the markets of large urban centres;The milk industry: the milk is sold to industries for further processing in the manufacture of dairy by-products;

- Merchant: after milking, the milk is sold to a person outside the herd who collects from various herds for final sale;

- **The number of cows:** indicates the number of cows (females older than 24 months) in the herd;
- **Presence of other animal species on the farm:** select affirmatively or negatively the existence of other animal species present on the farm;
- **Has fences:** select affirmatively or negatively the existence of fences;
- **There are footbaths on the farm:** select the affirmative or negative the existence of footbaths at the entrance to premises;
- **Control of the visitors:** select affirmatively or negatively the existence of a register forvisitors;
- **It has a sleeve, funnel or similar other:** select affirmatively or negatively the existence of places for cattle containment;
- **Origin of animals:** select the option according to the following conditions, depending on the origin of animals in the herd:
  - Farm itself: animals are born, grown, and bred in the farm itself;
  - Neighbour: animals were purchased from a nearby farm;
  - Commercial fair: animals were purchased at a commercial fair;
  - Exhibition fair: animals were purchased at an exhibition fair;
  - Merchant: animals were purchased from a cattle trader;
- **Other animals graze on their pastures:** select affirmatively or negatively if other livestock animals graze on its pastures (paddock rental modality);
- **Origin of the drinking water for animals:** select the option according to the following conditions, depending on the source of the drinking water, such as:
  - River and/or ditch: natural current of water that flows permanently and ends up in another, in a lake or in the sea, ditch or small channel that carries water, especially for irrigation;
  - Well and/or drinking water: deep holes made in the ground, especially to draw water from underground springs, water for human and animal consumption, that can be consumed without restriction for drinking or preparing food;
  - Rain: rainfalls;
  - Runoff: slope of a mountain or elevation of the land on any of its sides, it is a decline or place where the water runs. This is usually a sloping topographical surface, lying between high points (such as ridges, peaks, or ridges) and low points;

- **Has veterinary assistance:** select affirmatively or negatively, if a veterinary practitioner visits the farm;
- **Veterinary visit frequency**: select the option according to the following conditions, depending on the frequency of the veterinary practitioner's visit:
  - Weekly: the veterinary practitioner visits the farm every week;
  - Fortnightly: the veterinary practitioner visits the farm every 15 days;
  - Monthly: the veterinary practitioner visits the farm every month;
- **Sales of animal:** select affirmatively or negatively, if the cattle holder sells them to external parties;
- **What animal category does she/he sell:** select the option according to the following conditions, depending on the age category to which the most animals sold fall into:
  - Calves: calves, females, aged 1 day to 6 months;
  - Heifers: calve, females, aged 6 months to 18 months or until it gives birth to calf;
  - Cows: females that have already calved, generally older than 24 months;
  - Males: age category that includes calves, bulls, and bullocks;
- **Vaccination against brucellosis:** select affirmatively or negatively, depending on whether the cattle holder vaccinates against bovine brucellosis;
- **Which vaccine applied**: select the option, depending on the vaccine applied to the animals:
  - Strain 19: smooth, gram-negative strain that has its entire lipopolysaccharide, including the "O" chain, responsible for inducing antibodies that react with antigens for diagnosis;
  - RB51: elaborated from a rough strain of *Brucella abortus*. It lacks the "O" chain of lipopolysaccharide;
  - Do not know: when the owner knows that the brucellosis vaccination was performed but does not know the strain applied;
  - Both: when the owner applied both strains at different times;
- **Who performs the vaccination**: Select the option, depending on the person who administered the brucellosis-vaccine by injection:
  - Cattle holder/administrator: the owner or the person in charge of the herd administers the vaccine;
  - Public MV: the vaccine is administered by a state veterinarian;
  - Private MV: the vaccine is administered by a private veterinary practitioner;
- **Breeding system used**: select the option according to the following conditions, depending on the breeding system in force in the herd:

- Natural mating: consists of keeping the bull loose in the paddock, permanently with all the herd animals, so that it can interact freely with cows; Insemination: assisted reproduction technique;
- Mixed: when, according to the physical, economic, and environmental conditions, it is decided which technique (natural or artificial) will be applied according to the season;
- Embryo transfer: artificial breeding method that consists in collecting an embryo from a cow uterus, i.e., the donor, to introduce it into another female uterus, i.e., the recipient. The embryo will continue growing and developing until delivery;

- **Use a specific place for calving**: select the option according to the following conditions, depending on the place where females calve:
  - Pasture: place aimed for farming and cattle grazing, wide limit;
  - Corral: generally uncovered enclosure, next to the rural houses, which are used to keep domestic livestock, within a designated area;
  - Farrowing pens: traditional construction dedicated to calving, generally the last trimester of pregnancy;
- **Disinfect these places**: select affirmatively or negatively, if the owner of cows close to parturition disinfects the specific places for parturitions;
- **There have been reproductive problems in the last 2 years**: select affirmatively, negatively or if you do not know ("do not know") if there have been reproductive problems in the herd in the last 24 months;
- **Symptomatology presented (3 options):** select 3 relevant options presented:
  - Abortion: involuntary termination of pregnancy before the embryo or offspring is able to survive outside the womb;
  - Placental retention occurs after childbirth since the organ has not been expelled with the offspring;
  - Weak calf: clinically describes the calf that is born normally but is weak and slow to sit up and suckle; Affected animals progressively deteriorate and generally do not survive beyond a week of life;
  - Sterility: quality attributable to those biological organisms that cannot reproduce, either due to the malfunction of their sexual organs or because their gamete is defective;
  - Postpartum metritis: inflammation of the uterus usually due to a microbial infection that occurs during the 21 days (usually 10) after delivery. It is almost always seen after abnormal delivery or retained placenta;
  - Joint swelling: accumulation of fluid in the soft tissues surrounding the joint, due to its inflammation;
  - Epididymitis: inflammation of the epididymis, usually accompanied by redness and swelling of the scrotum;
  - Orchitis: inflammation of one or both testicles, often caused by a microbial infection, one of the causes of acute scrotum and azoospermia;
  - Anestrus: the period after calving during which cows show no behavioural signs of estrus, a state of sexual inactivity in females;
- **Destination of aborted material**: Select three relevant options presented:
  - Burial: the aborted material is buried;
  - Leave in place: if the aborted material is left on the site of abortion;
  - Food for other species: if the aborted material is fed to carnivorous species;
  - Garbage: if the aborted material is disposed of in garbage cans or left on vacant land to decay;
- **Entry with laboratory diagnosis**: select affirmatively or negatively, depending on whether animals newly-introduced in the herd are tested for brucellosis;
- **Have brucellosis tests been carried out in your herd**: select affirmatively or negatively if diagnostic tests for brucellosis have been carried out;
- **Name of the interviewer veterinary**: name and surname of the veterinarian or the person who conducted the survey; From column AZ to column BI is compiled the information on samples collected, with their respective results, according to the following parameters:
  - Earring: earring number of the sampled cow;
  - Age (months): age of the sampled cow in months;
  - Vaccine: type of vaccine administered to the cow;
  - Born on the farm: indicate affirmatively or negatively, if the sampled cow was born on the farm;
  - Calvings: indicate the number of calvings of the sampled cow;
  - Symptoms: Indicate affirmatively or negatively, if the cow has had symptoms of brucellosis in the last 24 months;
- **Report**: corresponds to the code of the AGROCALIDAD laboratory report from which the corresponding result was obtained;
- **Result**: The collected samples were analyzed using the characteristics of the laboratory test to be used for the analysis of samples

  (SVANOVIR® *Brucella*-Ab cELISA), which has a 99.5%-sensitivity and a 99.6%-specificity; those with a PI ≥ 30% will be considered as positive;

- **PI**: percentage of inhibition obtained from the reading of optical densities in an ELISA reader, for the interpretation of the results;
- **Observations**: corresponds to criteria issued from the results.

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Experimental section

Study 2

Assessment of brucellosis vaccination practices and quality control of vaccines registered and used in Ecuador

Submitted in P*athogens*

Ana Garrido-Haro, *Viviana Santafé-Huera, Paola Moreno-Caballeros, Euclides De La Torre-Medranda, Michelle Yugcha-Diaz, Hugo Rosero-Mayanquer, Jorge Berru-Roman, Marie-France Humblet, Jorge Ron-Román and Claude Saegerman*

Preamble

Vaccination is the most successful method to prevent and control animal brucellosis in endemic countries. In Ecuador, vaccination is not mandatory, and it is under the responsibility of cattle ranchers who own the herds. The objective of the study was to evaluate brucellosis management practices and conduct an analysis of the quality control of registered vaccines used in Ecuador. A national survey was conducted among veterinary professionals of AGROCALIDAD at the continental level in Ecuador to assess the knowledge and vaccination practices employed.

Additionally, to understand the handling of vaccines at points of sale, a survey was conducted among owners of commercial establishments (veterinary stores) in three of Ecuador's most productive provinces to examine the management of vaccines on shelves.

For the assessment of vaccine quality control, sampling was carried out with the support of the AGROCALIDAD to verify the quality of registered vaccines available in Ecuador. The analysis was performed at the vaccine quality control laboratory of AGROCALIDAD, and as a result of this study, two out of three of the vaccines available in Ecuador did not achieve satisfactory results.

The study provides a greater understanding of the most commonly associated brucellosis management and control practices, which can influence its epidemiology. Furthermore, it allows for the identification of both positive and negative aspects that could be considered for the national brucellosis program.

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Assessment of brucellosis vaccination practices and quality control of vaccines registered and used in Ecuador

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Abstract: Bovine brucellosis is a disease of great importance in the cattle industry, with significant economic losses and hampering trade and exportation. Brucellosis is also a zoonotic disease caused by members of the genus *Brucella*. In endemic country, the best way to prevent and control infection in cattle is vaccination. However, it goes hand in hand with proper diagnosis, effective biosecurity measures, control of animal movements and a culling strategy with compensation aimed at eliminating infected animals. Efforts to control bovine brucellosis could be undermined by low quality vaccines or inadequate vaccine implementation. In Ecuador, vaccination against *Brucella* is not mandatory. Until now, no adequate quality control of vaccines has been implemented to ensure the proper immunity of animals. The national reference laboratory developed a vaccine quality control analysis. Three vaccines distributed in Ecuador and registered in the Agency that supplies vaccines to Ecuador were tested, i.e. two corresponded to strain S19 and one to RB51; they were provided by the laboratory or distributor. The vaccines were resuspended, seeded on Tryptic Soy Agar (TSA) agar and the number of CFU was verified. The dissociation phase was determined by crystal violet staining and the identification of *Brucella abortus* was verified by Multiplex Bruce-ladder PCR. The results revealed that two out of three vaccines did not meet the quality control criteria in terms of bacterial count, which was not in the 50-100 × 10^9 CFU range (standard dose; subcutaneous way for strain S19) recommended by the World Organization for

Animal Health. In addition, the assessment of field vaccination practices (compliance) indicates a possible room for improvement. Indeed, a survey performed among veterinary professionals and technicians (n=95) in the 23 provinces of continental Ecuador and in veterinary stores (n=30) in three provinces of the Sierra region, indicated a 62.29% (S.E.: 3.93) and 56.86% (S.E.: 5.96) compliance, respectively. This compliance differed significantly between the two groups considered. From a strategic point of view, there results can guide the authorities for the management of an education campaign on good practices of vaccination in the country and to implement a systematic quality control procedure for vaccines used in Ecuador. Those activities should contribute to improve and refine the current bovine brucellosis national control program.

Keywords: *Brucella abortus*; cattle; vaccine; strain RB 51; strain S19; Buck 19; quality control; Ecuador

1. Introduction

Bovine brucellosis is an infectious disease caused by *Brucella abortus,* a facultative intracellular pathogen that causes persistent infection in animals and has been isolated from several species of livestock but mostly in cattle ([1]. Bovine brucellosis is a chronic infectious disease that causes abortions and sterility in cattle, resulting in significant economic losses for the cattle industry [2]. A regular and adequate surveillance is essential to determine the true prevalence of the disease especially in endemic areas [3].

The choice of strategic options to control brucellosis depends on its true prevalence in the animal reservoir, the socio-economic context, the animal health surveillance system in place and the policy set by the relevant health authorities [1]. In cattle, the vaccination with *Brucella abortus* Buck 19 (called B19 or S19) and RB51 is the most successful method for preventing and controlling the infection, whereas eradication can only be achieved by applying the "test and slaughter" strategy, combined with effective preventive measures such as biosecurity and the control of animal movements [1,4].

In Ecuador, the bovine brucellosis control program was adopted in 2008 (resolution 025). Ecuador has implemented a bovine brucellosis prevention and control program, which includes vaccination of susceptible animals, serological diagnosis of the disease in animals and sanitary slaughter of positive animals. In Ecuador, the brucellosis prevention and control program is managed by the *Agencia de Regulación y Control Fito y Zoosanitario* - AGROCALIDAD; it partly relies on the vaccination of females with S19 and/or RB51 vaccines. It is worth to mention that vaccination is not mandatory in Ecuador and is the responsibility of the cattle holder [5].

In Ecuador, bovine brucellosis is endemic; the apparent prevalence of bovine brucellosis was estimated to be 21.3% (95% CI: 16.8-26.6) at the herd level and 6.2% (95% CI: 5.5-7) at the animal level [6]. According to previous studies, the strains circulating in Ecuador are *Brucella abortus* biovar 1 [7] and *Brucella abortus* biovar 4 [8]. According to figures entered into the Social Surveillance System of the Ecuadorian Ministry of Public Health, the 2022-incidence of the disease in humans was 0.16 per 100,000 inhabitants; in 2023, one case was reported in the Azuay province [9].

Brucellosis is one of the most easily acquired laboratory infections, and strict biosafety precautions should be observed when working with the pathogen. Laboratory handling of *Brucella* spp. live cultures , including vaccine strains, is hazardous and must be performed within appropriate biosafety and containment level determined after a bio-risk analysis [10].

Vaccination has played an enormous role in reducing brucellosis prevalence in many countries [1]. It is a preeminent factor to prevent the disease [11]. A widely used vaccine for the prevention of brucellosis in cattle is *B. abortus* S19, which remains the reference vaccine compared to other vaccines [12]. It is a live vaccine normally administered to female calves aged between 3 and 6 months, as a single subcutaneous dose of 50–80 × 10^9 viable organisms [10]. With full doses, this strain was able to induce a protective immunity in cattle for their entire economic lifespan [1]. However, to overcome problem of transitional specific antibody response that hinders the distinction between vaccinated and truly infected animals by most serological tests [13]. Classical vaccination with S19 is restricted to female calves between 3 and 8 months of age; 60-90 × 10^9 CFU (colony-forming unit) are administrated subcutaneously, although some antibodies may persist in 1 to 2% of adults vaccinated when they were calves [14].

Brucella abortus strain RB5l is a naturally occurring, rugged, attenuated and stable mutant of strain 2308. Calves are vaccinated subcutaneously between 4 and 12 months of age with 10-34 × 109 viable organisms. Vaccination of cattle older than 12 months is only allowed under authorization, and the recommended dose is then 1-3 × 10^9 viable organisms [10]. This strain has interesting characteristics for a vaccine against bovine brucellosis [15]. However, the RB51 strain is resistant to rifampicin [16] which is a treatment of choice for human brucellosis [1]. *Brucella abortus* strain RB51 accumulates low amounts of cytoplasmic M-like OPS [17,18]. On the other hand, it has been documented that while most vaccinated calves develop placental infection after receiving full doses of *B. abortus strain* RB51 intravenously, a significant proportion of vaccinated animals also experience milk excretion; practical knowledge demonstrated that vaccinating pregnant animals may cause abortion and raise perinatal mortality [19].

Brucellosis vaccines must meet quality criteria that demonstrate the potency of the batch to be released. Suitable CFU counts are the following: 50-100 × 10^9 CFU (standard dose; subcutaneous route) and between 10-34 x 10^9 CFU (standard dose; subcutaneous route) for S19 and RB51, respectively [10].

In Ecuador, it is important to assess the vaccination process, the knowledge of veterinarians and technicians in charge of brucellosis vaccination and the quality of both national and imported vaccines, in order to provide results that support the national surveillance system and improve the field management of bovine brucellosis.

In this study, we aimed to assess both the quality of vaccines registered and used in Ecuador and the brucellosis vaccination practices in the field, based on scientific evidence.

2. Materials and Methods

The design summary of the study is presented in the **Figure 1**.

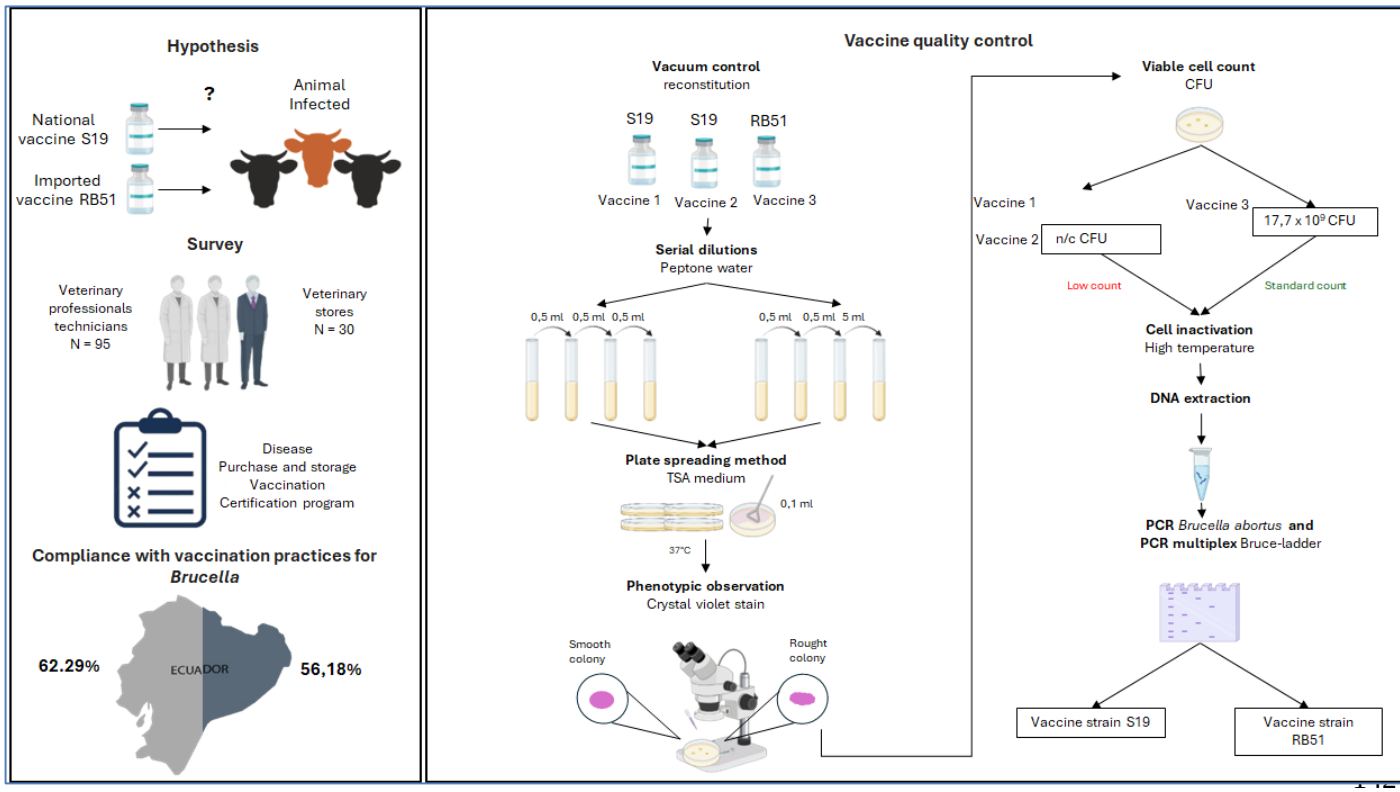

Figure 1. Design summary of the study

2.1. Study area

AGROCALIDAD is the official health control entity in Ecuador, responsible for establishing the procedures in the event confirmed positive or suspicious cases of animal diseases including bovine brucellosis. These procedures are performed according to guidelines of disease prevention and control.

The quality control of brucellosis vaccines was carried out at the AGROCALIDAD Laboratories in Tumbaco, Ecuador, in the containment laboratory of the Animal Diagnostics area (BSL2+). The vaccines evaluated were registered with the Agency according to the AGROCALIDAD manual for registration of companies and products for veterinary use [20], i.e. two national companies (S19) and one international (RB51).

A cross-sectional descriptive study on vaccination practices against bovine brucellosis in Ecuador was carried out through a survey among veterinary professionals and technicians of AGROCALIDAD in all provinces of continental Ecuador (N = 23) of the three natural regions; the coastal zone (i.e. Esmeraldas, Manabí, Los Ríos, Guayas, El Oro, Santa Elena, Santo Domingo de los Tsáchilas); the Andean Highlands zone (i.e. Carchi, Imbabura, Pichincha, Cotopaxi, Tungurahua, Chimborazo, Bolivar, Cañar, Azuay, Loja), and the Amazon region (i.e. Sucumbíos, Napo, Pastaza, Orellana, Morona Santiago and Zamora Chinchipe). In addition, a survey was conducted in the veterinary stores of three provinces of the Andean Highlands (i.e. Pichincha, Cotopaxi and Tungurahua). The Andean region (Sierra) gathers 53% of the cattle population [21].

2.2. Sampling method

Samples were collected in triplicate from each laboratory or importer of *Brucella* sp. vaccines as follows: 5 vaccines were placed in the first envelope, labeled as the laboratory sample (ML, acronym in Spanish), 3 vaccines were placed in the second envelope, labeled as counter sample 1 (CM1, acronym in Spanish), and 3 vaccines were placed in the third envelope, labeled as counter sample 2 (CM2, acronym in Spanish). All samples were taken from the same lot that was to be analyzed. Each sample was labeled with the lot number, collection date, and the sampler's name. Envelopes #1 (ML) and #2 (CM1) were sent to the AGROCALIDAD reference laboratory, specifically the Animal Diagnostic Laboratory and Vaccine Quality Control Laboratory, while envelope #3 (CM2) remained at the production laboratory or commercial supplier.

The samples were placed in a cooler at 4°C to maintain the cold chain and were transported as soon as possible to the laboratory. The CM1 was stored at the Agency for one year until disposed of. During sampling, the Agency technician was always accompanied by a technician or person from the vaccine manufacturing laboratory or the commercial representative authorized by the company. Upon entering the laboratory, the registration information, expiration date, lot number and temperature were verified.

2.3. Quality control analysis of vaccines

The following parameters were evaluated: vacuum levels, Petri dish count of viable microorganisms and verification of the appropriate number of CFU in the dose. In addition, a smoothness test was performed to determine the dissociation phase by observing colony formation. For identity testing, a PCR was performed to identify *Brucella abortus*. Finally, typing was performed using the Multiplex PCR assay, specifically the Bruce-ladder method [10,22,23].

2.3.1 Live bacterial count and verification of the vacuum

In each vaccine, the tablet aspect was checked: it should be white and yellowish. The vacuum was also measured with the help of a 10 ml-syringe. Each vaccine was reconstituted with its accompanying commercial diluent. Once the diluent was added, one waited 5 minutes and homogenized smoothly for 30 seconds in order to minimize the generation of aerosols.

2.3.1.1 Live bacterial count in vaccines

The container was opened in a biosafety cabinet in a way to minimize the generation of aerosols or liquid spillage. Four dilutions were performed, as indicated below:

- Dilution N# 1 (1:100): 0.5 ml of the vaccine was pipetted and added to tube N° 1 containing 49.5 ml of peptonized water and carefully shaken. The same procedure was followed for dilution N# 2 (1:10000), dilution N# 3 (1:1000000) and dilution N# 4 (1:100000000). Vials N° 1, 2 and 3 were discarded because they did not allow an adequate colony count. From dilution 4, 100 µl of tube 4 (dilution 1: 100000000) were placed on the surface of 4 Petri dishes; the plates contained agar (TSA).
- The tube contents was evenly dispersed with a disposable Drigalski spatula over the entire agar surface. It was left to stand for at least one minute. The plates were identified with the date and sample code and were incubated at 37 ± 2°C for 96 hours, under anaerobic conditions.

- For the bacterial count of RB51 vaccine, at dilution N° 4 (1:10000000), 5 ml of tube three were transferred to vial four containing 45 ml of peptonized water and shaken carefully; 100 µl of the tube 4 contents (dilution 1: 10000000) were seeded on the surface of 4 Petri flasks.

For reading, the colonies of the four seeded boxes of dilution # 4 were counted. The value obtained was multiplied by 20 to bring it to the application dose of 2 ml and multiplied by the respective dilution factor. The final values obtained were summed and averaged.

2.3.2 *Determination of dissociation phase (roughness)*

This procedure was performed with the seeded boxes from the plate count. The plates were observed under stereoscopic magnification (Henry Method) before and after staining with crystal violet; the colonies were classified into smooth (S), rough (R), mucous (M) and intermediate (I) (**Appendix A).** Crystal violet staining was performed on 3 of the seeded boxes. The crystal violet dye was diluted to a concentration of 1:40. The colonies were then coated with the diluted dye for 15 to 20 seconds. They were immediately observed under a stereomicroscope.

2.3.3 Identification of vaccine strains

2.3.3.1 Identification of *Brucella abortus* and typing with Multiplex PCR Assay Bruce-ladder

The seeded colonies were resuspended in 500 µl of molecular biology grade water. The vial was placed in the thermoblock at 95 °C for 25 minutes to inactivate the bacteria, then centrifuged for 10 minutes at 8000 rpm: the supernatant was sampled afterwards. The DNA obtained was stored at -20°C until use [24].

For the PCR of *Brucella abortus* identification, primers forward IS711: 5′- TGC CGA TCA CTT AAG GGC CTT CAT TGC CAG -3′ and reverse Abortus: 5′- GAC GAA CGG AAT TTT TCC AAT CCC -3′ were used to recognize the repetitive genetic element IS711 exclusive to the *Brucella* species [22]. The final component concentration of the PCR reaction was: buffer PCR 1X; dNTPs 0,2 mM; primers, 0.2 µM and 0,04U/ µL de la Enzima Platinum™ II Taq Hot-Start de Invitrogen; 5 µL of each sample DNA were employed in the reaction mix, with a 50 µl final reaction volume. The temperature profile at 95°C was 3 min, 34 cycles at 95°C for 1 min 15 sec, 55.5 °C for 2 min and 72 °C for 2 min. A final extension was at 72°C for 5 min. The reference material used was strain 11193 obtained from the brucellosis reference laboratories of Argentina, i.e. the *Servicio Nacional de Sanidad y Calidad Agroalimentaria* (SENASA).

The samples were also analysed using the Multiplex PCR Assay Bruce-ladder [23] (see table of **Appendix B**). The final concentration of the components for the PCR reaction was 3 mM $MgCl_2$, 0.3 µM of the primers 998 F, 998R, 752F, 752 R, 987F, 987R, 0.12 µM of the primers 535 F, 536R, 843F, 844R, 436F, 435R, 428F, 428R, 953F, 953R, 1X Go Taq Master mix Promega; 2 µL of each sample DNA were employed in the reaction mix. The final reaction volume was 25 µL PCR. The temperature profile was: 7 min at 95°C, 25 cycles at

95°C with 00:35 sec, 64°C for 00:45 sec, 72°C for 3 min and a final 6 min-extension at 72°C. The *Brucella suis* reference material was provided also by the Argentine brucellosis reference laboratories (SENASA).

2.4. Epidemiological surveys

A survey on bovine brucellosis vaccination practices in Ecuador was conducted by veterinary professionals and technicians (N=95). Data were collected with KoboToolbox. This survey included 61 questions. It focused on the vaccine administration, doses, practices and handling, types and age at vaccination. The survey was divided into five main categories: (i) classification data (8 variables), (ii) general knowledge on brucellosis vaccination process (9 variables), (iii) vaccine purchase and storage (11 variables), (iv) reconstitution, administration and disposal of vaccine waste (28 variables), and (v) certification program (5 variables). To assess the quality of the field vaccination procedure, 48 questions were selected and codified according to t **Table 1**. For each question, the number of modalities was calculated, and all modalities were placed in the good order, i.e. the lowest score was attributed to a bad practice and the highest score to the best practice (e.g. in a binary response, an answer close to the ideal situation, in terms of vaccination procedure quality, was 1 and null on the contrary).

Another survey, under the form of face-to-face interviews, targeted veterinary stores (N=30) and included 37 questions. It focused on knowledge, handling practices, storage, vaccine types and usage recommendations. The survey was divided into four main categories: (i) classification data (4 variables), (ii) vaccination process (6 variables), (iii) vaccine purchase and storage (25 variables), and (iv) certification program (2 variables). To assess the quality of the field vaccination procedure, 28 questions were selected and codified according to **Table 1**.

For both surveys, i.e. veterinary professionals/technicians and veterinary stores, the compliance rate of bovine brucellosis vaccination practices was calculated using the following sequential equations:

$$Global\ score\ observed\ (GSO) = \sum_{i=1}^{n} \frac{Score\ observed\ per\ each\ question}{Number\ of\ modalities\ per\ each\ question} \quad \text{(Equation 1)}$$

$$Theoritical\ minimal\ score\ (TMinS) = \sum_{i=1}^{n} = \frac{Theoritical\ minimal\ score\ per\ each\ question}{Number\ of\ modalities\ per\ each\ question} \quad \text{(Equation 2)}$$

$$Theoritical\ maximal\ score\ (TMaxS) = \sum_{i=1}^{n} = \frac{Theoritical\ maximal\ score\ per\ each\ question}{Number\ of\ modalities\ per\ each\ question} \quad \text{(Equation 3)}$$

$$Perentage\ of\ compliance = \sum_{i=1}^{n} = \frac{GSO}{\frac{TMinS+TMaxS}{100}}$$ (Equation 4)

The score of each question was divided by its number of modalities to avoid giving more importance for a question with more modalities (standardisation).

2.5. Statistical analysis

The distribution of compliance rates was assessed for normality using a Shapiro-Wilk test. The difference between the compliance rates for both groups, i.e. veterinary professionals/technicians vs. veterinary stores, was assessed using a two-sample Wilcoxon rank-sum (Mann-Whitney) test. All tests were performed in Stata SE 14.1® (StataCorp LP, College Station, TX, USA). Significance was claimed when p-value was < 0.05.

3. Results

3.1. Live bacterial count, smoothness and verification of the vacuum

In the live bacterial count, a total of 20 x 10^9 CFU/2ml was obtained in S29 vaccine #1, showing no satisfactory results since the amounts were lower compared to WOAH recommendations (50-100 x 10^9 CFU, standard dose, subcutaneous route). It is worth mentioning that the supplier mentions, in its documentation, that each dose contains 50-80X10^9 live *Brucella abortus* germs (S19). Regarding colony roughness, 100% of them were smooth; these colonies must indeed be at 95% in a smooth phase and not absorb the dye. That specific parameter was fulfilled based on the analysis performed and the colonies. However, it did not comply with the live bacteria count per dose (**Table 2**): in vaccine #2, 0 CFU of live bacteria was obtained in 2 ml of S19-vaccine, showing no satisfactory result. Indeed, it does not comply with the WOAH recommendation, i.e. between 50-100 x 10^9 CFU. As no colonies were identified in the final dilution, roughness parameters could not be analysed, resulting in no satisfactory result (**Table 2**). In vaccine #3, 17.7 x 10^9 CFU of live bacteria were isolated in 2 ml; it complies with the WOAH recommendations which are between 10 and 34 x 10^9 CFU in RB51 vaccines. The colonies were 100% in the rough phase (**Table 2**).

3.2. Identification of vaccine strains

A band of 498 bp was obtained in the amplification product, indicating the detection of *Brucella abortus* DNA in vaccines #1 and #3. The diluted vaccine #2 was directly seeded to identify the vaccine strain; it amplified a 498 bp-band of molecular weight.

In the typing performed to identify the vaccine strain, the molecular weights corresponding to *Brucella abortus* S19 were obtained by amplifying fragments 1682, 794, 450 and 152 bp in vaccines #1 and #2. For vaccine #3, the typing result was *Brucella abortus* Strain RB51, by amplifying fragments 2524, 794, 587, 450 and152 bp. Molecular weights reported in [23] were taken into account to interpret typing results.

3.3. Assessment of bovine brucellosis vaccination practices

Detailed calculation is collationed in **Supplementary material S1**. The compliance rates were normally distributed for both surveys, i.e. veterinary professionals/technicians (N=95; Shapiro-Wilk test: p-value = 0.09; mean = 62.29; SE = 3.93) and veterinary stores (N=30; Shapiro-Wilk test; p-value = 0.23; mean = 56.86; SE = 5.96) (**Figure 2**). The compliance rate of veterinary stores was significantly lower compared to veterinary professionals/technicians (two-sample Wilcoxon rank-sum test: p-value < 0.0001) (**Figure 3**).

The margin of improvement corresponds to the proportional difference between the theoretical maximal score that can be reached and the proportion observed for each group of actors. The average margin of improvement reached around 38% for veterinary professionals/technicians and 43% veterinary stores.

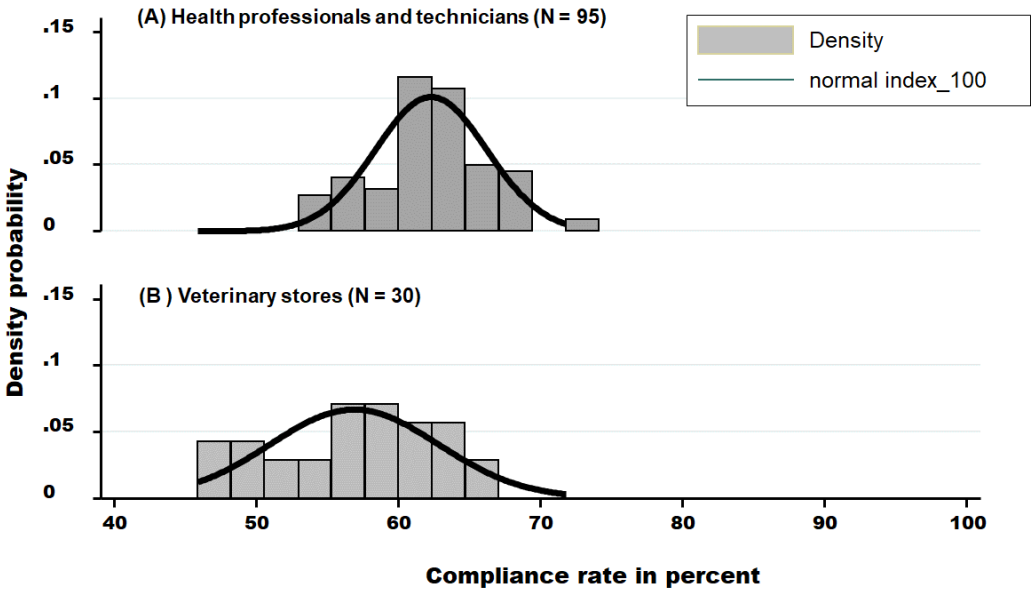

Figure 2. Normal distribution of compliance rates for both veterinary professionals/technicians (A) and veterinary stores B), regarding the

bovine brucellosis vaccination practices.

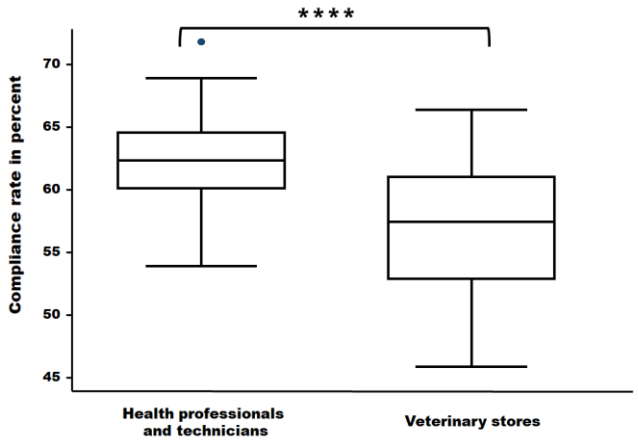

Figure 3. Compliance rates for both veterinary professionals/technicians (A) and veterinary stores (B).

Legend: **** two-sample Wilcoxon rank-sum test; p-value < 0.0001.

Table 1. Quality control of the vaccination process against bovine brucellosis in Ecuador, for veterinary professionals/technicians (A) and veterinary stores (B).

Quality control variable	Code	Score (min)	Score (max)	Modalities (N°)	Modalities (description)
A) Veterinary professionals and technicians					
Academic level completed	Q01	1	2	2	1: Third level; 2: Fourth Level
Years of experience	Q02	1	5	5	1: Less than 2 years; 2: 2 to 5 years; 3: 6 to 9 years; 4: 10 to 15 years; 5: More than 15 years
Do you know if there is an effective treatment to cure cattle of brucellosis?	Q03	0	1	2	No=1; Yes=0
Is vaccination against bovine brucellosis in Ecuador mandatory?	Q04	0	1	2	No=0; Yes=1
Do you know if there is an official calendar for bovine vaccination against brucellosis in Ecuador?	Q05	0	1	2	No=0; Yes=1
What is the main benefit of vaccination against brucellosis in animals?	Q06	1	4	4	1: Certification of free land; 2: Export of animals and products of animal origin; 3: Reduction in the excretion of *Brucella* spp. in animals; 4: Reduction of disease transmission between farms
Do you know if there are vaccines against brucellosis in other animal species?	Q07	0	1	2	No=0; Yes=1
Is the bovine brucellosis vaccine type?	Q08	1	3	3	1: Not know; 2:Dead; 3:Alive
In the case of vaccination against two diseases, is a different syringe used for the vaccines to be administered?	Q09	0	1	2	No=0; Yes=1

What is the main source of infection of a brucellosis-free farm?	Q10	1	9	9	1: Other domestic animals; 2: Vaccination (default of attenuation); 3: Wildlife; 4: Staff health; 5: Artificial insemination; 6: Colostrum; 7: Resurgence of the disease (due to lack of elimination of infected animals on the farm); 8: Contaminated food, pastures or materials; 9 : Introduction of sick animals;
Have you seen any reproductive problems or side effects after administration of bovine brucellosis vaccine? Specify which	Q11	0	2	3	0: No; 1: Fever; 2: Abortions
What is your most important criterium to choice bovine brucellosis vaccine?	Q12	1	6	6	1: Other; 2: Avaibility ; 3: Price; 4: Origin; 5: Efficacy ; 6: Strain type
Who purchase bovine brucellosis vaccine?	Q13	1	4	4	1: Local agricultural warehouse; 2: The commercial house; 3: The owner; 4: The Ecuadorian State
How often do you acquire bovine brucellosis vaccine?	Q14	1	4	4	1: Every 2 or 3 months; 2: Once a month; 3: Once every 15 days; 4: Once a week
Where do you store the acquired bovine brucellosis vaccine?	Q15	1	3	3	1: At home; 2: On the farm; 3: At the veterinary clinic
What is the storage place of the vaccine?	Q16	1	5	5	1: Other; 2: To the environment; 3: In a refrigerated box; 4: Freezer (-20º C); 5: Refrigerator (up to 4º C)
How do you check that the vaccine storage temperature is adequate?	Q17	1	5	5	1: Other; 2: Does not take temperatura; 3: Read the temperature on the refrigerator dial; 4: Add a thermometer in the refrigerator freezer; 5: Add a thermometer in the refrigerator
Do you periodically check the temperature of the refrigerator	Q18	0	1	2	No=0; Yes=1
What type of brucellosis vaccine presentation do you generally use	Q19	1	2	2	1: Not_freeze_dried; 2: Freeze_dried
Is the expiration date of the bovine brucellosis vaccine checked before administration?	Q20	0	1	2	No=0; Yes=1

Who is in charge of administering the bovine brucellosis vaccine on the farms you have visited?	Q21	1	5	5	1: Other; 2: Cowboy; 3: Property administrator; 4: Agronomist or agricultural engineer; 5: Veterinary doctor
From the farms you have visited, is vaccination against bovine brucellosis on the farm based on a strategy?	Q22	0	2	3	0: No strategy; 1: Batch immunization of animals; 2: Individual immunization
According to sex, which cattle are vaccinated against brucellosis on the farm?	Q23	1	3	3	1: Only males; 2: Both; 3: Only females
From your experience, is vaccination against bovine brucellosis on the farm carried out on sick animals?	Q24	0	1	2	No=1; Yes=0
What is the age of the first vaccination against bovine brucellosis?	Q25	1	3	3	1: More than 8 months; 2: Less than 3 months old; 3: Between 3 and 8 months
In your experience, what is the strain (type) of vaccine used in the first vaccination against bovine brucellosis?	Q26	0	2	3	0: Not known; 1: RB 51; 2: S19
What is the age for bovine brucellosis revaccination?	Q27	1	3	3	1: Other; 2: Before service; 3: 12 months
What is the strain (type) of the vaccine used for revaccination against bovine brucellosis?	Q28	1	3	3	1: Indistinctly; 2: S19; 3: RB51
Do you know if pregnant females are revaccinated?	Q29	0	1	2	No=0; Yes=1
What is the route of administration of the vaccine against bovine brucellosis?	Q30	0	1	2	0: Other; 1: Subcutaneous
Do you keep a record of animals vaccinated against brucellosis?	Q31	0	1	2	No=0; Yes=1
Do you perform an adequate immobilization of the animals, for a correct vaccination?	Q32	0	1	2	No=0; Yes=1
Do you change syringes and/or needles before vaccinating other cattle against brucellosis?	Q33	0	1	2	No=0; Yes=1
Do you, as an Agency technician, check that the vaccines have a vacuum prior to application?	Q34	0	1	2	No=0; Yes=1
Do you as an Agency technician/physician make sure there is no air in the syringe before injecting the vaccine?	Q35	0	1	2	No=0; Yes=1
Do you store vaccine doses once prepared (reconstituted), to be administered in subsequent days to other cattle?	Q36	0	1	2	No=1; Yes=0

How many days do you store the reconstituted vaccine, for later use?	Q37	1	3	3	1: More than 2 weeks; 2: Between 1 and 2 weeks; 3: Less than 1 week
Do you maintain the cold chain during the period of preparation and administration of the vaccine against brucellosis to cattle?	Q38	0	1	2	No=0; Yes=1
Once the vaccine is reconstituted and prepared according to the manufacturer's instructions, is it administered immediately?	Q39	0	1	2	No=0; Yes=1
Do you generally homogenize vaccine vials during vaccination?	Q40	0	1	2	No=0; Yes=1
How do you dispose of waste (vials, syringes, needle) generated during the bovine brucellosis vaccination process?	Q41	1	4	4	1: Common household garbage; 2: Common farm garbage; 3: Buried; 4: Incineration
What type of clothing do you wear during the brucellosis vaccination process?	Q42	1	3	3	1: Not use protective clothing; 2: Common protective clothing (overalls, boots); 3: Special protective clothing (gloves, glasses, mask)
Observation of accidents during the application of the vaccine to the animal (e.g.: pricking with the syringe, splashing of drops or aerosols into the nose/eye)?	Q43	0	1	2	No=1; Yes=0
In the case of brucellosis vaccine accidents, did you have any symptoms?	Q44	0	1	2	No=1; Yes=0
Do you think there should be adequate quality control of the vaccine against bovine brucellosis?	Q45	0	1	2	No=0; Yes=1
Have the farmers been audited to determine the conditions of the vaccination process?	Q46	0	1	2	No=0; Yes=1
Have you received any training on bovine brucellosis and vaccination?	Q47	0	1	2	No=0; Yes=1
What was the duration of the training course received?	Q48	1	4	4	1: 1 hour; 2: 2 hours; 3: between more than 2 hours and 1day; 4: More than 1 day
Total	48	21	113	140	

B) Veterinary stores					
Years of warehouse operation	Q01	1	5	5	1: Less than 2 years; 2: 2 to 5 years; 3: 6 to 9 years; 4: 10 to 15 year; 5: More than 15 years
Do you know if there is an effective treatment to cure cattle of brucellosis?	Q02	0	1	2	No=1; Yes=0
Is vaccination against bovine brucellosis mandatory in Ecuador?	Q03	0	1	2	No=0; Yes=1
Is there an official schedule for bovine vaccination against brucellosis in Ecuador?	Q04	0	1	2	No=0; Yes=1
What is the main benefit of vaccination against brucellosis in animals?	Q05	1	4	4	1: Certification of free land; 2: Export of animals and products of animal origin; 3: Reduction in the excretion of *Brucella* spp. in animals; 4: Reduction of disease transmission between farms
Do you know if there are vaccines against brucellosis in other animal species?	Q06	0	1	2	No=0; Yes=1
What is the type of vaccine against bovine brucellosis?	Q07	1	3	3	1: Not know; 2:Dead; 3:Alive
Is the refrigerator exclusively used for storing vaccines?	Q08	0	1	2	No=0; Yes=1
The refrigerator have an alarm device to detect failures in the electrical supply?	Q09	0	1	2	No=0; Yes=1
Is the refrigerator where you store the brucellosis vaccine connected to an emergency circuit so that in the event of a power outage it continues to receive power?	Q10	0	1	2	No=0; Yes=1
Is temperature control carried out?	Q11	1	5	5	1: Not done; 2: Once a month; 3: Once a week; 4: Once a day; 5: Once in the morning and once in the afternoon
Where is the vaccine stored?	Q12	1	5	5	1: Other; 2: To the environment; 3: In a refrigerated box; 4: Freezer (-20º C); 5: Refrigerator (up to 4º C)
During the transportation of the vaccine to the warehouse, does your vaccine supplier ensure the cold chain?	Q13	0	1	2	No=0; Yes=1

How often is the order for vaccines made?	Q14	1	4	4	1: Other; 2: Monthly; 3: Biweekly; 4: Weekly
What is your most important criterium to choice bovine brucellosis vaccine?	Q15	1	6	6	1: Other; 2: Avaibility ; 3: Price; 4: Origin; 5: Efficacy ; 6: Strain type
Where do you purchase the vaccine against bovine brucellosis?	Q16	1	5	5	1: Unknown; 2: Other; 3:Commercial house; 4: Veterinary warehouse; 5: Direct importator
How often do you get the bovine brucellosis vaccine?	Q17	1	4	4	1: Every 2 or 3 months; 2: Once a month; 3: Once every 15 days; 4: Once a week
What is the recommended age for the first vaccination against bovine brucellosis used?	Q18	1	4	4	1: Other; 2: More than 8 months; 3: Less than 3 months old; 4: Between 3 and 8 months
What strain (type) of vaccine do you recommend for the first vaccination against bovine brucellosis?	Q19	1	3	3	1: Indistinctly; 2: RB 51; 3: S19
What is the age at which you recommend revaccination against bovine brucellosis (RB51) should be used?	Q20	1	3	3	1: Other; 2: Before service; 3: 12 months
What is the strain (type) of the vaccine that you acquire for revaccination against bovine brucellosis?	Q21	1	3	3	1: Indistinctly; 2: S19; 3: RB51
Have you been asked for a recommendation on the route of administration of the vaccine against bovine brucellosis?	Q22	0	1	2	0: Other; 1: Subcutaneous
Do you maintain a sales record (register) of the vaccines you sell?	Q23	0	1	2	No=0; Yes=1
How do you dispose of expired vaccine waste?	Q24	1	5	5	1: Other way; 2: Common household garbage; 3: Common farm garbage; 4: Buried; 5: Incineration
Do you require a medical prescription to sell brucellosis vaccines?	Q25	0	2	3	0: No; 1: Sometimes; 2: Yes
In general, which people come to purchase vaccines from your warehouse?	Q26	1	5	5	1: Other; 2: Livestock farmers; 3: Agronomists; 4: Agricultural engineers; 5: Veterinarians
Do you think that adequate quality control should be carried out on the vaccine against bovine brucellosis?	Q27	0	1	2	No=0; Yes=1
Have you received any training on bovine brucellosis and vaccination?	Q28	0	1	2	No=0; Yes=1
Total	28	15	78	91	

Table 2. Quality control results of brucellosis vaccines commercialized in Ecuador

Vaccine identification	Concentration reported (2 ml dose)	Vacuum	Plate count					Smoothness	
			Nº Plate	CFU	Doses µl/ µl dispensed (2000/100)	Dilution factor	Total	Negative (CFU that do no stain)	Positive (CFU with shades of red purple)
Vaccine No. 1 S19	50-80 x 10^9	12.5 ml	1	10	20	10^8	20 x 10^9	PCR	-
			2	10	20	10^8	20 x 10^9	10	-
			3	11	20	10^8	22 x 10^9	11	-
			4	9	20	10^8	18 x 10^9	9	-
			Total				80 x 10^9	-	-
			Average				20 x 10^9	-	-
Vaccine No. 2 S19	50 x 10^9	10 ml	1	0	20	10^8	0	-	-
			2	0	20	10^8	0	-	-
			3	0	20	10^8	0	-	-
			4	0	20	10^8	0	-	-
			Total				0	-	-
			Average				0	-	-
Vaccine No. 3 RB51	10-34 x 10^9	14.5 ml	1	113	20	10^7	22.6 x 10^9	-	PCR
			2	75	20	10^7	15 x 10^9	-	75
			3	84	20	10^7	16.8 x 10^9	-	84
			4	82	20	10^7	16.4 x 10^9	-	82
			Total				70.8 x 10^9	-	-
			Average				17.7 x 10^9	-	-

Legend: *PCR: colonies used for molecular identification; CFU = colony forming units.

4. Discussion

In Ecuador, cattle population is 3.9 million heads. The Sierra region has the largest number of cattle, with 2.0 million heads, which represents 53% of the population, followed by the Coast, with 38.6% [21]. In the first semester of 2023, 53 new cases of *Brucella abortus* were reported in continental Ecuador [25]. Indeed, continental Ecuador can be considered as an endemic country for brucellosis, while Galapagos Islands are considered as free of the disease without vaccination [26]. In this context, diagnosis and vaccination are essential elements for the prevention and control of bovine brucellosis in continental Ecuador [27]. The vaccines recommended by WOAH are S19 and RB51 [10]. According to the Ecuadorian National Brucellosis Program, these two vaccines are used to prevent brucellosis in cattle [5]. In Ecuador,

vaccination is not mandatory, but AGROCALIDAD has registered 5 vaccines against bovine brucellosis since 1999, i.e. four S19 and one RB51 [20]. In the past, these vaccines were evaluated but only on the basis of the documentation provided by the companies and using the national Manual for the Registration of Companies and Products for Veterinary Use. It is worth mentioning that, out of the five companies registered, one was removed from the registry for lack of adequate documentation and another one could not be evaluated because it has not imported vaccine to date (unpublished data). Indeed, the vaccine quality control documental analyses were only carried out in the AGROCALIDAD official laboratory for the three remaining companies.

4.1. Quality control of bovine brucellosis vaccines

The results of this study indicate that two of these companies (commercialised S19) did not comply with vaccine quality control. In consequence, these two companies have been cancelled until they can comply with vaccine quality control in order to ensure proper immunization of animals. In general, the introduction of vaccines with a low protection index is a major drawback and is one reason of failure of control programs (e.g., [28-32]). The use of low efficacy vaccines has long-term consequences, as ineffective vaccines can promote a fertile niche in poorly immune hosts allowing the transmission of virulent *Brucella* through vaccinated animals [31,33-35]. Indeed, for brucellosis, perhaps more than for other infectious diseases, "a bad vaccine is worse than no vaccine" as it provides a false sense of security [12,31].

The brucellosis eradication programs may be very expensive in developing countries [36]. In this context, a progressive step-by-step approach is recommended [1]. In endemic countries, a first step is the registration of farms and animals, a first estimation of the true prevalence in animal and human populations, and the determination of *Brucella* spp. circulating strain(s). These go along with an appropriate vaccination campaign of animals at risk in order to decrease the disease transmission between herds and indirectly to decrease the incidence in the human compartment. The Veterinary Authority should consider several aspects when establishing a vaccination program. These include (e.g.): the disease epidemiology, incidence and prevalence, zoonotic potential, density of the exposed animal population, population immunity, risk for specific subpopulations, farm and animal identification systems, diagnostic capacity, control measures such as slaughter and compensation, legislation, availability of safe vaccines, and human, financial and material resources. In addition, in a One Health approach, a cost-benefit analysis should be carried out, considering the impact on trade and public health [37]. This implies good collaboration between the Ministries of Agriculture and Health.

In Ecuador, the lack of a strategic plan for cattle immunization forces the owners of each farm to buy the vaccine available in veterinary stores, so the animals of a farm are immunized at different ages and with different vaccines. It is crucial to emphasize that Ecuador should implement a mandatory vaccination plan and monitor it by regular serological randomized surveys to assess its achievement. The quality control of vaccine batches must be

mandatory, including the quality of seed strain, and their genetic and biochemical characteristics [38]. This quality control must be performed by a national reference laboratory inside AGROCALIDAD.

According to the Animal and Plant Health Inspection Service (APHIS), the estimated vaccine coverage is sufficient to promote herd immunity in the target population [39]. However, further studies should evaluate the immunization status of vaccinated animals one-month post-vaccination to ensure sustained protection. Aznar et al. [4] classified a farm as "well vaccinated" if the proportion of seropositive vaccinated animals was not significantly lower than the ideal 95%, a threshold considered necessary to achieve effective herd immunity. This level of immunization coverage is closely linked to vaccine quality, as vaccines with lower efficacy may not induce a strong enough immune response across the population. Therefore, evaluating the efficacy of the vaccine is crucial for determining overall coverage and the long-term success of disease control programs [37].

However, a good vaccine does not ensure a good coverage if vaccination practices are not appropriate. For example, a good vaccine stored in bad conditions (e.g. no respect of the cold chain) can have a detrimental effect of animal immunization [4]).

4.2. Compliance of bovine brucellosis vaccination practices

The assessment of bovine brucellosis vaccination practices indicated a compliance rate close to 62% and 57%, for veterinary professionals/technicians and veterinary stores, respectively. Few studies on the brucellosis vaccination procedure exist (e.g. [4]). Results are not comparable as the methodology is not completely the same. Recently, the S19 vaccine strain was isolated from in a goat in Ecuador, possibly due to the re-use of a syringe previously used in the farm to vaccinate a cow. It raised early warnings on the low level of knowledge and application of biosecurity standards in the use and labelling of vaccines [40].

In the present study, considering a theoretical optimal compliance of 100%, there is room for improvement. Education programs on vaccination procedures dedicated to persons in charge of field vaccination should be organised by AGROCALIDAD. In addition, these compliance rates were significantly different. According to the APHIS manual of procedure on bovine brucellosis, persons authorized to vaccinate must be trained and supervised [39]. Data summarised in **Supplementary material S1** can be used to contextualize the training which should be theoretical (main principles) and practical.

4.3. Limitations of the study

Concerning quality control, for budgetary reason, only a cross-sectional sampling of vaccines was implemented. It should be interesting to render this vaccine quality control mandatory for all producers who commercialise the vaccine (lot by lot). This quality control should be performed by

AGROCALIDAD and at the producers' expense. Only the vaccines with satisfactory results should be commercialised and used in the field.

The two surveys were based on a questionnaire filled by both veterinary professionals/technicians and veterinary stores. Anonymity of each actor was ensured to obtain responses corresponding to the true field reality. In rare cases, an actor had failed to respond to one or few questions (only 2% of observations); the imputed missing value was the theoretical lower score for this question. In a further study, it should be also interesting to triangulate the results of this study with observational studies during field vaccinations.

5. Conclusions

The lack of quality control for vaccines and vaccination procedures can lead to a feeling of false security. This study evidenced a serious lack of vaccine quality control for two companies, among the three companies that produce and commercialise a bovine brucellosis vaccine in Ecuador; the efficacy of vaccination is thus questionable. This observation, along with the lack of action plan against bovine brucellosis, can partially explain the prevalence of bovine brucellosis in continental Ecuador. Also, a mandatory registration of vaccination is of prime importance to better differentiate vaccinated and infected animals but also to monitor its effect (if good vaccine and vaccination procedures are in place). The results of the present study can be delivered to AGROCALIDAD, to the bovine technical committee (*Mesa Técnica Bovina*), and to veterinary professionals/technicians, veterinary stores and famers to raise awareness of all actors on their responsibilities and to improve their bovine brucellosis vaccination practices.

Author Contributions: Conceptualization, A.G.-H, J.R.-R., V.S-H, P.M.-C., J.B-R, E.D.LT.-M, M.Y.-D., H.R-M, and C.S.; methodology A.G.-H, V.S-H, J.R.-R, M.Y.-D., M.F. H. and C.S.; software, A.G.H and C.S.; validation, J.R.-R and C.S.; formal analysis methodology A.G.-H , V.S-H, J.R.-R, M.Y.-D., and C.S ; investigation, A.G-H.; resources, A.G.H , J.R.-R, M.Y.-D., E.D.LT, and C.S ; data curation, A.G-H, J.R.-R and C.S.; writing—original draft preparation, A.G-H.; writing—review and editing, J.R.-R, M.F. H.; E.D.LT.M, P.M.-C., and C.S.; visualisation, A.G-H, J.R.-R. and C.S.; supervision, J.R.-R and C.S.; project administration E.D.LT.-M, J.R.-R., P.M.-C., and C.S. All authors have read and agreed to the published version of the manuscript

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Institutional Review Board Statement: Through Memorandum No. AGR-AGROCALIDAD/CDL-2023-000777-M and Memorandum No. AGR-AGROCALIDAD/CDL-2023-000838-M, requesting the sampling of bovine brucellosis vaccines for quality control analysis to fine-tune the vaccine evaluation process to be implemented in the Agency.

Informed Consent Statement: The surveys were carried out by anonym AGROCALIDAD technicians that agreed to fill the survey. Sampling of the laboratories producing vaccines and importers was carried out with the consent of AGROCALIDAD, i.e. the Agency of Regulation and Control in charge of ensuring the quality of veterinary products.

Data Availability Statement: Data supporting the study findings are available in supplementary material S1.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Colony readings (WOAH, 2022)

Colony	Appearance of colonies before staining	Appearance of colonies after staining	Acceptance result	
			Vaccine S19	Vaccine RB51
S	Round, shiny, blue to blue greenish in colour.	No staining	99%	NA
R	Dry and granular appearance, yellowish-white matte colour.	Dissociated colonies (I, M or R) show various shades of red and purple, the surface may show radial cracks.	NA	100%
M	Transparent and greyish, and can be distinguished by their viscous consistency when touched, with a sowing handle.			
I	Intermediate in appearance between S and R forms. Slightly opaque and more granular than S colonies.			

Legend: NA = not applicable.

Appendix B. Primers used in Bruce-ladder PCR (López-Goñi et al., 2008)

Primers	Sequence	Amplicon size	DNA Target	Source of genetic difference
BMEI0998f	ATC CTA TTG CCC CGA TAA GG	1,682	Glycosyltransferase, gene wboA	IS711 insertion in BMEI0998 in *B. abortus* RB51 and deletion of 15,079 bp in BMEI0993BMEI1012 in *B. ovis*
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC			
BMEI0535f	GCG CAT TCT TCG GTT ATG AA	450 (1,320)	Immunodominant antigen, gene bp26	IS711 insertion in BMEI0535BMEI0536 in *Brucella* strains isolated from marine mammals
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA			
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1,071	Outer membrane protein, gene omp31	Deletion of 25,061 bp in BMEII826–BMEII0850 in *B. abortus*
BMEII0844r	GCG TCC AGT TGT TGT TGA TG			
BMEI1436f	ACG CAG ACG ACC TTC GGT AT	794	Polysaccharide deacetylase	Deletion of 976 bp in BMEI1435 in *B. canis*
BMEI1435r	TTT ATC CAT CGC CCT GTC AC			
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587	Erythritol catabolism, gene eryC (Derythrulose-1phosphate dehydrogenase)	Deletion of 702 bp in BMEII0427–BMEII0428 in *B. abortus* S19
BMEII0428r	AAT GAC TTC ACG GTC GTT CG			
BR0953f	GGA ACA CTA CGC CAC CTT GT	272	ABC transporter binding protein	Deletion of 2,653 bp in BR0951BR0955 in *B. melitensis* and *B. abortus*
BR0953r	GAT GGA GCA AAC GCT GAA G			
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218	Ribosomal protein S12, gene rpsL	Point mutation in BMEI0752 and *B. melitensis* Rev.1
BMEI0752r	GAT GTG GTA ACG CAC ACC AA			
BMEII0987f	CGC AGA CAG TGA CCA TCA AA	152	Transcriptional regulator, CRP family	Deletion of 2,203 bp in BMEII0986–BMEII0988 in *B. neotomae*
BMEII0987r	GTA TTC AGC CCC CGT TAC CT			

Supplementary material S1. Raw data of the study (add a link in the website of Pathogens)

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Experimental section

Study 3

Determination and characterization of (novel) circulating strains of Brucella sp. within the National Bovine Brucellosis Control Program in Ecuador

Submited in *pathogens*

Ana Garrido-Haro, Mercy- Falconi, *Paola Moreno-Caballeros, Michelle Yugcha- Díaz,* María Elena-Rovalino, *Hugo Rosero-Mayanquer, David Fretin, Constance Wielick, Claude Saegerman, Jorge Ron-Roman*

Preamble

This study focused on the cultivation and identification of circulating *Brucella* strains from retromammary lymph nodes and milk samples collected over the past three years as part of Ecuador's National Brucellosis Control Program. Cultures were performed on specific media, followed by molecular identification using PCR and MLVA techniques. A total of 25 retromammary lymph node samples from a slaughterhouse and 50 milk samples from farms previously selected by serology were analyzed. Of the samples tested, 19 retromammary lymph nodes and 35 milk samples tested positive for *Brucella abortus* by PCR. For the MLVA-16 analysis, 29 DNA samples previously tested by PCR were selected, with at least one sample per province chosen for genotyping. The MLVA-16 analysis accurately differentiated the strains present in Ecuador, which is crucial for epidemiological studies, outbreak tracking, and the design of control strategies. This study confirmed the presence of biovars 1 and 4, and for the first time, biovar 2 in Ecuador. The precise detection of *Brucella* sp. and the implementation of advanced genotyping techniques, such as MLVA, are essential for controlling this disease in the country.

Article

Determination and characterization of (novel) circulating strains of *Brucella* sp. within the National Bovine Brucellosis Control Program in Ecuador

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Abstract: Brucellosis is a zoonosis caused by bacteria of the species *Brucella* that affects both animals and humans. This infectious disease represents a significant public health and economic challenge in many regions of the world, including Ecuador. *Brucella abortus* is the most common species in cattle. This bacterium is responsible for bovine brucellosis, a disease characterized primarily by spontaneous abortions in the last third of pregnancy, retained fetal membranes, and decreased milk production. Infected animals can carry the bacteria asymptomatically and serve as a source of infection for other animals and humans. Transmission occurs primarily through direct contact with secretions, aborted fetuses, or contaminated reproductive fluids. The present study focused on the culture and isolation of circulating *Brucella* strains from retromammary lymph nodes and milk samples collected during the three last years from six provinces of continental Ecuador and within the National Brucellosis Program of Ecuador. *Brucella* culture was performed in two specific media, CITA and Farrel and their subsequent molecular identification using PCR and MLVA diagnostic techniques. For this purpose, 25 retromammary lymph node samples were collected from animals destined for slaughter at authorized slaughterhouses in each province and 50 milk samples were collected from farms. Milk samples coming from serologically positive animals. Samples were collected as part of the Agency's passive surveillance under the National Brucellosis Program, then cultured and isolated. Of the 50 isolated milks, 35 positive samples and 19 retromammary nodes

were obtained and identified as *Brucella abortus* by PCR. The Multi-Locus Variable Number Tandem Repeat Analysis 16 (MLVA-16), allowed us to accurately discriminate among the strains present in Ecuador, which is crucial for epidemiologic studies, outbreak tracing and the design of control strategies. This study confirmed the presence of *Brucella* strains of biovars 1 and 4 and, for the first time, the presence of biovar 2 in Ecuador. The isolation and accurate detection of *Brucella,* along with the implementation of advanced genotyping techniques such as MLVA, are essential components in the fight against this disease in Ecuador.

Keywords: *Brucella* spp.*;* cattle; Ecuador; MLVA-16; PCR.

1. Introduction

Brucellosis is an economically important disease in production animals worldwide (Corbel, 1997). Brucellosis, caused by species of the Gram-negative bacterium *Brucella*, members of the genus *Brucella*, is a facultative intracellular pathogen (Saegerman et al., 2010). Worldwide, brucellosis remains a major source of disease in humans and livestock. The prevalence of brucellosis in the animal reservoir directly determines its incidence in human compartment (Saegerman et al., 2010). The genus *Brucella* is remarkably homogeneous, exhibiting over 90% DNA/DNA homology (Le Flèche et al., 2006). *Brucella'*s genus is classified according to several phenotypic, genotypic, and ecological characteristics. Traditionally, *Brucella* species are identified according to their preferred host and certain biochemical and serological characteristics (Moreno and Moriyón, 2006). Brucellosis is reportable disease in the World Animal Health Information System (WOAH, 2022).

Bacteriological culture is considered the gold standard for brucellosis diagnosis due to its high specificity and ability to identify different species and biovars. This method is crucial for epidemiological studies, as it enables the determination of how the disease spreads in a region and identifies possible sources of infection (Minharro et al., 2013; Whatmore, 2009). However, this process is laborious and requires specific biosafety conditions due to the risk of infection for laboratory personnel (Godfroid et al., 2011). The genus *Brucella* consists of coccobacilli, or short bacilli, that are 0.6 to 1.5 μm long and 0.5 to 0.7 μm wide. They are usually observed as isolated cells, although occasionally they may occur in pairs or small groups. The morphology of microorganisms of this genus is fairly uniform. However, pleomorphic forms may occur in older cultures. *Brucella* is not a motile bacterium, does not form spores, and lacks true fimbriae or capsules (WOAH, 2022). Serologic assays are essential tools for the diagnosis of brucellosis, especially in resource-limited settings. The most commonly used methods include the Rose Bengal agglutination test, complement fixation test, and enzyme-linked immunosorbent assay (ELISA). These methods allow rapid and relatively inexpensive detection of antibodies directed to *Brucella*. However, they can have sensitivity and specificity issues (Nielsen and Yu, 2010).

The detection of *Brucella abortus* by polymerase chain reaction (PCR) has established itself as an effective technique due to its high sensitivity and specificity. The IS711 gene is an insertion sequence widely used in the identification of different species of the genus *Brucella* (Bricker y Halling, 1994). The Bruce-ladder is a multiplex PCR technique developed for the identification and differentiation of *Brucella* species. The Bruce-ladder was described in detail by López-Goñi et al. in 2006 and provides a robust tool for diagnosing different *Brucella* species, including those used in vaccines (López-Goñi et al., 2008). The Multiple tandem repeat variability analysis (MLVA) allows discrimination between strains, which is crucial for epidemiological studies, traceability of outbreaks and design of control strategies (Le Flèche et al., 2006). This technique is based on the amplification of specific regions of *Brucella* DNA containing tandem repeats, followed by quantification of the number of repeats present (Whatmore, 2009).

Twenty-five species have been described within genus *Brucella* and this genus is closely related to genus *Bartonella*. From a phylogenetic point of view, *B. abortus, B. canis, B. ceti, B. melitensis, B. neotomae, B. ovis, B. pinnipedialis* and *B. suis* represent the same species (*B. melitensis*), but due to different host specificities, they have been affiliated with different species (VetBact, 2024). The *Brucella* species of clinical importance to humans are *B. melitensis, B. abortus, B. suis,* and *B. canis. B. melitensis* is the most virulent and most commonly associated with human infection and is transmitted by sheep and goats. *B. abortus* is associated with cattle, *B. suis* with pigs, and *B. canis* with dogs. Classically, these species are subdivided in biovars: *B. melitensis* includes three biovars (1, 2 and 3), *B. abortus* eight (1, 2, 3, 4, 5, 6, 7 and 9), and *B. suis* five (1-5) (Bricker y Halling, 1994; Hanot Mambres et al., 2017).

Brucellosis is an important zoonotic disease in Latin America, where it is a public health and economic concern. The disease is endemic in several countries in the region and affects both animals and humans. Factors contributing to its prevalence include lack of adequate control and eradication in livestock, consumption of unpasteurized dairy products, and direct contact with infected animals, farms located in regions bordering forest areas, and in some areas, the difficulty to access the veterinary service. In addition, extensive beef herds raised at pastures with different age and productive groups inter-mingled, and induce concerns regarding hygiene practices and disease prevention measures (Bonilla-Aldana et al., 2023). The agricultural sector in Ecuador plays an important role in sustaining food sovereignty and contributes significantly to the country economy (Eras and Lalangui, 2021). In Ecuador, the national study carried out by AGROCALIDA, reported that the apparent prevalence of bovine brucellosis was 21.3% (95% CI: 16.8-26.6) at the herd level and 6.2% (95% CI: 5.5-7) at the animal level (Garrido-Haro et al., 2023). Risk factors identified after multivariate analysis were area in ha per herd > 70 ha (OR = 2.73, 95% CI: 1.18-6.32) and number of calves per animal (two or more with OR ≥ 1.8 and p-value ≤ 0.047) (Garrido-Haro et al., 2023). In the northwest of the country, the seroprevalence of brucellosis in humans was estimated at 1.88% (95% CI:

1.48-2.38), with the circulating strain being *Brucella abortus* biovar 4 (Ron-Román et al., 2014) and *Brucella abortus* biovar 1 (Ron-Román et al., 2012). The effort to control brucellosis in Ecuador is a major challenge, as it requires greater coordination between the public health and veterinary sectors, due to the lack of mandatory vaccination program, low biosecurity measures and inadequate infrastructure on farms, lack of resources, in addition to low awareness in some rural areas, hinder the effective control of the disease.

The present study aims to implement the necessary methodology in the national reference laboratory, such as the culture and isolation of *Brucella* of samples stored from 2022 to 2024, in addition to the molecular typing of strains using the MLVA.

This methodology can be associated with the speciation and even the geographical distribution and dispersion of outbreaks of a disease, contributing to its epidemiological traceability in Ecuador.

2. Materials and Methods

2.1. Study area

Brucellosis is WOAH notifiable disease that needs a follow-up and monitoring done within the National Brucellosis Program, which is the responsibility of the Agency for Regulation and Control of Animal and Plant Health- AGROCALIDAD. In order to implement the isolation and culture technique inside the agency, samples of milk and retromammary lymph nodes were stored from the years 2022 to 2024. It is important to mention that this program is covered by Resolution 131, "Manual of procedures for the control of bovine brucellosis" (AGROCALIDAD, 2016), which aims to apply control measures when animals or farms are found with confirmed cases of the disease. Indeed, when bovine animals are serologically positive (Rose Bengal and competitive ELISA), the slaughter of these animals is required. The samples analyzed correspond to the following six provinces: Morona Santiago, Azuay, Cotopaxi, Santo Domingo, Pichincha, Tungurahua (**Figure 1 and Table 1**).

2.2. Bacterial isolates

A total of 25 retromammary lymph nodes and 50 milk samples were inoculated in the BSL2+ laboratory of AGROCALIDAD, following the biosecurity measures implemented according to the pathogen of interest. Samples were inoculated in duplicate on Farrel selective medium and CITA medium (Centro de Investigación y Tecnología Agroalimentaria, Zaragoza, Spain), following the recommendations of the WOAH (2022) and De Miguel et al. (2001). Although the Farrel medium inhibiting most contaminating microorganisms (Farrell, 1974), this medium also inhibits the growth of *B. ovis* and some *B. melitensis* and *B. abortus strains*. While

the CITA medium allows the isolation of the major *Brucella* species, including *B. suis* (De Miguel et al., 2011; WOAH, 2022).

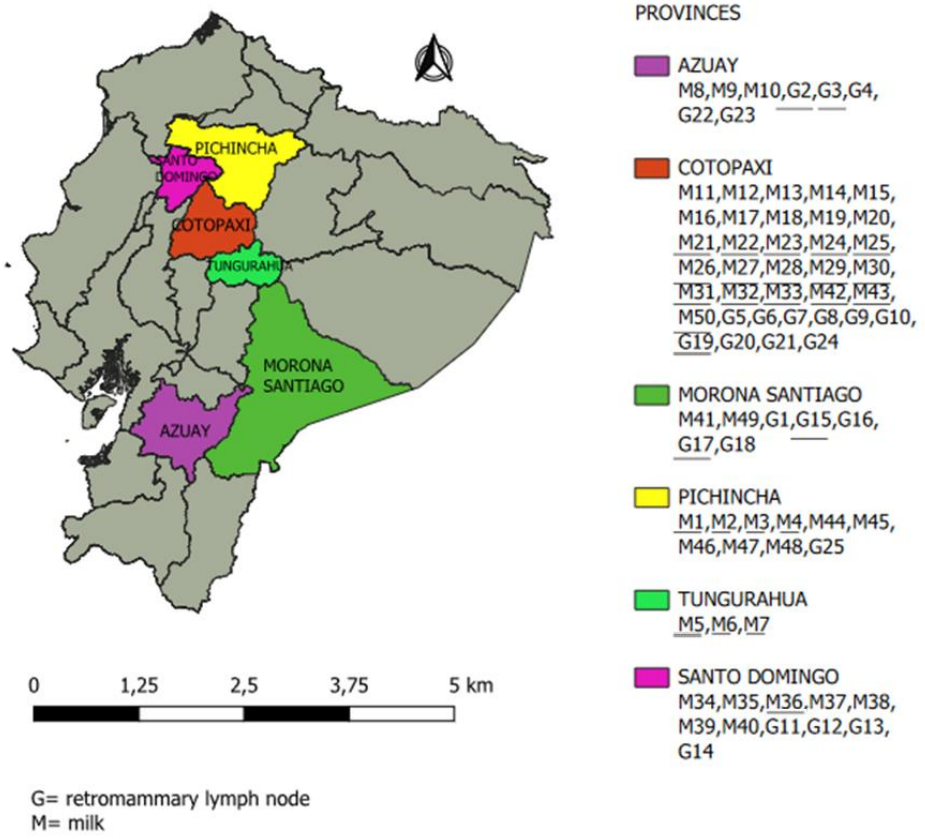

Figure 1: Map of continental Ecuador showing the six provinces sampled and milk (M) and retromammary lymph node (G) samples in each province.

Legend: Samples underlined are those for which a strain of *Brucella abortus* was isolated with an MLVA-16 profile.

2.2.1 *Sample Preparation*

Retromammary lymph nodes: Samples were aseptically collected using sterile instruments. The tissue samples were surface disinfected by immersion in 95% ethanol, followed by a brief flaming. Once disinfected, fat was removed, and the tissue was cut into small pieces. The tissue fragments were placed in sterile Stomacher bags, taking care to avoid breaking the bag. The samples were then homogenized for 1 minute in a small amount of phosphate-buffered saline (PBS) to prevent excessive dilution, which could reduce the sensitivity of the culture. The Stomacher equipment was placed inside a biosafety cabinet during the process. Subsequently, 0.1-1 ml of the homogenized sample was plated onto Farrell and CITA media to enhance sensitivity. The plates were incubated under appropriate conditions, 15 days with high humidity (near saturation) and 5-10% CO_2.

Milk samples: Once the sample arrived at the laboratory, it was refrigerated until phase separation became evident, ensuring that it was not left for too long before inoculation. A volume of 400 µL from the upper lipid layer of the sample was inoculated onto culture plates using a sterile swab or bacteriological loop, streaking for isolation. The plates were incubated with 5 to 10% CO_2 at 37°C ± 1°C for 15 days.

2.2.2 *Characterization, morphological and biochemical*

Brucella colonies were observed under a stereomicroscope. Cultures were examined on the third day. In cases where the presence of *Brucella* was confirmed but a pure culture could not be isolated, the colonies were transferred to non-selective media, namely tryptic soy agar (TSA), for at least 48 hours. Biochemical tests for oxidase, catalase and urease were performed according to the protocols described by Alton (Alton et al., 1988).

2.4. Extraction of DNA

Before DNA extraction, the *Brucella* strains were inactivated by carefully transferring approximately 6 colonies into 500 µl of molecular biology-grade water, dissolving them, and then placing the solution in a thermal block at 100°C for 20 minutes, then centrifuged for 10 minutes at 8000 rpm, the supernatant was taken. DNA extraction from the obtained *Brucella* colonies was performed with the DNA extraction kit Pure link Genomic Mini kit from Invitrogen ref K 1820-02, the obtained DNA was quantified in Thermo Scientific™ NanoDrop™ 2000 spectrophotometer and stored at -70°C for further analysis.

2.5. PCR

For the PCR of *Brucella abortus* identification, primer forward IS711: 5′- TGC CGA TCA CTT AAG GGC CTT CAT TGC CAG -3′, primer reverse Abortus: 5′- GAC GAA CGG AAT TTT TCC AAT CCC -3′, that recognize a repetitive genetic element IS711 exclusive to the species of *Brucella* (Bricker y Halling, 1994). The final concentration of the components of the diagnostic PCR reaction was: Promega Go Taq Green Master mix 1X

Buffer PCR 1X; 0.2 μM; primers forward and reverse, with a final volume of 50 μl. The temperature profile was 3min, 95°C; 34 cycles of at 95°C with 1min:15 sec, 55.5°C for 2 min, 72°C for 2 min with a final extension of 5min at 72°C. The reference material used was strain 11193, from the Reference Laboratory for Brucellosis, SENSA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, Argentina).

The samples were also analyzed using the Multiplex PCR Assay Bruce-ladder (López-Goñi et al., 2008). The final concentration of the components of the diagnostic PCR reaction was 3 mM MgCl2, 0.3 μM of the primers: 998 F, 998R, 752F, 752 R, 987F, 987R; 0.12 μM of the primers: 535 F, 536R, 843F, 844R, 436F, 435R, 428F, 428R, 955F, 953R, 1X Go Taq Master mix Promega. PCR amplification was performed in a total volume of 25 μL The temperature profile was 7 min, 95°C; 25 cycles of at 95°C with 00:35 sec, 64°C for 00:45 sec, 72°C for 3 min with a final extension of 6 min at 72°C. The reference material used was strain *Brucella suis*, from the Reference Laboratory for Brucellosis SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, Argentina).

2.6. MLVA genotyping

PCR amplification was performed in a total volume of 20 μL, the final concentration of the components of the diagnostic PCR was Go Taq 1X *Master mix Promega* and 0.3 μM of each MLVA 16 *(*Al Dahouk et al., 2007; Le Flèche et al., 2006*)*. The following PCR program was used: an initial denaturating step at 95°C during 5 min followed by 30 cycles of 96°C for 30 sec, 60°C for 30 sec, 70°C for 1 min and a final extension step of 70°C for 10 min. Genotyping was carried out using a combination of minisatellite and microsatellite repeats, following the scheme :The tandem-repeat loci so-called MLVA16 were divided into three groups, as previously mentioned: eight minisatellite loci in panel 1 (bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55), three microsatellite loci in panel 2A (bruce18, bruce19, and bruce21) and five microsatellite loci in panel 2B (bruce04, bruce07, bruce09, bruce16, and bruce30) (Le Flèche et al., 2006; Al Dahouk et al., 2007; Hanot Mambres et al., 2017). A 100 bp molecular weight marker (Invitrogen and Promega) was used in the electrophoresis, and then the size of each PCR product was converted into a corresponding tandem repeat number, as described by Le Fleche et al. (2006). The reference strain used was *Brucella* strain 2308. Genotypes obtained were compared to those deposited in the MLVA bank for Bacterial Genotyping (https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/61/ - *Brucella* V4_6_5).

2.7. Statistical analysis

The MLVA profile of isolates was subjected to minimum spanning tree (MST) analysis using BioNumerics version 6.6 software. The tree illustrates diversity within clusters based on single locus variation (SLV) (Hanot Mambres et al., 2017). Units (and not sizes) of each marker were considered in the analysis. In addition, MLVA-16 dendrogram of *Brucella abortus* analysis by geographic location in Latine America and biovars was

generated using R software version 4.4.1 on a windows operating system (https://cran.r-project.org/bin/windows/base/). The following libraries were used to generate the dendrogram: ape, phangorn, ggtree, readxl, and ggplot2.

3. Results

3.1. Characterization, morphological

The *Brucella* colonies observed under a stereomicroscope with backlighting exhibited a morphology characterized by a diameter of 1–2 mm, translucent appearance, and pale honey color. When viewed under the stereomicroscope with backlighting, the colonies displayed well-defined edges and were slightly convex (WOAH, 2022). Biochemical tests showed that all isolates were positive to oxidase and catalase with intermediate urease enzyme activity (the reaction occurs approximately after 3h of incubation).The results of *Brucella* culture on Farrel and CITA selective media show that of the 25 retromammary lymph nodes inoculated, 11 grew on Farrel but 15 on CITA. Similarly, of the milks inoculated on the two selective media, 13 grew on Farrel medium but 33 on CITA medium, see results (**Tables 1 and 2**). In total, *Brucella* culture positive was found 2-fold more with the CITA medium (N = 48) versus Farrel mediaum (N = 24). For 6 samples (including 4 retromammary lymph nodes) the Farrel was positive with negative results with CITA medium. On the opposite, 30 samples was positive in CITA medium and negative in Farrel medium.

3.2. MLVA characterization

Figure 2 shows the minimum spanning tree obtained with MLVA-16 data of Ecuadorian bovine isolates, where it can be seen that there are different genotypes isolated within the Ecuadorian territory. The samples corresponding to Pichincha (M1,M2,M3,M4) (1 herd), Cotopaxi (M42) (1 herd) and Azuay (G2) (1 herd) genotypically related. The Cotopaxi isolates (M21, M22, M23, M24, M30, M31, M32, M50) (3 herds) are also genotypically related. The samples from Tunguragua (M5,M6,M7) (1 herd), Cotopaxi (M25,M26,M27,M28,M29) (2 herds), Azuay (G3) (same herd as G2) are genotypically close. For the Cotopaxi samples (M43, G19) (2 herds) they share a similar genotype and finally for the Santo Domingo (M36) (1 herd), Morona Santiago (G15, G17) (2 herds) samples they are identified in the tree branch as close biovars.

Figure 3 shows the dendogram of *Brucella abortus* analysis in relation to geographic location in America and correspondant biovar. The analysis was performed according to MLVA-16 bank (https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/61) allowed us to identify the species and genotypes of isolates as *B. abortus* biovar 1 (9 isolates, 4 genotypes, 3 herds), *B. abortus* biovar 2 (9 isolates, 1 genotype, 3 herds), *B. abortus* group C-SRR309419 (6 isolates, 1 genotype,

3 herds), *B. abortus* C-Hernández-Mora 2017 (3 isolates, 2 genotypes, 3 herds), *B. abortus* bv 4 (2 isolates, 1 genotype, 2 herds) (**Table 3**). Regarding the geographic relationship of the different genotypes present in Ecuador in relation to those reported in the MLVA-16 bank, it can be observed that the isolates from Pichincha (M1,M2,M3,M4) - Ec_Pich , Cotopaxi (M42) - Ec_Cotop and Azuay (G2) - Ec_Azuay are genotypically close to the samples reported in the United States of America, identified as *Brucella abortus* SRR3096419, which in the tree is close to *Brucella abortus* bv 1. For the isolates from Cotopaxi (M21,M22,M23,M24,M30,M31,M32,M50) - Ec_Cotop, geographically at the American level are genotypically related to *Brucella abortus* bv. 2 genotypes from Brazil (Corbel et al., 2006; Lucero et al., 2008; Minharro et al., 2013). For the isolates from Tunguragua (M5,M6,M7) - Ec_Tung, Cotopaxi (M25,M26,M27,M28,M29) - Ec_Cotop, Azuay (G3) - Ec_Azu samples, they are closely related to *Brucella aboratus* bv. 1 isolates from Brazil and close to those reported in Argentina and the United States of America.

For the samples Cotopaxi (M43, G19) - Ec_Cotop, they share a similar genotype and finally for the samples, Santo Domingo (M36) - Ec_SD, Morona Santiago (G15, G17) - Ec_MS are identified in the tree branch as biovars relate to the genotypes reported by Hernandez_Mora2017 from Costa Rica (Hernández-Mora et al., 2017).

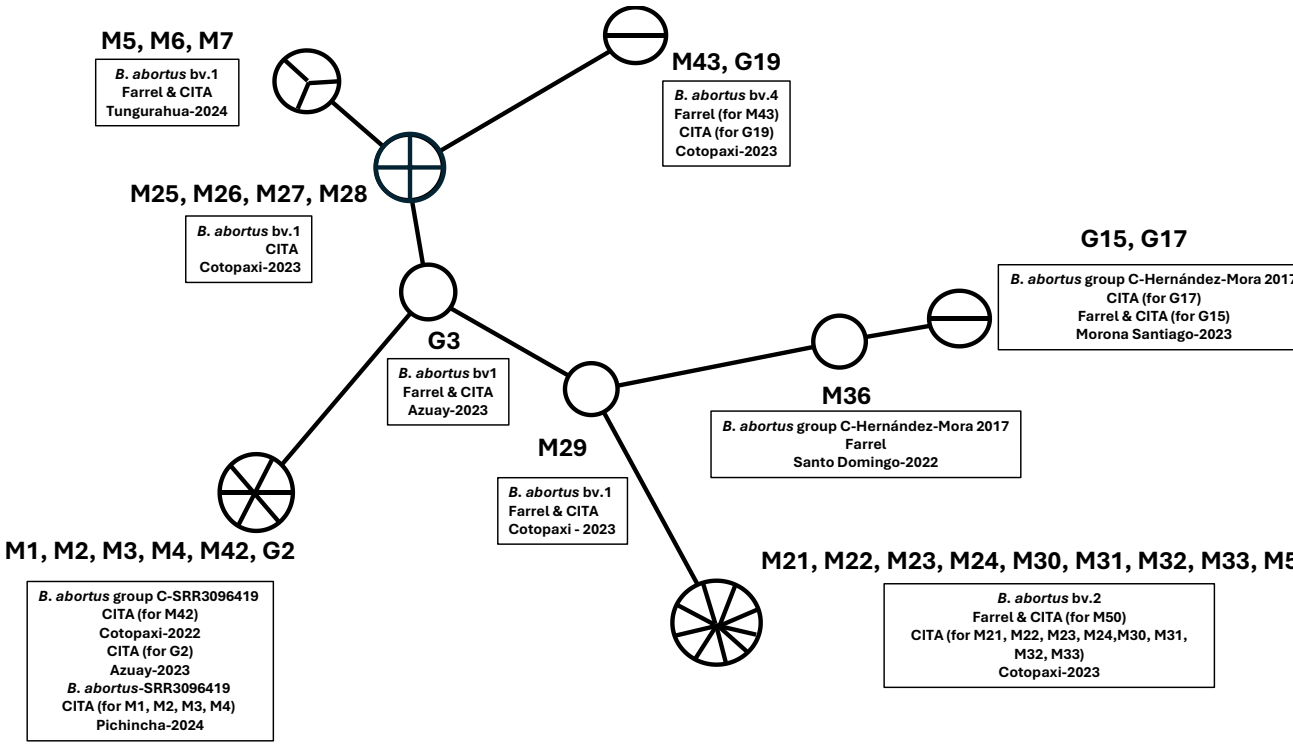

Figure 2. Minimumspanning tree obtained with MLVA-16 data of Ecuadorian bovine isolates.

Legend: Each strain is represented by a circle. The large circles group strains with identical genotypes, with the size proportional to the number of entries. The lines, whose length is based on the number of mutations (VNTR variants - variable number of tandem repeats), connect the different genotypes. The tree illustrates the genetic relationships between *Brucella abortus* strains collected from various provinces of mainland Ecuador. The presence of nine different genotypes can be identified. The connections between the nodes highlight possible connection patterns between provinces.

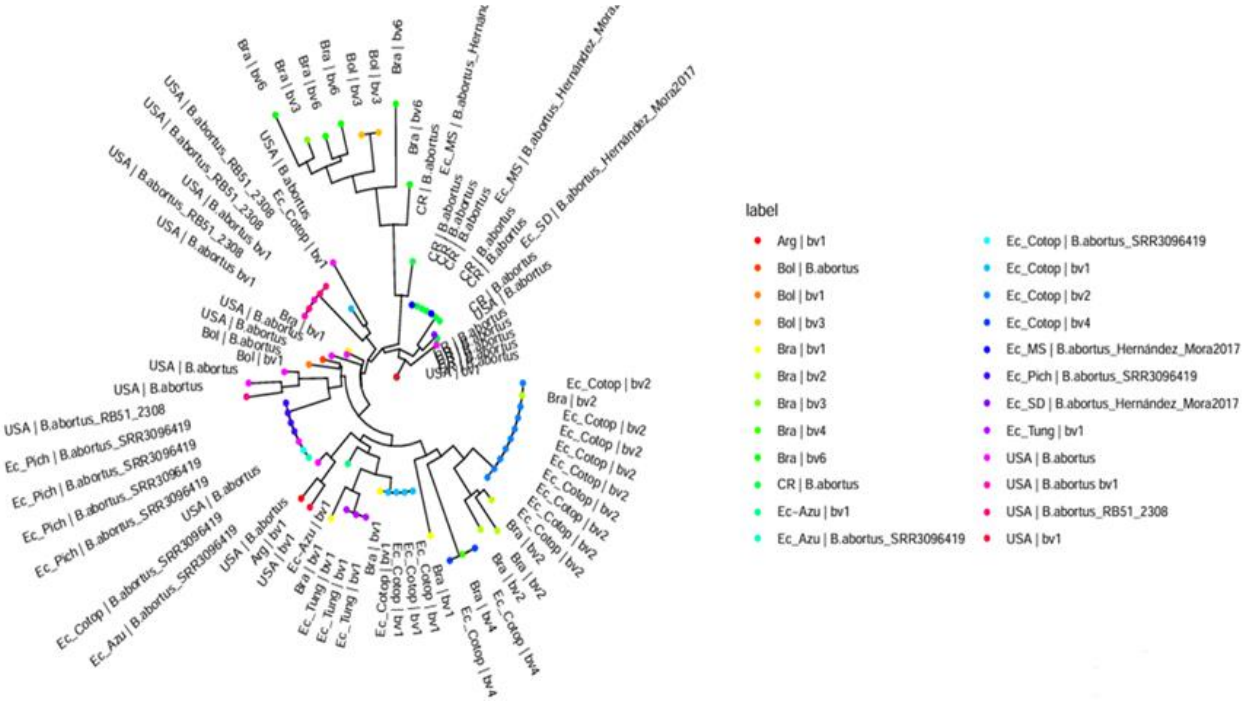

Figure 3. MLVA-16 Dendogram of *Brucella abortus* analysis related to geographic location in America and biovar. The analysis was performed according to: https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/61 (last access: 21 August 2024).

Legend: Arg, Argentina; Bol, Bolivia; Bra, Brazil, CR, Costa Rica; Ec_Azu, Azuay province of Ecuador; Ec_Cotop, Cotopaxi province of Ecuador; EC_MS, Morona Santiago province of Ecuador; Ec_Pich, Pichincha province of Ecuador; Ec_SD, Santo Domingo province of Ecuador; Ec_Tung, Tungurahua province of Ecuador; USA, United States of America; bv1, B. abortus bv1; bv2, B. abortus bv2; bv3, B. abortus bv3; bv4, B. abortus bv4; bv6, B. abortus bv6; B. abortus_SRR3096419, reference strain from USA; B. abortus_Hernández_Mora2017, refrence strain from Costa Rica.

Table 1. Results of milk samples inoculated on Farrel and CITA media and results of *Brucella abortus* and Multiplex Bruce-ladder PCR identification.

ID Number	Sample	Province	Date of sample	Farrel Growth	CITA Growth	PCR *Brucella abortus*	Bruce-ladder
M1	Milk	Pichincha	2024	0	1	1	*Brucella abortus*
M2	Milk	Pichincha	2024	0	1	1	*Brucella abortus*
M3	Milk	Pichincha	2024	0	1	1	*Brucella abortus*
M4	Milk	Pichincha	2024	0	1	1	*Brucella abortus*
M5	Milk	Tungurahua	2024	1	1	1	*Brucella abortus*
M6	Milk	Tungurahua	2024	1	1	1	*Brucella abortus*
M7	Milk	Tungurahua	2024	1	1	1	*Brucella abortus*
M8	Milk	Azuay	2023	0	0	0	0

M9	Milk	Azuay	2023	0	0	0	0
M10	Milk	Azuay	2023	1	1	1	*Brucella abortus*
M11	Milk	Cotopaxi	2023	0	0	0	0
M12	Milk	Cotopaxi	2023	0	0	0	0
M13	Milk	Cotopaxi	2023	0	0	0	0
M14	Milk	Cotopaxi	2023	0	0	0	0
M15	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M16	Milk	Cotopaxi	2023	1	1	1	*Brucella abortus*
M17	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M18	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M19	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M20	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M21	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M22	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M23	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M24	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M25	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M26	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M27	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M28	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M29	Milk	Cotopaxi	2023	1	1	1	*Brucella abortus*
M30	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M31	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M32	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M33	Milk	Cotopaxi	2023	0	1	1	*Brucella abortus*
M34	Milk	Santo Domingo	2022	1	1	1	*Brucella abortus*
M35	Milk	Santo Domingo	2022	0	0	0	0
M36	Milk	Santo Domingo	2022	1	0	1	*Brucella abortus*
M37	Milk	Santo Domingo	2022	1	1	1	*Brucella abortus*
M38	Milk	Santo Domingo	2022	0	0	0	0
M39	Milk	Santo Domingo	2022	0	0	0	0
M40	Milk	Santo Domingo	2022	1	1	1	*Brucella abortus*
M41	Milk	Morona Santiago	2023	0	0	0	0
M42	Milk	Cotopaxi	2022	0	1	1	*Brucella abortus*
M43	Milk	Cotopaxi	2023	1	0	1	*Brucella abortus*
M44	Milk	Pichincha	2023	1	1	1	*Brucella abortus*
M45	Milk	Pichincha	2023	0	0	0	0
M46	Milk	Pichincha	2024	0	0	0	0
M47	Milk	Pichincha	2023	0	0	0	0
M48	Milk	Pichincha	2023	0	0	0	0
M49	Milk	Morona Santiago	2023	0	0	0	0
M50	Milk	Cotopaxi	2023	1	1	1	*Brucella abortus*

Legend: ID, identification; 0, absence ; 1, presence.

Table 2. Results of milk samples inoculated on Farrel and CITA media and results of *Brucella abortus* and Multiplex Bruce-ladder PCR identification.

Number ID	Sample	Province	Date of sample	Farrel Growth	CITA Growth	PCR *Brucella abortus*	Bruceladder
G1	Retromammary node	Morona Santiago	2023	1	1	1	*Brucella abortus*
G2	Retromammary node	Azuay	2023	0	1	1	*Brucella abortus*
G3	Retromammary node	Azuay	2023	1	1	1	*Brucella abortus*
G4	Retromammary node	Azuay	2023	1	0	1	*Brucella abortus*
G5	Retromammary node	Cotopaxi	2023	1	1	1	*Brucella abortus*
G6	Retromammary node	Cotopaxi	2023	1	0	1	*Brucella abortus*
G7	Retromammary node	Cotopaxi	2023	0	1	1	*Brucella abortus*
G8	Retromammary node	Cotopaxi	2023	0	0	0	0
G9	Retromammary node	Cotopaxi	2023	0	1	1	*Brucella abortus*
G10	Retromammary node	Cotopaxi	2023	0	0	0	0
G11	Retromammary node	Santo Domingo	2022	1	0	1	*Brucella abortus*
G12	Retromammary node	Santo Domingo	2022	1	1	1	*Brucella abortus*
G13	Retromammary node	Santo Domingo	2022	1	1	1	*Brucella abortus*
G14	Retromammary node	Santo Domingo	2022	1	1	1	*Brucella abortus*
G15	Retromammary node	Morona Santiago	2023	1	1	1	*Brucella abortus*
G16	Retromammary node	Morona Santiago	2023	0	0	0	0
G17	Retromammary node	Morona Santiago	2023	0	1	1	*Brucella abortus*
G18	Retromammary node	Morona Santiago	2023	0	0	0	0
G19	Retromammary node	Cotopaxi	2023	0	1	1	*Brucella abortus*
G20	Retromammary node	Cotopaxi	2023	1	0	1	*Brucella abortus*
G21	Retromammary node	Cotopaxi	2023	0	1	1	*Brucella abortus*
G22	Retromammary node	Azuay	2023	0	0	0	0
G23	Retromammary node	Azuay	2023	0	1	1	*Brucella abortus*
G24	Retromammary node	Cotopaxi	2023	0	1	1	*Brucella abortus*
G25	Retromammary node	Pichincha	2024	0	0	0	0

Legend: ID = identification; 0 = absence; 1 = presence.

Table 3. MLVA16 genotype of the Ecuadorian cattle *Brucella* strains.

ID Number	Province	Date of sample	Host	Bruce Number																Biovar of *B. abortus*
				06	08	11	12	42	43	45	55	18	19	21	04	07	09	16	30	
M1	Pichincha	2024	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B. abortus*-SRR3096419
M2	Pichincha	2024	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B. abortus*-SRR3096419
M3	Pichincha	2024	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B. abortus*-SRR3096419
M4	Pichincha	2024	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B. abortus*-SRR3096419
M5	Tungurahua	2024	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	7	*B.abortus bv1*
M6	Tungurahua	2024	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	7	*B.abortus bv1*
M7	Tungurahua	2024	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	7	*B.abortus bv1*
M21	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M22	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M23	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M24	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M25	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	6	*B.abortus* bv1
M26	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	6	*B.abortus* bv1
M27	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	6	*B.abortus* bv1
M28	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	6	*B.abortus* bv1
M29	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	3	6	3	4	5	*B.abortus* bv1
M30	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M31	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M32	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M33	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
M36	Santo Domingo	2022	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	5	3	3	5	*B.abortus* group C-Hernández-Mora2017
M42	Cotopaxi	2022	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B.abortus group C-SRR3096419*
M43	Cotopaxi	2023	Cattle	4	5	4	12	1	2	3	3	6	45	8	1	4	3	4	6	*B.abortus* bv4
M50	Cotopaxi	2023	Cattle	4	5	4	12	2	2	3	3	6	45	7	3	5	3	4	5	*B.abortus* bv2
G2	Azuay	2023	Cattle	4	5	3	12	2	2	3	3	6	43	8	3	6	3	5	6	*B.abortus* group C-SRR3096419

G3	Azuay	2023	Cattle	4	5	4	12	2	2	3	3	6	43	8	3	4	3	4	6	*B.abortus* bv1
G15	Morona Santiago	2023	Cattle	4	5	3	12	2	3	3	3	6	43	8	3	5	3	3	5	*B.abortus* group C-Hernández-Mora2017
G17	Morona Santiago	2023	Cattle	4	5	3	12	2	3	3	3	6	43	8	3	5	3	3	5	*B.abortus* group C-Hernández-Mora2017
G19	Cotopaxi	2023	Cattle	4	5	4	12	1	2	3	3	6	45	8	1	4	3	4	6	*B.abortus* bv4

Legend: bv, biovar; Biovar of *B. abortus* was assigned based on the comparison of the results obtained for each locus with the MLVA Bank *Brucella* v4_6_5 public database (https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/61/). When a biovar was not reported in the literature, the group to which it belongs according to MLVA Bank *Brucella* v4_6_5 was added and solved with the supporting reference. SRR3096419: sample name (https://www.ncbi.nlm.nih.gov/sra/SRR3096419).

4. Discussion

The use of two selective isolation media, such as Farrell's and CITA's media, for the culture of *Brucella* is of great importance in the microbiological diagnosis of this bacterium (Alton et al., 1988; Godfroid et al., 2010; WOAH, 2022). In Farrel's medium (Stack et al., 2002), the concentrations of nalidixic acid and bacitracin used have inhibitory effects on some strains of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis* and some strains including the vaccine strain RB51. Indeed, it is advisable to use in addition, the CITA medium, which contains vancomycin, colistin, nystatin, nitrofurantoin and amphotericin B. The CITA medium appears to be more sensitive to a more large panel of main *Brucella* species that could be present in the samples analyzed (Marín et al., 1996; Hornsby et al., 2000; De Miguel et al., 2011; WOAH, 2022).

Throughout the world, *B. abortus* biovar 1 is the most widely isolated in cattle (Acha y Szyfres, 2003). In Latin America, *Brucella abortus* biovar 1 is the most commonly reported, with its presence documented in countries such as Argentina, Brazil and Colombia. In Argentina, biovar 1 has historically been predominant and is associated with the high prevalence of bovine brucellosis, a disease that represents an animal and public health concern of national importance (Samartino, 2002; Hollender et al., 2013). Similarly, in Brazil and Colombia, Chile, Cuba, México and Venezuela, *B. abortus* biovar 4 is frequently reported (Lucero et al., 2008; Hollender et al., 2013; Minharro, Silva Mol, et al., 2013; Torres Higuera et al., 2019). In Ecuador, *B. abortus* biovars 1 and 4 have been identified in previous studies (Ron-Román et al., 2012; Ron-Román et al., 2014). In the present study, using the multi-locus VNTR analysis (MLVA) method, biovar 2 was identified for the first time in milk samples collected from the province of Cotopaxi. This biovar has been observed in natural infections in cattle, associated with the "abortion storm" in infected pregnant animals, as well as the birth of calves that die within hours, and the occurrence of "stillbirths," where calves are born full-term but lifeless (Hollender et al., 2013). The typing of *B. abortus* strains isolated from cattle in Ecuador confirmed the presence of *B. abortus* biovar 2 in the country, supporting the surveillance of the pathogen within the national program for the control and eradication of bovine brucellosis. In neighboring countries like Bolivia and Peru, available information is even scarcer, which may be related to the limited diagnostic infrastructure and access to advanced typing techniques in these regions (Rossetti et al., 2017). Collaboration within regional countries must be stimulated in order to have a more complete and understandable epidemiological brucellosis picture.

The presence of *Brucella abortus* in Ecuador, particularly strains associated with biovar 1,2 and 4 field strains, presents a significant challenge for the control of bovine brucellosis in the country. This finding, confirmed by molecular identification techniques such as MLVA, underscores the urgent need to improve the efficacy of existing vaccines and control programs. Bovine brucellosis, caused mainly by *Brucella abortus*, is a zoonotic disease that affects both public and animal health.

The identification of *Brucella abortus* biovar 2 strains in Ecuadorian cattle reveals a genetic diversity that can complicate the design of control and prevention strategies. Genetic variability between strains can affect vaccine effectiveness (Moreno et al., 2002), highlighting the importance of tailoring vaccines to local strains to maximize their effectiveness. Currently, Ecuador faces critical problems related to the quality of brucellosis vaccines and effectiveness of field vaccination.

Understanding the dominant species and biovars of *Brucella* affecting livestock is essential for developing effective strategies to prevent and control brucellosis in animal populations (Ocholi et al., 2004). Due to the presence of different biovars and genotypes within the same species of *Brucella*, it is necessary to implement adapted surveillance and control strategy in different parts of the country to achieve effective detection and characterization of the genotypes present. Regions with more intensive surveillance can report greater genetic diversity and thus make known the effective measures to be taken within the brucellosis program (Godfroid et al., 2011).

Nine genotypes, represented by their MLVA profiles, can be identified in the table *3*. These genotypes are mainly grouped by province and year of sampling.

The genotypes found in Pichincha (M1-M4), Cotopaxi (M42 and G2) have the same profile as certain genotypes reported by the United States Department of Agriculture (USDA) and analyzed by ILLUMINA methodology (Illumina MiSeq) of *Brucella abortus*, in a sample named Sample B11-0148 - "SRR3096419" and analyzed in 2016. This suggests that these are similar strains to those found in the present study (NCBI, 2024).

In the case of milk samples from Tungurahua (M5, M6, and M7), the genotype of the samples matches with a bovine isolates reported by Minharro et al. (2013) in NCBI (https://microbesgenotyping.i2bc.paris-saclay.fr/databases/view/61/) as 2013Minharro#095 from Brazil. In addition, other studies have identified *Brucella* spp. biovar 1 in Brazil (Megid et al., 2005; Minharro et al., 2013; Poester et al., 2002). According to the records of the Animal Health Certification Department of AGROCALIDAD, Ecuador has trade agreements for the exchange of breeding cattle and breeding embryos, which could indicate the introduction of *Brucella* in Ecuador from Brazil (AGROCALIDAD, 2024*)*. The importation of infected animals from different regions or countries can introduce new genotypes into a particular region (Moreno et al., 2002). In the case of samples from Cotopaxi (M25, M26, M27 and M28), when comparing them with the MLVA Bank of *Brucella*, it can be observed that the samples have a genotype similar to those reported in Minharro et al. (2013), with a profile corresponding to the sample reported as 2013Minharro#085, which also corresponds to *B. abrotus* biovar 1. In addtion, the sample from Azuay (G3) is also concordant with *B. abortus* biovar 1 in the MLVA Bank of *Brucella*. This sample is found to match with the genotype of the sample identified as 2013Minharro#128. As can be seen in the country, there are three genotypes of *Brucella abortus* 1. This may be due to the fact that *Brucella* is a bacterium that can adapt to different hosts and environments by mechanisms such as mutation and genetic recombination, resulting in the emergence of new genotypes in the

same geographical area. Genetic mutation and recombination can create genetic diversity within a a same biovar (Moreno and Moriyón, 2006; Whatmore, 2009). Strains of *B. abortus* bv. 1 from humans in Ecuador presented certain peculiarities during typing, in particular the inhibition of growth in medium colored with safranin (100 μg/ml) and fuchsin (20 μg/ml) (Ron-Román et al, 2014). This could have anticipated the existence of different genotypes of *B. abortus* bv. 1, as evidenced by the results of MLVA-16.

The samples isolated from Cotopaxi province (M21, M22, M23, M24, M30, M31, M32, M33, and M50) were genotyped as *Brucella abortus* biovar 2, which was compared with this reported as 2013Minharro#020, 2013Minharro#022 in the MLVA Bank of *Brucella*. Continued importation of cattle from areas where this biovar is endemic may explain the occurrence and isolation of *B. abortus* biovar 2 in this study (Corbel et al., 2006; Lucero et al., 2008; Minharro et al., 2013). In addition this biovar 2 was also report in Argentina (Hollender et al., 2013).

The samples isolated in Cotopaxi province (M43 and G19) were genotyped as *Brucella abortus* biovar 4, which was compared to those from samples reported in 2013 by Minharro et al. (2013). The MLVA-16 profiles found in the present study match with the same as called Minharro#088 the MLVA Bank of *Brucella*, version v4_6_5. Although *B. abortus* biovar 4 is not the most common in Latin America. Its presence has been documented in some regions like Brazil, Argentina (Minharro et al., 2013) and Ecuador (Ron-Román et al., 2014). Its monitoring need a broader brucellosis surveillance efforts on the continent.

The samples isolated in Santo Domingo (M36), Morona Santiago (G15) and Cotopaxi (G17) provinces present the same genotypes to a strain circulating in Costa Rica (Hernadez-Mora et al., 2017). In the last, it is mentioned that the relationship of local strain with *B. abortus* found in Costa Rica is related to the circulating strain from North America and Brazil, considering that the cattle in Costa Rica came from these countries, proving that the existing relationship of circulating strains in the Americas are comparable to those of Ecuador (Hernández-Mora et al., 2017).

Looking at the frequencies of the loci, it can be observed that certain loci such as Bruce06=4, Bruce08=5, Bruce11=3 or 4, and Bruce12=12 are quite common in the strains found in Ecuador. These common loci may be indicators of specific genetic characteristics of *Brucella abortus* strains present in the country. The repetition of the profiles between different provinces suggests that the strains have been spread through cattle movements or other routes of transmission within the country. In countries with a high prevalence of brucellosis, the transport of infected animals from one region to another can facilitate the spread of the disease both locally and nationally. This problem is exacerbated in areas where identification of infected animals is not effective and/or veterinary controls are inadequate (Godfroid et al., 2013). The unmonitored movement of infected animals can spread *Brucella* to new regions, introducing the disease into previously unaffected populations. This poses animal health risks, has economic consequences and endangers public health, as brucellosis can be transmitted to humans through contact with infected animals or contaminated products (Corbel et al., 2006). In addition, the

lack of effective control and the movement of animals without adequate regulation perpetuate a cycle of infection that makes it difficult to eradicate the disease. Indeed, the government must inject the appropriate budget into the National Brucellosis Control Program. This control program could include mandatory vaccination, compensation for the slaughter of infected animals, appropriate quarantine at introduction of animals, capacity building and education. In addition, identification and comparison of local circulating genotypes of *Brucella* spp. with global strains may help to understand the epidemiology of brucellosis in Ecuador and to establish more effective control measures.

The lack of rigorous quality control has been identified as a key deficiency in Ecuador. Recent studies indicate that vaccines available in the country do not always meet the standards recommended by international organizations, which may result in inadequate immunization of animals (Garrido-Haro et al., submitted). The low efficacy of vaccines allows virulent strains of *Brucella abortus* to persist in the bovine population, increasing the incidence of the disease and complicating its eradication. Furthermore, the absence of a mandatory and standardized vaccination program contributes to the spread of brucellosis. Without a systematic and effective vaccination strategy, the ability to control the disease is severely compromised. To improve the situation, it is essential to implement a mandatory annual vaccination program for all females at risk, along with rigorous quality control of vaccines and training for veterinary professionals.

In Ecuador, brucellosis is a significant public and veterinary health concern due to less regulated agricultural and livestock practices that contribute to a higher prevalence of the disease in livestock, in addition to several predominant risk factors for the development of the disease such as the lack of resources for vaccination and control, as well as inadequate education on livestock management practices, facilitate the persistence and spread of the disease (Garrido-Haro et al., 2023). The results of the present study can be shared to AGROCALIDAD, to the bovine technical committee (*Mesa Técnica Bovina*), and to veterinary professionals and technicians, veterinary stores and famers to sensibilize all actors on the epidemiology on bovine brucellosis and to improve their bovine brucellosis management practices.

5. Conclusions

The present study allowed the identification of Ecuadorian isolates of bovine *B. abortus* at the biovar level using conventional and molecular techniques, from samples stored at AGROCALIDAD, from the years 2022-2024 and originating from 6 provinces of continental Ecuador. This analysis highlights the importance of continue surveillance and detailed genetic analysis to understand the epidemiology of *Brucella* in different regions. The unexpected identification of new *B. abortus* biovar 2 for the first time in the province of Cotopaxi in Ecuador has important public health and livestock implications. The use of Farrell and CITA media in a complementary manner maximizes the efficiency, specificity and reliability of *Brucella* isolation, which is crucial for a rapid and accurate

diagnosis of brucellosis. Due to their taxonomic and epidemiological relevance, the identification of these isolates down to the biovar level will provide genetic resources that can be used as reference strains in future research. These findings can be considered as a basis for the identification of biotypes not documented in the country and could be used in monitoring and follow-up programs of bovine brucellosis in Ecuador. Despite its cost, it is recommended to perform whole genome sequencing on several strains, which is the most specific test to identify *Brucella* spp., and to consider a new national sampling to confirm the presence of biovars that may be present and have not previously been identified.

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Institutional Review Board Statement: In Ecuador, animal health management, including bovine brucellosis, is regulated by several laws and resolutions issued by AGROCALIDAD. One of the most important regulations in this area is Resolution No. 131, which details the protocols to be followed for the sampling of bovine animals suspected of having brucellosis, as well as the conditions under which infected animals may be slaughtered. According to this resolution, sampling must be carried out using standardized techniques that guarantee the representativeness and accuracy of the results. These procedures must be carried out by official veterinarians and the results must be reported in time to allow a rapid and appropriate response.

Informed Consent Statement: The sampling was free of charge for the farmers, and the animal owners agreed with the sampling.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 4 - General discussion, recommendations, perspectives and conclusions

General discussion

Representativeness of data

As in many other Latin American countries, bovine brucellosis poses a significant challenge to animal health and public safety in Ecuador. Therefore, control of this disease is critical from both a veterinary and public health perspective. Brucellosis control and eradication is a multifaceted task that requires the proactive participation of various stakeholders, including government agencies, farmers, veterinarians, laboratories and others.

Ecuador needs to promote sustainable livestock development, which requires the implementation of effective policies. However, the lack of budget and political instability, the crisis of political security, the quality and legitimacy of democracy in the country have deprived such measures of effectiveness. The importance of state and regional investment in brucellosis control remains one of the main limiting factors due to the low budget allocated to combat this disease (AGROCALIDAD, 2024b; Dadar et al., 2021; Wolff y Dressler, 2024).

In domestic livestock, brucellosis stands as a major factor contributing to abortion and reduced fertility, significantly hindering animal production. Lack of awareness about this disease renders at-risk groups including livestock producers and their families, veterinarians, laboratory personnel, as well as dairy and slaughterhouse workers (Moriyón et al., 2023). The general public is mainly affected by the consumption of raw milk and unpasteurized dairy products and, to a lesser extent, raw organ meats, blood and offal (Corbel et al., 2006).

Agriculture in Ecuador is one of the most far-reaching strategic sectors, given its important contribution to the trade balance, the Gross Domestic Product (GDP), imports and exports. In addition, it aims to guarantee food sovereignty and security while providing employment opportunities to the economically active population (Ministerio de Agricultura y Ganadería, 2021). In Ecuador, cattle lead the livestock sector with a total of 3.9 millions head nationwide**, t**he Sierra region boasts the highest cattle population, totaling 2.0 million, which accounts for 53% of the country's total herd. Following closely behind is the Coast region, with 38.6% of the national herd (INEC, 2022). The Ecuadorian family has an average of 4 members, so it could be said that about 1.71 million people are directly or indirectly involved in cattle raising, which shows the social and economic importance of the sector (Ministerio de Agricultura y Ganadería, 2021)**.**

Although AGROCALIDAD has a national brucellosis control program, not all cattle ranchers are willing to participate in the program and obtain brucellosis-free certification, which guarantees that their cattle and by-products are free of the disease and pose no risk to human health (AGROCALIDAD, 2020). In 2022, according to AGROCALIDAD data, there were 1,750 certified brucellosis-free farms (unpublished data).

Certification of brucellosis-free herds has proven effective in disease control. To achieve this, rigorous national surveillance programs are essential to identify infected herds and implement timely measures. Effective control also involves animal surveillance, prevention of transmission to uninfected herds, and removal of carriers like dogs, cats, and rodents (Herrera et al., 2008; Renukaradhya et al., 2002b). Farmer cooperation is essential for long-term control and eradication programs. Veterinary organizations should raise farmer awareness of prevention strategies and transmission through educational programs. Ensuring adequate veterinary resources and services is essential for prevention (Dadar et al., 2021).

In Ecuador, **study 1** showed an apparent prevalence of bovine brucellosis at the herd level of 21.3% and at the individual animal level of 6.2%, using cELISA for the detection of antibodies against *Brucella*. The study conducted in Ecuador identified several risk factors associated with the presence of bovine brucellosis, such as herd size (>70 ha), the number of calves born per animal and the low level of knowledge and biosecurity on the farm. In addition, it is important to consider the study of Carbonero et al. (2018), knowing that Ecuador has provinces with extensive dairy herds and that these were identified as significant risk factors, and the lack of adequate biosecurity practices suggest that dairy herd management should have strict on-farm biosecurity measures to control brucellosis (Carbonero et al., 2018). Studies by Camus (1980) and Sanogo et al. (2012) showed that brucellosis incidence varies proportionally with herd size, which correlates with farm area (Camus, 1980; Sanogo et al., 2012).

It should be noted that no single serological test is suitable in all epidemiological situations of brucellosis, nor all animal species. All have limitations. For a particular interpretation or diagnostic application, all factors influencing the suitability of the analytical method and isolated test results should be considered (WOAH, 2022). For example, the study by Paucar et al. (2021) mentions that although serologic tests are useful, the accuracy may vary depending on the test and the population studied. In addition, their study showed that small and medium cattle owners in Ecuador face particular challenges in the diagnosis and control of brucellosis (Paucar et al., 2021).

The variability in seroprevalence rates and test accuracy highlights the importance of adapting control strategies to local conditions and herd type. The implementation of biosecurity and control measures, together with the appropriate selection of diagnostic tests, is essential for the effective management of brucellosis in Ecuador (Sanogo et al., 2013).

The control and management of bovine brucellosis is a critical animal health issue, particularly in countries such as Ecuador where the disease is endemic (Garrido-Haro et al., 2023). The implementation of control programs, such as vaccination with *Brucella abortus* S19 and RB51 strains, aims to reduce the spread of this disease, which affects both cattle and humans. However, the effectiveness of these programs depends largely on the quality of the vaccines and the vaccination practices used in the field.

This study found that two of the three laboratories supplying vaccines to Ecuador did not meet the quality control parameters established by the World Organization for Animal Health (WOAH), particularly with regard to the number of colony-forming units (CFU) in the standard subcutaneous dose. This finding underscores an important concern, as a vaccine that does not meet quality standards may not induce adequate immunity in animals, thereby compromising brucellosis control efforts (WOAH, 2022).

Vaccine quality is a critical aspect of effective brucellosis prevention. Vaccines that do not meet quality criteria may result in inadequate protection, which could lead to disease outbreaks, even in vaccinated animals. In addition, field vaccine management practices, such as proper reconstitution and administration of vaccines, are equally important. The absence of specific vaccination programs in endemic regions, or the fact that they are incomplete, are some of the reasons why the prevalence of brucellosis in certain herds does not decrease. For example, if only some animals are vaccinated, or if vaccination is delayed, it is easier for the rest of the herd to remain exposed to the disease (Gobierno de Mexico, 2019). Surveys of veterinarians and technicians in Ecuador revealed deficiencies in these practices that may contribute to the persistence of brucellosis in the country.

The review of brucellosis management and control practices in Ecuador also highlights the need for better training and supervision of technicians and veterinarians responsible for vaccination. It is essential that these professionals are well informed and trained in the handling and administration of vaccines, as well as in the biosecurity measures necessary to prevent the transmission of brucellosis (Dadar et al., 2021).

The lack of vaccines that meet vaccine quality standards in Ecuador, in addition to vaccination management practices, are deficiencies that may compromise efforts to control and eventually eradicate bovine brucellosis in the country. It is essential that animal health authorities, such as AGROCALIDAD, strengthen vaccine quality control processes and improve the training of technicians and veterinarians involved in vaccination programs, in addition to the urgent need for Ecuador to implement mandatory mass vaccination (Aznar et al., 2017; Garrido-Haro et al., 2023; Ron-Román et al., 2014).

In addition, it is necessary to implement continuous monitoring of the effectiveness of vaccines in use and to ensure that vaccination practices follow international guidelines. Further studies are also recommended to evaluate the long-term impact of vaccines that do not meet quality standards and to explore possible improvements in vaccination and brucellosis control strategies in Ecuador **(study 2).**

The third study (**study 3**) presented in the research focused on the determination of circulating strains of *Brucella* sp. in positive cattle of the national bovine brucellosis control program in Ecuador, The research involved the isolation of *Brucella* strains from milk and retromammary lymph nodes collected between 2022 and 2024, using culture and molecular identification techniques such as PCR Bruceladder and MLVA-16. The findings confirmed the presence of *Brucella* biovars 1, 4 in Ecuador and the identification for the first time of *Brucella abortus* biovars 2 in milk samples from the province

of Cotopaxi in Ecuador. *Brucella abortus* is divided into 1 ,2, 3 ,4 ,5 ,6 and 9 biovars, of which biovars 1 to 3 are the most frequently represented biovars in strains isolated from humans (Zhijun Zhong, et al., 2012), hence the importance of maintaining adequate genetic surveillance at the country level since these *Brucella abortus* strains represent significant challenges for public and veterinary health.

In the fourth study **(study 4)** found that the actual rate of bovine brucellosis varies greatly depending on whether cows are vaccinated and which vaccine is used. The S19 vaccine (true animal prevalence of 0.97%), appears to be more effective than RB51(true animal prevalence of 2.75%), as the prevalence of disease in cows receiving the S19 vaccine was much lower.

The study shows that the implementation of compulsory mass vaccination against bovine brucellosis in Ecuador is indeed necessary and depends on the commitment of the government. The vaccination rates achieved were (only 13% at farm level and 24% at animal level), in the Coast and Highlands regions.

The iELISA test (**study presented in appendix 1**) with a new specific antigen analyzed had respectively a sensitivity and a specificity of around 94% and 98% in the detection of bovine brucellosis and could be proposed as an alternative test within the national brucellosis program.

Bovine brucellosis has a significant impact on both animal health and public health, underscoring the need to address it from a “One Health” approach. Despite having a National Brucellosis Control Program, factors such as lack of investment, low participation from farmers, and issues with vaccine quality hinder control and eradication efforts. This disease considerably affects livestock production, a key sector in the country’s economy, highlighting the urgency of implementing more effective policies, such as mass vaccination and improved biosecurity practices. These challenges require greater government commitment and collaboration among all involved stakeholders, including the Ministry of Agriculture, the Ministry of Health, and the Ministry of Environment. The latter is crucial, as brucellosis can affect multiple species, making it essential to conduct monitoring in both terrestrial and marine wildlife. Only through an integrated and collaborative approach can a sustainable and effective control of this disease be ensured.

Control program

Ecuador's National Brucellosis Program, managed by AGROCALIDAD, aims to prevent, control, and eradicate bovine brucellosis. The program involves rigorous epidemiological surveillance, both passive (through public reporting of suspected cases) and active (via planned monitoring of cattle populations). AGROCALIDAD is responsible for inspecting, diagnosing, and quarantining affected farms. When cases are suspected or confirmed, they are immediately reported, initiating containment measures such as marking infected animals, enforcing biosecurity protocols, and ultimately culling positive cases. Additionally, restrictions on animal movement and public health measures are implemented to ensure control and prevent further spread of the disease. It is important to note that AGROCALIDAD is not responsible for cattle vaccination; this responsibility falls on individual farm owners. AGROCALIDAD also serves as the regulatory body for the registration of biological products. The agency outlines procedures for the approval of live or attenuated bacterial vaccines, adhering to international standards set by WOAH-CAMEVET for the control of finished veterinary biological products (AGROCALIDAD, 2016).

Because of animal movement between countries, the situation of brucellosis in the bordering countries of Ecuador is of importance. In the case of Colombia, bovine brucellosis is endemic, except three self-declared zones free of bovine brucellosis. According to a study from 2006 to 2012, the average prevalence found was 22 to 23% at herd level and 4.7 to 4. 6% at animal level (Cárdenas et al., 2018). In addition, a study carried out in 2015 and 2016 identify the risk factors associated with the disease. These risk factors are the reintroduction of brucellosis into herds previously free of brucellosis through the introduction of replacement animals and bulls from farms with unknown or positive brucellosis status and artificial insemination with non-certified semen (Cárdenas et al., 2019). The study of Ramirez (2020) highlights the importance of developing effective strategies to reduce the prevalence of the disease due to the high seroprevalence in animals and at the herd level (Ramirez et al., 2020). Indeed, it is necessary to reformulate the control program, review vaccination strategies, culling strategies and education plans focused on the cost of the disease.

In the case of Peru, studies reported indicate that the country needs to conduct a brucellosis study with more emphasis on cattle to determine the current true situation in Peru (Meza et al., 2010; Rossi, 2018; Ventocilla et al., 2009; Zavala et al., 2011).

Brazil is the country with the largest territory in Latin America, followed by Argentina, so it is important to review their control and eradication program of this disease. In Brazil, bovine brucellosis caused by *Brucella abortus*, is present throughout the country, with prevalence varying between states and within the productive areas of the states (Clementino and Azevedo, 2016). The data from prevalence studies were consolidated to determine the practices that could be associated with the

risk of brucellosis. It was obtained as a conclusion that the activities that increase the prevalence, increase the brucellosis transmission and influence the dynamics of the disease are the frequency of animal replacement, the difficulty of individualized animal management and problems related to sanitary control, number of females per herd, trade without appropriate conditions, artificial insemination with semen of low quality control standards (de Alencar Mota et al., 2016).

In the case of Argentina, the National Service of Health and Agrifood Quality (SENASA), in its last prevalence study conducted in 2014 in the area of major cattle production, obtained an animal prevalence of 0. 81% and a herd prevalence of 12.35% in the eleven provinces included in the study (SENASA, 2014). Another study in the provinces of La Pampa and San Luis have determined the prevalence of 1.8% at animal level and of 19.7% at farm level (Aznar et al., 2015). There is no significant difference of prevalence in the two previous studies. In addition, the vaccination is well implemented, but the practices of vaccinators should be improved, cattle producers should review the symptomatology associated with the disease, acquire cattle from farms officially free of brucellosis, perform diagnostic tests, implement quarantine procedures before introducing animals into the herd, implement adequate immunization by the way of massive vaccination campaigns in the study district (Aznar et al., 2017).

In 2020, some regions of Chile were classified as free of bovine brucellosis, which correspond to the regions of the northern macrozone, from the region of Arica and Parinacota in the north to the region of Coquimbo and the southern regions. Some regions are not-free of bovine brucellosis, which correspond to the Central and Southern Chile (Servicio Agrícola y Ganadero, 2020). Chile has a annual percentage incidence of bovine brucellosis of 0.024 in 2015 (Ferreira, 2018).

The diagnostic tests used in these countries are based on initial screening and confirmatory diagnosis. In the case of Ecuador, the national program mentions the use of the Rose Bengal test as screening and the cELISA test as confirmatory. However, among the diagnostic tests carried out by the Agency for Regulation and Control, there are SAT test, polarized fluorescence test, and with the support of Belgian cooperation it has been possible to implement in 2024 the culture and isolation of *Brucella* and its subsequent molecular identification by PCR, Bruce-ladder and use of MLVA, for strain genotyping. Similarly, for Colombia it is mentioned that the same serological tests are used, and they also have *Brucella* isolation. In the case of Peru, the studies reviewed indicate the use of Rose Bengal and milk ring test as screening test, and complement fixation and indirect ELISA as confirmatory test. In Argentina, Rose Bengal, 2-mercaptoethanol (2ME), SAT test, ELISA are officially used. Besides, in Argentina there is a Reference Laboratory for Brucellosis, so they have a Laboratory with good level of biosafety where the isolation of *Brucella*, and the elaboration of vaccine strain 19 were done. In addition, they perform quality control of vaccines. In Chile, the Rose Bengal, the complement fixation test and the cELISA are officially recommended.

In these countries, S19 and/or RB51 vaccines are administrated subcutaneously. The estimated age of vaccination of the females is 3 to 8 months. In adults, a boost with RB51 is sometimes performed (PAHO, 2023).

In all the Latin American countries mentioned before, the importance of strengthening brucellosis control programs has been identified in order to reduce the prevalence in animals and herds, limit animal movements and stress quarantine procedures before introduction of animals into the herd, make surveillance of animals, wildlife and humans, promote appropriate immunization and mass vaccination campaign, apply procedure to limit the risk of infection by artificial insemination (safe origin of the semen), and promote a personalized health education program on main principles of management against brucellosis.

Strengthening veterinary services and the capacity of the livestock sector is based on good risk management, so veterinary services and control agencies must be unified for joint attention based on "One Health". To this end, it is important to work on the allocation of economic resources, an efficient eradication policy and effective disease control (Godfroid et al., 2011a; Pappas et al., 2006; Aznar et al., 2017).

Importance of identification and typing of circulating strains of *Brucella* in cattle

The identification and typing of prevalent *Brucella* strains in cattle is essential for the effective control of bovine brucellosis, a zoonotic disease of high public and animal health relevance.

Brucella strain identification and typing allows veterinary authorities to develop more precise and effective control and eradication strategies. Strain identification provides insight into transmission dynamics and allows vaccination and biosecurity programs to be adapted to the specific epidemiological characteristics of each region (WOAH, 2022).

Brucella strain typing provides valuable information for epidemiologic surveillance, which is essential for early detection of outbreaks and prevention of their spread. As noted by Garofolo et al. (2017), molecular identification of strains allows tracking of transmission routes and identification of sources of infection, which is essential for real-time decision making during an outbreak (Saegerman et al., 2010; Garofolo et al., 2013).

Bovine brucellosis, due to its zoonotic nature, has a significant impact on public health, as the prevalent strains in livestock can help to predict and prevent potential human infections. Accurate identification of these strains is essential for assessing the risk of transmission to agricultural workers and the general population (Moreno et al., 2002). In addition, the development and effectiveness of vaccines against brucellosis depend on the knowledge of local strains, as genetic variability can influence their efficacy, according to (Khurana et al., 2021).

The implementation of advanced identification methods should be a priority for health authorities in regions where brucellosis is endemic.

Cattle vaccination

In the case of Ecuador, vaccination against bovine brucellosis is not mandatory and is under the responsibility of the farmer (AGROCALIDAD, 2016). This is an obvious problem facing the country (Ron-Román et al., 2014). Historically, vaccination has been recognized as the most straightforward and powerful way to control infectious diseases. The most effective programs for brucellosis in livestock (cattle) have been achieved through the use of live attenuated vaccines such as strain 19 (Zriba et al., 2019). *Brucella abortus* strain S19 has historically been the reference vaccine for bovine brucellosis prevention due to its high efficacy and ability to induce long-lasting immunity (WOAH, 2022).

The results of the Bayesian analysis (see appendix – study 4) evaluated the diagnostic sensitivity and specificity of a cELISA and an iELISA with a synthetic antigen, and the true prevalence of bovine brucellosis in different subpopulations (unvaccinated, vaccinated with the S19 strain and vaccinated with the RB51 strain). It was found that the prevalence in S19 vaccinated animals was lower than that obtained for RB51 vaccine. The S19 vaccine has been shown to be more effective than the RB51 vaccine in eradicating brucellosis by protecting cattle against bovine brucellosis (Blasco et al., 2023). While S19 has been successful in eradicating bovine brucellosis in large-scale programs, there is no convincing evidence that RB51 contributes significantly to these efforts (Blasco et al., 2023).

The subcutaneous administration of S19 vaccine to young calves has been shown to be effective in inducing protective immunity, which is a major contributor to bovine brucellosis control programs. An ideal vaccine against brucellosis should: (i) be live and stimulate a strong Th1 immune response; (ii) not interfere with diagnostic serological tests; (iii) be attenuated, avoiding disease or persistent infection in animals and not be pathogenic to humans; (iv) provide strong, long-lasting protection, preventing systemic and uterine infection and abortion with a single dose, even in pregnant animals; (v) not cause seroconversion upon revaccination; (vi) remain stable without reverting to virulence; and (vii) be inexpensive, easy to produce, and to administrate (Corbel, 1997; Saegerman et al., 2010; Dorneles et al., 2015).

Brucellosis control strategies

Brucellosis control in low-income countries faces many challenges, including limited resources, poor sanitation infrastructure, and difficulties in implementing large-scale control programs.

To reduce the prevalence of bovine brucellosis in Ecuador, it is essential to adopt mandatory annual vaccination of all female bovines before service. Vaccination should only be administered by veterinarians and technicians certified by the relevant control agency. Additionally, it is crucial to

implement strict quality control for vaccines and regulate their sale and administration. Studies should be conducted every four years to determine the prevalence of bovine brucellosis, and the control program should be continuously evaluated. Simultaneously, nationwide educational campaigns should be carried out to raise awareness about bovine brucellosis and its implications for human health, thereby strengthening the control program. Lastly, follow-up on the properties included in the study is necessary to ensure the effective implementation of these measures (Nielsen, 2017).

Mass vaccination is one of the most effective strategies to control bovine brucellosis in endemic countries. In low-income countries, the use of live attenuated vaccines such as strain 19, which have been shown to be effective in preventing the disease, is recommended. However, it is crucial to adapt vaccination to local strains and ensure that vaccines are available at an affordable cost to farmers (Moreno et al., 2002; 2022).

Educating veterinarians, farm workers, and farmers about the importance of brucellosis control and biosecurity practices is essential. Education can improve vaccination rates and compliance with preventive measures (Schurig et al., 2002).

In low-income countries, where it may be difficult to control the movement of livestock, it is essential to implement strict measures to limit the spread of brucellosis. This may include the use of quarantines, certification of brucellosis-free animals prior to movement, and the establishment of brucellosis-free zones. In situations where brucellosis is widespread and other measures are not effective, it may be necessary to resort to the slaughter of infected animals. This measure, although drastic, should be accompanied by fair compensation programs for farmers to ensure cooperation and minimize economic losses (Moreno et al., 2002; Nielsen, 2017).

Brucellosis control must be approached from a "One Health" perspective, involving collaboration between the animal health, human health and environmental sectors. This strategy is essential for effective control of brucellosis due to its zoonotic nature. The development of joint control programmes combining animal and human surveillance can significantly improve early detection and rapid response to outbreaks, thereby strengthening disease prevention and control measures (Ko and Splitter, 2003).

As brucellosis is a disease of global importance, low-income countries such as Ecuador can benefit from the support of international organizations such as the World Organization for Animal Health (OIE) or the World Health Organization (WHO), or apply to international projects such as the BruTryp project supported by the Belgian Cooperation, which can provide funding, technical resources and training. An effective strategy to maximize the use of available resources could be the implementation of pilot projects funded in specific areas to test different control strategies before scaling them up on a national scale.

The presence of *Brucella abortus* in Ecuador, particularly strains associated with biovar 1,2 and 4 field strains, presents a significant challenge for the control of bovine brucellosis in the country. This finding, confirmed by molecular identification techniques such as multilocus variable number tandem

repeat analysis (MLVA), underscores the urgent need to improve the efficacy of existing vaccines and control programs. Bovine brucellosis, caused mainly by *Brucella abortus*, is a zoonotic disease that affects both public and animal health.

The identification *of Brucella abortus* biovar 2 strains in Ecuadorian cattle reveals a genetic diversity that can complicate the design of control and prevention strategies. Genetic variability between strains can affect vaccine effectiveness. Currently, Ecuador faces critical problems related to the quality and effectiveness of brucellosis vaccines. The lack of rigorous quality control has been identified as a key deficiency. Recent studies indicate that vaccines available in the country do not always meet the standards recommended by international organizations, which may result in inadequate immunization of animals. The low efficacy of vaccines allows virulent strains of *Brucella abortus* to persist in the bovine population, increasing the incidence of the disease and complicating its eradication. Furthermore, the absence of a mandatory and standardized vaccination program contributes to the spread of brucellosis. Without a systematic and effective vaccination strategy, the ability to control the disease is severely compromised. To improve the situation, it is essential to implement a mandatory annual vaccination program for all females, along with rigorous quality control of vaccines and training for veterinary professionals.

Recommendations

Based on the results of this study and knowledge of the national situation, the following recommendations are made for each actor involved.

Recommendations to the control entity

It is essential that vaccination against brucellosis be made mandatory at the national level. Experience in other countries has shown that systematic vaccination is one of the most effective tools for reducing the prevalence of this disease. This measure should be accompanied by educational campaigns to make farmers aware of the importance of vaccination (Godfroid et al., 2010; USDA, 2024).

The control entity must put in place a strict system for recording the delivery of vaccine doses, certifying and evaluating the efficacy of the vaccines used to prevent brucellosis. This includes purchasing high-quality vaccines and ensuring their distribution and storage are strictly supervised to ensure efficacy (Blasco et al., 2022). In addition, a supervision of the implementation of vaccination campaigns is of prime importance.

In order to promote the elimination of contaminated animals and enhance adherence to management protocols, a compensation scheme for farmers must be established. Successful brucellosis

eradication strategies frequently compensate for culling diseased animals (Narrod et al., 2012; Roth et al., 2003).

Although there is a reference laboratory with technical capacity, it is essential to review and update the diagnostic methods used. This will ensure that the most accurate and effective techniques are used to detect brucellosis, which is essential for the control and eradication of the disease (Nielsen, 2017; WOAH, 2022). In addition to the previously mentioned recommendations, it is important that the control entity actively participates in proficiency testing exercises. These exercises allow to evaluate and improve the accuracy and consistency of the laboratories performing brucellosis diagnostics. Through participation in international proficiency testing programs, the national reference laboratory can ensure that the diagnostic methods used in the country meet international standards and are comparable to those used in other regions of the world. This is crucial to maintain the credibility and effectiveness of disease control and eradication measures (Wiegers, 2004).

Finally, it is recommended that the control Authority strengthen its cooperation with international and regional organizations in order to exchange experiences and obtain technical and financial support. In addition, scientific development agreements should be made with the scientific community. In addition to collaborating with the Ministries of Health and Environment, with the goal of working together under the "One Health" approach. This will facilitate the implementation of more effective strategies and allow for a more coordinated response to brucellosis (McDermott and Arimi, 2002).

Recommendations for veterinarians and technicians

Veterinarians should work closely with the regulatory authority to ensure proper brucellosis surveillance. To this end, it is essential that they become accredited under the brucellosis control program, which will allow them to actively participate in surveillance, case reporting, and disease control and eradication programs. This collaboration with health authorities is essential to coordinate efforts and optimize brucellosis management strategies (CDC, 2024).

Regular diagnostic testing of the herd by a veterinarian is essential, especially in areas where brucellosis is an endemic disease. Early diagnosis allows the identification and isolation of infected animals, which is critical to preventing the spread of the disease (Khurana et al., 2021).

Vaccination and herd management are two examples of control techniques that veterinarians should oversee and implement. Strict biosecurity procedures must be implemented, including quarantine of newly acquired animals and isolation of those showing symptoms of disease (Blasco et al., 2022).

It is essential that veterinarians educate farmers about the importance of controlling and preventing brucellosis. This includes explaining the risks associated with the disease, preventive measures, and the importance of reporting suspected cases (e.g. abortion) to the appropriate health authorities (Corbel et al., 2006).

When handling sick animals, veterinarians should ensure that they are isolated and, if necessary, safely disposed of in accordance with the Regulatory Agency's guidelines. To protect itself, use of individual protective equipment is recommended. To prevent the disease from spreading to humans, it is also important to safely handle meat and dairy products from these animals (USDA, 2022).

It is important for veterinarians to stay current on advances in the diagnosis, treatment, and prevention of brucellosis. Participation in continuing education programs and review of the scientific literature are key to ensuring the application of best practices.

Recommendations to livestock farmers

Although there is no compulsory vaccination against brucellosis in Ecuador, producers must protect their cattle, for which it is essential to vaccinate young females between 3 and 8 months with the S19 strain (WOAH, 2022). Once vaccination has been carried out, appropriate follow-up should be carried out according to the guidelines of veterinarians and the regulatory body (MSD, 2024).

When using artificial insemination, ensure that the semen is from bulls that have been confirmed to be brucellosis-free. In addition, avoid purchasing diseased animals by performing diagnostic testing to confirm they are free of the disease before introducing them to your herd (Acha y Szyfres, 2003). Periodically perform diagnostic tests on animals in the herd and monitor those that have experienced abortions or have shown prominent signs of the disease (Nicoletti, 1980). Keep newly acquired animals in quarantine until they are confirmed to be free of brucellosis. Additionally, immediately isolate any animal showing signs of the disease and notify health authorities (FAO, 2024).

Farmers are advised to regularly clean and disinfect facilities, especially calving areas and isolation pens. In addition, limit access to the farm and prevent the entry of vehicles and people who may have been in contact with infected animals (FAO, 2014b).

Educate farm workers on the signs of brucellosis, preventive measures and the importance of good hygiene. In addition, it is recommended to educate the community on the importance of brucellosis control to prevent the spread of the disease (Corbel, 2006; Moriyón et al., 2023).

Keep detailed records of vaccinations, diagnostic tests, and any incidence of disease within the herd, and report any suspected cases to health authorities for prompt action. Ensure compliance with local regulations on brucellosis, including control and eradication measures imposed by authorities, and participate in national or regional brucellosis control and eradication programs whenever possible (USDA, 2024; WOAH, 2022).

Recommendations for vaccine dispensers

Vaccine distributors should provide clear recommendations on proper usage, including correct dosage, ideal administration timing (such as the animal's age), and necessary precautions before and after vaccination. They should also emphasize the importance of following veterinary guidance (Corbel et al., 2006). It is essential that distributors have a thorough understanding of the various products they handle, including the different vaccines available, their dosage recommendations, and administration guidelines. This knowledge will enable them to recommend the most appropriate product according to the customer's needs and local regulations (USDA, 2024).

Proper vaccine storage is critical for maintaining the efficacy of vaccine. *Brucella* vaccines must be stored at appropriate temperature, typically under refrigeration, and distributors must ensure that the cold chain conditions are maintained at all times. Additionally, it is essential to regularly monitor the temperature of refrigeration equipment to prevent any loss of vaccine effectiveness (OIE, 2020).

It is important that retailers do not sell expired products, as this could jeopardize animal health and negatively impact brucellosis control efforts. When vaccines expire, they must be disposed of safely and responsibly in accordance with local regulations and the manufacturer's instructions (WHO, 2014).

The vaccines against *Brucella* should only be sold with a veterinarian's prescription. This ensures that the vaccination is administered under the supervision of a qualified professional who can assess the patient's health situation and choose the most appropriate vaccination plan. The sale of vaccinations under prescription also helps to prevent the indiscriminate or improper use of vaccinations (CAMEVET, 2017).

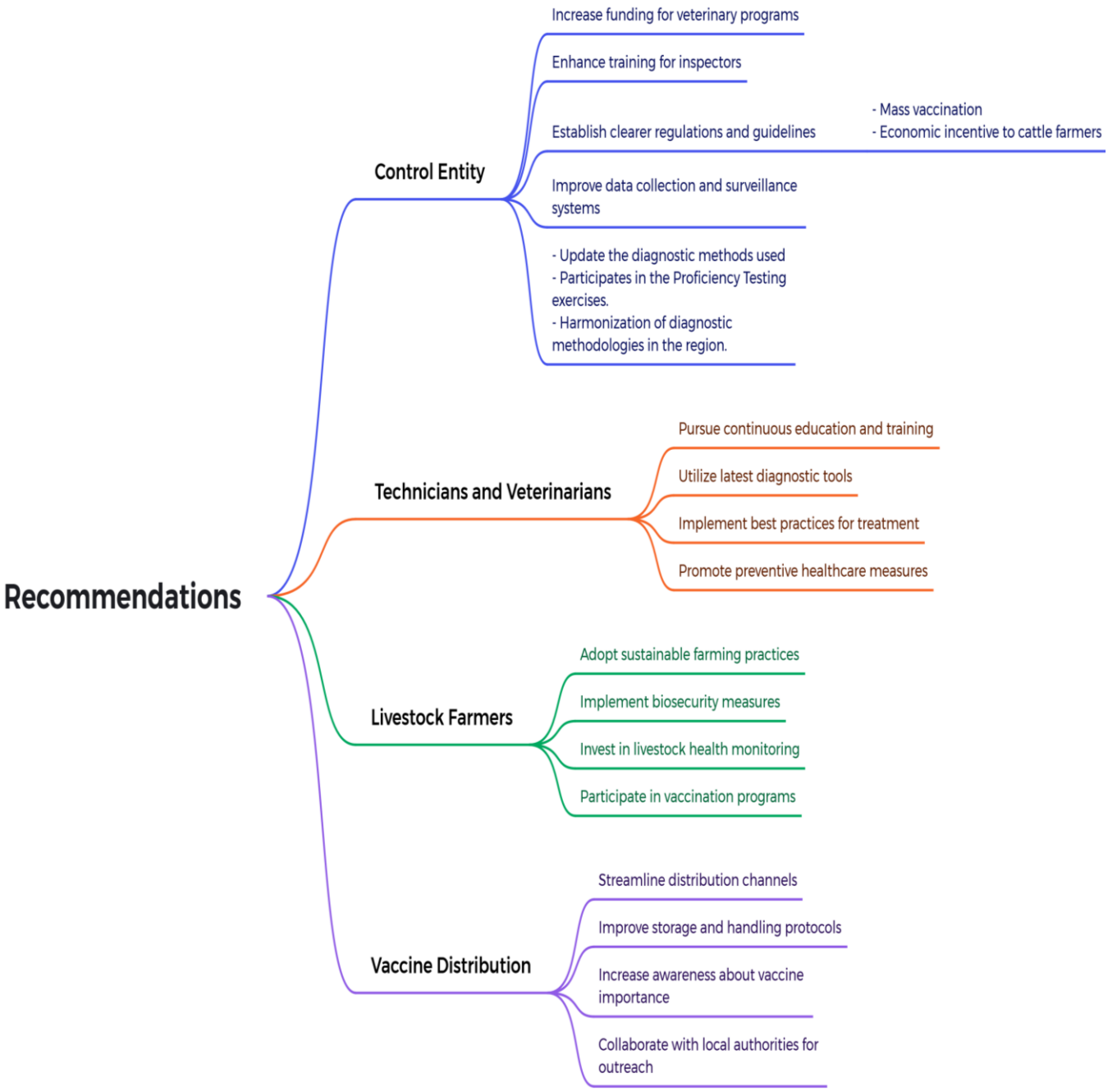

Figure 7 *:* Summary of general recommendations for the different stakeholders.

Implementation of recommendations in the short, long and medium terms

For a country like Ecuador, with a high prevalence of the disease, and especially considering the limited resources available, rigorous planning with short, medium and long-term goals is required. The following is a proposal for the Regulatory and Control Agency and other key stakeholders involved, justifying each recommendation and detailing the estimated timeframe for its implementation, considering the economic situation of the country and the high prevalence of the disease. This plan takes into account the need for efficient use of resources, focusing initially on low-cost, high-impact interventions, and then consolidating and expanding interventions as results are achieved and program sustainability is ensured. This proposal should be presented and discussed in the new platform of exchange of information between actors created inside the project BruTryp (*Mesa Técnica Consultiva Bovina*).

Short-term (1-2 years)

Objective: Identification, initial control, and awareness.

- **Intensify diagnosis and epidemiologic surveillance**

Action: Implement mass diagnostic campaigns using detection methods such as Rose Bengal test and ELISA, prioritizing high prevalence areas according to local studies (Carbonero et al., 2018; Garrido-Haro et al., 2023; Paucar et al., 2021). The gold standard test is culture and isolation, for which the laboratory must be continuously prepared (WOAH, 2022).

Justification: These tests are relatively inexpensive and effective in identifying foci of infection, which is crucial to target interventions efficiently. AGROCALIDAD has the capacity to conduct passive and active surveillance, so nationally representative sampling is needed to strengthen epidemiologic surveillance.

Estimated time: 6-12 months for implementation in the main areas of high prevalence.

- **Education and awareness campaigns**

Action: Develop educational programs for livestock farmers, veterinarians, the general population, and vaccine distributors, focusing on the transfer of knowledge about brucellosis. The program should train field technicians who will then be responsible for educating the community (AGROCALIDAD, 2024a).

Justification: Adequate knowledge of brucellosis transmission and prevention is essential for communities to accept and participate in control measures.

Estimated time: 3-6 months for design and initial implementation of campaigns.

- **Management and control of positive animals**

Action: Establish protocols for the management of positive animals, including quarantine and culling in severe cases, accompanied by a program of economic compensation for affected livestock owners (Lladó, 2006; Moriyón et al., 2023).

Justification: This measure, although costly, is essential to prevent the spread of the disease to other animals and eventually to humans.

Estimated time: 12-18 months for implementation in priority areas.

Medium Term (2-5 years)

Objective: Consolidate control and reduce prevalence.

- **Systematic vaccination**

Action: Implement a systematic vaccination program with B19 vaccine, prioritizing areas with the highest prevalence of brucellosis (Blasco et al., 2022; Khurana et al., 2021).

Justification: Vaccination is an effective and sustainable measure to reduce the incidence of the disease in cattle and, consequently, its transmission to humans (Saegerman et al., 2010).

Estimated time: 3-4 years to achieve significant vaccination coverage.

- **Strengthen diagnostic and control infrastructure**

Action: Improve laboratory and veterinary services infrastructure to ensure accurate and timely diagnosis. In addition, establish agreements with universities to support research on brucellosis. In addition to establishing diagnostic networks to monitor the disease. It is also important to consider that laboratories must be recognized worldwide, so accreditation of techniques under the ISO17025 standard, which confers technical competence, is proposed (Colling et al., 2008).

Justification: A robust diagnostic system is fundamental to the control and eventual eradication of brucellosis, and laboratories are a key pillar in this process.

Estimated time: 2-3 years to improve local capacity.

- **Strengthen policies and regulations**

Action: Review and update health policies and regulations related to brucellosis and ensure their proper implementation and enforcement (EUROPEAN COMMISSION, 2009).

Justification Regulatory updates and compliance are essential to maintain long-term control of the disease.

Estimated time: 2-3 years for review and update.

Long-term (5-10 years)

Objective: Eradication and sustainable prevention.

- **Eradication program:**

Action: Initiate national eradication programs based on a combination of diagnosis, culling, compensation, and sustained vaccination (EUROPEAN COMMISSION, 2009; USDA, 2022).

Justification: Eradicating brucellosis requires a comprehensive strategy combining several controlling measures to be effective in the long term.

Estimated time: 5-10 years for full implementation and visible results.

- **Ongoing monitoring and evaluation**

Action: Establish ongoing monitoring and evaluation systems to ensure the sustainability of implemented activities. Monitoring through active and passive surveillance (AGROCALIDAD, 2016).

Justification: Continuous monitoring is key to preventing resurgence and ensuring that the gains in brucellosis control are maintained.

Estimated time: Periodic evaluations every 2-3 years.

- **Regional integration and international cooperationAction**: Work with neighbouring countries and international organizations to harmonize brucellosis control policies and programs (FAO, 2014a).

Justification: International cooperation is critical for eradication in regions with high livestock mobility and can strengthen national capacity through knowledge and technology transfer.

Estimated time frame: 5-10 years, with ongoing coordination activities.

Feasibility of implementing brucellosis control in Ecuador

Given Ecuador's high brucellosis prevalence and limited resources, the proposed recommendations require careful planning over short, medium, and long-term phases.

Short-term (1-2 years)

- **Diagnosis and Surveillance:** Feasible with low-cost tests (Rose Bengal, ELISA) in high-prevalence areas, but success depends on funding and logistical coordination.
- **Education Campaigns:** Quick and cost-effective, but requires strong community engagement.
- **Control of Positive Animals:** Requires compensation programs for farmers, which could face delays due to financial constraints.

Medium Term (2-5 years)

- **Vaccination:** Effective but needs coordinated national efforts and sustained vaccine supply.
- **Strengthening Infrastructure:** Requires investments in laboratories and technical expertise, depending on partnerships and funding.
- **Policy Updates:** Achievable but may face bureaucratic delays.

Long-term (5-10 years)

- **Eradication Program:** Requires long-term commitment to diagnostics, culling, and vaccination. High costs and sustainability are challenges.
- **Monitoring:** Continuous but depends on stable funding and infrastructure.
- **International Cooperation:** Critical for success, though coordination with neighboring countries is key.

Short-term goals are viable with minimal costs, but medium and long-term measures, such as vaccination and eradication, require sustained resources, funding, and **international cooperation.**

Perspectives

Ecuador is facing a major challenge in the control and eradication of bovine brucellosis, a disease with profound economic and public health implications. The increasing number of outbreaks in recent years underscores the urgent need for a coordinated and sustained multi-sectoral effort. Future perspectives include improving diagnostic capabilities, enhancing vaccination strategies, and strengthening biosecurity measures. In the short term, the focus should be on the intensification of diagnostic efforts, the implementation of mass vaccination programs, and the raising of awareness among farmers and veterinarians. Medium-term strategies include consolidating these efforts by vaccinating herds, improving laboratory infrastructure, and ensuring strict sanitary policies and regulations. The long-term goal is to control/eradicate brucellosis through sustained vaccination, robust surveillance and regional cooperation. By adhering to international standards and collaborating with neighboring countries and global health organizations, Ecuador can significantly reduce bovine brucellosis prevalence, protecting livestock and public health.

Figure 8 : Summary of short-, long- and medium-term implementation of recommendations and outlook

Limitations

In order to carry out this research, it was essential to ensure the proper maintenance of the equipment in AGROCALDAD's containment 2 plus laboratory, especially the type 2 biosafety cabinets, incubators, air filters and pressure systems. Since the handling of *Brucella* requires Biosafety Level 3 conditions, the Bru Tryp project provided support for the maintenance of the cabinet and incubator, critical equipment for culture, isolation of *Brucella* and evaluation of vaccines registered in Ecuador. Likewise, with the collaboration of the Agency, the air filters of the laboratory were maintained, and adequate pressure was ensured, among other equipment necessary for the success of the project, guaranteeing a safe environment for the handling of this bacterium.

Another significant limitation was the rapid acquisition of reagents and materials needed to implement methodologies, both in the evaluation of vaccines and in the development of techniques such as bacterial culture, considered the "gold standard". In addition, specific reagents and materials were required for the implementation of molecular diagnostic techniques, strain typing and genotyping (MLVA). The limited availability of economic resources was a critical factor in the development of these activities. Nevertheless, despite the difficulties, we were able to complete the research proposed in this thesis with the support of BruTryp project (joint collaboration between ARES, ESPE and AGROCALIDAD).

An additional constraint was to obtain reference material of *Brucella* strains for the implementation of the Bruce-ladder. This process required time, management and budget to coordinate the request with the reference laboratory for South America, SENASA Argentina, who kindly supported us and sent the reference material to the Agency under the appropriate IATA biosafety conditions. This allowed us to strengthen our diagnostic capacity.

Political instability and budget constraints in Ecuador have limited the ability to conduct comprehensive surveys and ensure complete coverage of areas with the highest prevalence of brucellosis. These limitations affect data collection and the full implementation of effective control programs.

These limitations were compounded by the impact of the COVID-19 pandemic, which delayed various research activities due to global constraints. However, the pandemic also led to the use of new technologies, such as the internet, to facilitate communication and project continuity.

In my case, I had to take months without salary and with deduction of my holidays to be able to stay in Belgium, so that for future projects it will be necessary to review the conditions inside AGROCALIDAD in order to be able to offer to the thesis candidate an adequate stability of time, since the work carried out included both the institutional work and the realization of the project.

Despite all the limitations encountered, we always found the collaboration of all the actors, colleagues and institutions who contributed with great enthusiasm to the realization of each of the objectives.

Conclusions

In conclusion, bovine brucellosis control in Ecuador faces significant challenges due to factors such as the lack of highly effective vaccines, limited certification of disease-free farms and limited government investment, especially in the regional context. To address these issues, a comprehensive "One Health" approach involving both the Ministry of Agriculture and the Ministry of Health is essential to improve the sustainability of the livestock sector at the national and regional levels.

The results of the reviewed studies provide a solid basis for strengthening the national brucellosis control program in Ecuador. This includes the implementation of more advanced diagnostics, continuous improvement of vaccine quality, and strengthening of biosecurity measures at all stages of livestock production. In addition, it is essential to promote the education of farmers and veterinarians to ensure the adoption of better sanitary practices.

In the long term, Ecuador must align its efforts with international standards and promote regional cooperation, which will not only contribute to the eradication of brucellosis, but also improve animal and human health and strengthen the competitiveness of the agricultural sector in the global market.

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Chapter 6 – Appendixes

Study 4

Bayesian estimation of the true Ecuadorian prevalence of bovine brucellosis in non-vaccinated and vaccinated cattle sub-population, sensitivity and specificity of a competitive ELISA and an indirect ELISA based on a new synthetic antigen

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Abstract

Ecuador has four natural regions (Coast, Highlands, Amazon and Galapagos). The Agencia de Regulación y Control Fito y Zoosanitario- AGROCALIDAD - is in charge of the National Brucellosis (bB) Control Program, which was implemented in 2008. Brucellosis is endemic on the continent and the Galapagos Islands have been declared free of bB. In Ecuador, vaccination against bB is not mandatory; it is the responsibility of the farm owner. The vaccines currently used are strains S19 and RB51. From the data obtained on the percentage of vaccination in farms and animals in the Coast and Highlands, it was 13% at farm and 24% at animal levels. When present, the clinical presentation of bB

can be diverse and not pathognomonic. Indeed, serology is the most convenient method for detecting bB. The use of such tests in bB control requires evaluation of diagnostic performance and discriminative ability in different epidemiological settings. The objective of this study was to assess the performance

of a competitive (cELISA) and an indirect ELISA based on new synthetic antigen (iELISA). A random stratified sample of continental Ecuadorian cattle population was performed (3299 bovines older than 24 months coming form 223 farms). A Bayesian approach was used to evaluate the two tests by estimating their respective diagnostic sensitivities and specificities and the true prevalence of bB in different sub-populations (non-vaccinated, vaccinated with strain S19 and vaccinated with strain RB51). Globally, in Ecuadorian context, the correlation-adjusted sensitivity of the cELISA and the iELISA was similar and estimated as 94.02% (credibility interval [CrI], 88.56–97.78) and 94.05% (CrI, 88.73–97.77), respectively. The correlation-adjusted specificity of the cELISA and the iELISA was different and estimated as 95.85% (CrI, 95.05–96.76) and 98.10% (CrI, 97.03–98.92), respectively. The true prevalence of bB was 1.63% (95% CrI, 0.56-2.54) in non-vaccinated cattle and drop to 0.97% (95% CrI, 0.005-2.54) in S19-vaccinated cattle and was 2.75% (95% CrI, 0.50-5.32) in RB51-vaccinated cattle. Because no significant difference in Se and Sp were observed in regions where both vaccines are used, more study is needed to understand why the true prevalence is higher in case of vaccination with RB51 versus Buck 19 (not correct use of the vaccination protocol is the most plausible hypothesis). The level of agreement between the two tests was evaluated using indices of agreement. High agreement was found for negative results (97.46%; 95% confidence interval [CI], 97.05–97.84). In addition, the agreement between ELISAs was moderate for sera testing positive (44.44% with 95% CI: 38.62–50.39). The results of this study indicate that with similar sensitivity and higher specificity, the iELISA based on innovative synthetic antigen (more standardizable) should be proposed as a screening test for bB in Ecuador. In addition, the approach proposed gives insights on the quality of the vaccination campaign and claims for refining of the Ecuadorian national brucellosis control program.

Keywords: Bovine brucellosis; Ecuador; Bayesian approach; ELISA, Test performance; Synthetic oligosaccharides; Vaccine.

1. Introduction

Bovine brucellosis (bB) is a worldwide zoonotic disease mainly due to *Brucella abortus*, a facultative intracellular pathogen mostly associated with cattle, its natural or primary host (Godfroid et al., 2013a; Pappas et al., 2005). Cattle become infected after close contact with infected animals, contact with uterine secretions or aborted fetuses, by vertical and sexual transmission and after ingesting contaminated food, milk/colostrum, forage and water (Godfroid et al., 2013a; Ragan et al., 2013). The disease causes substantial economic losses due to abortion mostly in the last trimester of pregnancy, mastitis and reduced milk production in female animals, and orchitis and epididymitis in male animals. Infertility can occur in both male and female animals (De Figueiredo et al., 2015). The annual loss by bB has been estimated at $600 million in Latin America [Khurana et al., 2021]. In addition, a 20–30% decrease in milk production has been estimated in bB-affected herds (Dadar et al., 2021; Herrera et al.,

2008). Economic losses in the livestock herds of San Pedro de Suma in the province of Manabí, in the Coastal region, were estimate between US$ 1,922 and 3,843 (Paredes, 2021).

In humans, it is an occupational disease. Transmission to humans is mainly through close contact with contaminated placenta, urine, excrement, blood and aborted fetuses, affecting mainly workers who handle domestic ruminants such as veterinarians, veterinary assistants, farmers, slaughterhouse workers and butchers, as well as laboratory workers (Guerrier et al., 2011; Mia et al., 2022).

Bovine brucellosis has been reported in Latin America since the first decade of the 20th century and remains up to now a major zoonosis despite campaigns for its control. The Galapagos Islands (with few populations of cattle) were recognized as free of brucellosis without vaccination [Gioia et al., 2018]. However, bB remains endemic in the continental Ecuador [Paucar et al., 2021].

In Ecuador, the Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD) is the institution in charge of the national bB control program, which started activities in 2008, and is based on the vaccination of females with S19 and/or RB51 strains (voluntary vaccination), serological diagnosis, the slaughter of positive animals and certification of herds as free of bB (AGROCALIDAD, 2020). Herds with bB certificates receive a bonus of US$ 1 cent per liter of milk by pasteurizers [Agrocalidad, 2021].

When present, the clinical presentation of bB is diverse and not pathognomonic in cattle. Indeed, serology is the most convenient method for detecting bB. The use of such tests in bB control requires evaluation of diagnostic performance and discriminative ability in different epidemiological settings. Competitive ELISA was recognized for testing bB in different animal and human species and was generally considered as highly specific but indirect ELISA as highly sensitive [Saegerman et al., 2010; Godfroid et al., 2010]. For both ELISA a critical point is the difficulty to maintain a constant quality of the lipopolysaccharide of *Brucella* coated in the plate. Recent discovery of synthetic oligosaccharide antigens representing the capping M epitope elements of *Brucella* O-polysaccharides offers the opportunity to standardize more easily the quality of the antigen used and to increase the specificity of the brucellosis serodiagnosis [McGiven et al., 2015; David et al., 2017].

In bB, the isolation and identification of *Brucella* spp. is considered as the reference standard method; a positive test result provides an unequivocal diagnosis of a positive case of brucellosis [World Animal Health Organization, 2018; Sanogo et al., 2013]. However, these methods are not always feasible in diagnostic investigations. Therefore, diagnosis is frequently based on imperfect serological methods, such as ELISA tests, which are WOAH prescribed tests for trade and are commonly used in combination for the diagnosis of brucellosis [World Organization for Animal Health, 2022; Saegerman et al., 2010; Sanogo et al., 2014]. In addition, the WOAH accept Bayesian approach to estimate the

diagnostic sensitivity and the diagnostic specificity of such tests [World Organization for Animal Health, 2018]

The aims of this study were to estimate the characteristics (sensitivities and specificities) of a competitive ELISA and an indirect ELISA based on a new synthetic antigen using a Bayesian approach and to determine the true prevalence of bB in continental Ecuador in different epidemiological settings (vaccinated and non-vaccinated bovines).

2. Materials and methods

2.1. Study area

Ecuador covers 281,341 km^2, it is divided into four regions, in which 24 provinces are distributed (Ministerio de Asuntos Exteriores, Unión Europea y Cooperación, 2021). Three continental regions are the Coastal region in western part, the Highlands in the middle part and the Eastern region (i.e. Amazonia). The fourth region constitutes the Galapagos Islands.

In Ecuador, the agricultural sector contributes to the gross national product by 8% (Ministerio de Agricultura y Ganadería, s. f.) and 5.7 million litres of milk are produced per day at national level, generating employment for 1,140,000 Ecuadorians (AGROCALIDAD, 2022). From the national cadastre, Ecuador counts 4,525,183 heads of cattle originate from 285,579 farms (with around 41% of animals in Coastal region, 49% in Highlands and the rest in Eastern region) [Panaftosa - OPS/OMS, 2021].

Ecuadorian climate varies from one region to another, due to differences in altitude, proximity to the equator and proximity to the Pacific Ocean [World Bank, 2021]. The Coastal region varies between the wet season from December to May and the dry season from June to November, with temperatures range from 23ºC and 26ºC. The climate in Highlands (Andes Mountains) is cold and rainy from November to April and dry from May to October with temperatures range from 13ºC and 18ºC. In the eastern region (Amazonia), the climate is rainy and humid between January and September, with temperatures range between 23ºC and 26ºC, and it is dry between October and December. The Galapagos Islands have temperate climate with temperatures range from 22ºC and 32ºC

2.2. Animal samples

The 23 provinces of mainland Ecuador were surveyed between May and June 2018. Blood samples were obtained in tubes without anticoagulant by puncturing the coccygeal vein of each animal. The samples were transferred to the AGROCALIDAD laboratories, preserving the cold chain (4 to 8°C). To separate the blood serum, the samples were centrifuged for 5 minutes at 5,000 rpm. The serum was kept at a temperature of 4 to 8°C until analysis in AGROCALIDAD's serology laboratory. The blood samples were obtained with the prior consent of the herd owner and did not involve any cost to them.

A two-stage sampling size determination was used for this study. The first stage was at herd level. Parameters considered were a confidence level of 95%, a Se and Sp of 95%, and an estimated prevalence of 15% with 5% of precision. The second stage was at animal level; parameters considered were a confidence level of 95%, sensitivity (Se) and specificity (Sp) of 95%, and estimated prevalence of 10% with 5% of precision. To minimize the occurrence of false positive serological reaction (FPSR) due to the brucellosis vaccination in Ecuador, 24-month-old female bovines were sampled (N = 3299 bovines coming from 223 herds).

2.3. Diagnostic tests

Two diagnostic tests were used in parallel. The first test was a competitive ELISA test (cELISA) and the second one an indirect ELISA using a synthetic antigen (iELISA).

2.3.1 Competitive ELISA (cELISA)

Greiner 762021 F8 microplates were coated overnight at 4°C with a *Brucella abortus* lipopolysaccharide (LPS) preparation. This antigen was provided by the Belgian Sciensano Institute. The optimal coating dilution of the LPS was determined by checkerboard titration by varying the dilution of the LPS and the dilution of the conjugate. Briefly, the serum samples were diluted 1/2 for testing as indicated by the manufacturer. The negative and positive controls were always included when serum samples were examined. Fifty microliters of sample were necessary. After a 120-min incubation period at 37 °C +/−3 °C, the plate was washed with washing solution before the addition of conjugate (Monoclonal A76-12B12 coupled to the Raifort peroxidase). After a second incubation at 37°C +/−3 °C for half an hour more, the plate is revealed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) for 10 min at room temperature. The reaction is stopped by the addition of a blocking solution. The results were expressed as % inhibition using the following equation:

$$\frac{OD\ neg - OD\ sample}{OD\ neg}\ x\ 100 \qquad \text{(Equation 1)}$$

With: OD, the optical density and neg, the negative control sample.

The cut-off of the blocking ELISA kit was calculated to be in line with the official reference serum provided by the Belgian federal reference laboratory Sciensano (determined as 70% inhibition) (see also **Appendix A1**).

2.3.2. Indirect ELISA using a synthetic antigen (iELISA)

Greiner 762021 F8 microplates were coated overnight at 4°C with a synthetic antigen at 2.5 µgr/ml. This synthetic antigen is M-tetrasaccharide coupled to BSA (M-epitope) supplied by the Animal and Plant Health Agency (APHA Scientific, UK) [Bundle & McGiven, 2017]. The M-epitope is an α1,3-linked D-Rha4NFo disaccharide. The tetrasaccharide encompasses the disaccharide structure with one additional OPS sugar unit on either side. This maintains the unique *Brucella* OPS epitope but includes side structures that increase the size of the antibody epitope. Multiple tetrasaccharides are conjugated to a protein carrier (BSA) to enable passive adsorption to ELISA plates.

The plates were then saturated with casein hydrolysate solution, dried, and stored in aluminum bags with desiccant. Briefly, the serum samples were diluted 1/100 for testing as indicated by the manufacturer. The negative and positive controls were always included when serum samples were examined. Twenty microliters of sample were necessary. After a 60 min incubation period at room temperature (21 °C +/−3 °C), the plate was washed with washing solution before the addition of protein G conjugated antibody coupled to the Raifort peroxidase. After a second incubation at room temperature (21 °C +/−3 °C) for one more hour, the plate is revealed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) for 10 min at room temperature. The reaction is stopped by the addition of a blocking solution. The results were expressed in optical density S (serum)/P (positive control) ratio and % S/P.

The cut-off of the iELISA kit based on the use of synthetic antigen was calculated to be in line with the official reference provided by the Belgian federal reference laboratory Sciensano (determined as 36 % S/P) (see also **Appendix A1**).

2.3.3. Repeatability and reproducibility of cELISA and iELISA used

The intra-assay repeatability was estimated using a weak sample (internal reference serum diluted 1:4 for cELISA and 1:400 for iELISA) was tested 288 times on three different plates. The intra-laboratory reproducibility was estimated using four dilutions (1/4; 1/8; 1/16 for cELISA and 1/50; 1/100; 1/200; 1/400 for iELISA) of the same sample in the linear range of the test. These dilutions were tested in three replicates on six different runs performed by 2 operators on different days.

2.4. Bayesian analysis

The methodology developed by Sanogo et al. [Sanogo et al., 2013; Sanogo et al., 2014] was applied.

2.4.1. Estimation of test sensitivity and specificity and true prevalence in different settings

In the absence of a 'gold standard', a Bayesian approach was used to evaluate the performance of the cELISA and the iELISA by estimating Se and Sp [Branscum et al., 2005; Berkvens et al., 2006]. In addition, given both tests are based on antibody detection, they can be considered conditionally dependent, i.e. the results of the two tests for a given animal are correlated [Sanogo et al., 2013]. Indeed, a Bayesian model was developed considering the correlation between the tests on infected and non-infected animals. The approach facilitated the inclusion of both field data and prior expert information in the same model to estimate test characteristics (Se and Sp), and prevalence. Based on the scientific literature and using realistic prior information relating to prevalence (uniform distribution between 0 and 0.1) [Paucar et al., 2021; Lucero et al., 2008], cELISA and iELISA data (Se between 0.95 and 1, translated as beta distribution characterized by an alpha = 96 and a beta = 6; Sp= uniform distribution between 0.95 and 1) [Saegerman et al., 2010; Godfroid et al., 2010], and covariance [Branscum et al., 2005], the model was established within WinBUGS [Spiegelhalter et al., 2003] (see **Appendix A2**). Three parameters were monitored during the analysis: (i) the deviance information criterion (DIC), (ii) the effective number of estimated parameter (pD), and (iii) the Bayesian *p*-value [Praet et al., 2006]. In brief, the DIC and Bayesian *p*-value were used to check if the prior information was in conflict with testing data results (i.e. based on the likelihood of observations). The DIC is a generalization of the Akaike Information Criterion (AIC) for a multinomial model [Berkvens et al, 2006]. The value of DIC must be positive and low as possible. The pD of the model was used to assess the impact of the constraints. Using the following formula, the optimal pD can be calculated [Berkvens et al., 2006]:

$$2^n - 1 \qquad \text{(Equation 2)}$$

For two tests, the optimal pD value is three.

The model used three chains, a 'burn-in' of 10,000 iterations, and an additional 10,000 iterations to obtain the posterior distributions. Trace plots were simultaneously combined with autocorrelation plots to explore the convergence of the model. If the trace plot indicated good mixing and the autocorrelation plot little or no correlation among samples, then convergence was claimed. Where autocorrelations were still high after the first few lags, 'thinning' was applied where the thinning coefficient was determined by the number of lags at which the autocorrelations significantly dropped to zero. A more formal test for convergence, the Brooks–Gelman–Rubin (BGR) statistic was also used [Gelman and Rubin, 1992]. The quality of a model (with appropriated priors) was claimed when a good

mixing of chains was observed (BGR statistic), the Bayes *p*-value was close to 0.5, the pD value close to 3, and posterior density distribution clearly identified (e.g. distribution with clear shape).

2.4.2. Prior and sensitivity analysis

The parameter estimates obtained using the Bayesian model with conditional dependence between tests vary with the prior distributions [Branscum et al., 2005]. Therefore, in order to assess the influence of the proposed prior distributions on the estimated parameters, a sensitivity analysis was performed which consisted in using less informative priors [Enoe et al., 2000; Branscum et al., 2005]: uniform distribution between 0 and 0.2 for the prevalence (prevalence of bB varies considerably from one country to another in Latin America with rates ranging from 0.5% to 10%), and with a Se= 50% and translate as a beta distribution characterized by an alpha = 51 and a beta = 51; Sp= uniform distribution between 0.5 and 1 (if a test characteristics is below 0.5, the test is generally not commercialised). For each set of alternative prior distributions considered for the parameters, the model was run with the same number of chains and similar diagnostics were performed.

2.5. Assessment of agreement between the tests

The two tests were compared using concordance analysis to assess their agreement with our results. Firstly, the global level of agreement was expressed using the Kappa coefficient [Petrie and Watson, 2013]. Secondly, the level of agreement was expressed in terms of indices of positive and negative agreement [Cicchetti and Feinstein, 1990], i.e. respectively the observed agreement proportion for positive and negative test results. Confidence intervals were calculated according to the method of [Graham and Bull, 1998]. Using a 'two-by-two' contingency table (**Table 1**), the two indices of positive agreement (p_{pos}) and negative agreement (p_{neg}) were respectively:

$$\boldsymbol{p_{pos}} = \frac{\boldsymbol{2a}}{\boldsymbol{2a+b+c}} \text{(Equation 3)}$$

$$\boldsymbol{p_{neg}} = \frac{\boldsymbol{2d}}{\boldsymbol{2d+b+c}} \quad \text{(Equation 4)}$$

where p_{pos} and p_{neg} were the indices of positive and negative agreement, respectively (parameters a, b, c and d are detailed in **Table 1**).

Table 1. Contingency table showing results for the two diagnostic tests (cELISA and iELISA)

	cELISA Positive	Negative	Total
iELISA			
Positive	*a*	*b*	*a + b*
Negative	*c*	*d*	*c + d*

Total	$a + c$	$b + d$	N

Legend: N, total of samples tested by both tests ($a + b + c + d$).

3. Results

3.1. Repeatability and reproducibility of ELISA tests

The intra-assay repeatability (coefficient of variation, CV) was 6.03% and 7.02% for cELISA and iELISA, respectively. It is below to the required criteria (CV<10%). The intra-laboratory reproducibility varies from 4.44 and 11.02 and 2.02% to 9.27% for cELISA and iELISA, respectively. It is below to the required criteria (CV <15%).

3.2. Serology

Considering the two-by-two contingency table (**Table 1**), cross-classified test results of 3299 random samples resulted in a = 64, b = 117, c = 43 and d = 3075. A total of 107 (3.2%) of serum samples tested positive on the cELISA, whereas 181 (5.5%) were positive using the iELISA. Both tests gave the same results in the case of 3139 samples (95.15%). Evidence of difference between the interpretation of results of both tests in serial and in parallel was demonstrated (Fisher's exact test; *p*-value < 0.001) with more sensitive interpretation in parallel as expected.

3.3. Indices of agreement between tests

The cross-classified test results were used to calculate the indices of agreement between the two assays. Both results were the same for 95.15% of samples (95% CI: 94.36-95.86). The concordance between the two tests was estimated as moderate agreement using Kappa coefficient = 0.42 with 95% CI: 0.39-0.45. The agreement of the positive results to both tests (p_{pos}) was estimated to be 44.44% (95% CI: 38.62–50.39), whereas the agreement on negative test results (p_{neg}) was estimated to be 97.46% (95% CI: 97.05–97.84). These indices provide evidence that the test outcomes for animals are correlated.

3.4. Estimated true prevalence, test sensitivity and specificity

The estimation of the true prevalence and test Se and Sp were performed using all animals (n=3299; model 1) and in three different settings: non-vaccinated animals (n=2506; model 2), vaccinated animals with Buck 19 (n=383; model 3) and vaccinated animals with RB51 (n=392; model 4) (**Table 2**).

After some pre-testing and following application of a thinning coefficient of 100, all models used in this study appeared to converge as the chains were properly mixed and the autocorrelations were zero. The BGR plots also corroborated these findings. The estimated Bayesian *p*-value of our models was 0.49, 0.49, 0.52 and 0.47 for the model 1 to 4, respectively and indicating no particular problems with model fit (i.e. values are close to 0.5). The pD estimated from the multinomial probabilities was 2.88, 2.82, 2.58 and 2.62 for the model 1 to 4, respectively and were quite relatively close to the optimal

value of 3. The DIC value was estimated as 23.92, 23.10, 16.11 and 17.68 for the model 1 to 4, respectively, which were quite close to optimal values (must be positive and small as possible) (**Table 3**).

The estimated values of Se and Sp for both the cELISA and iELISA are summarized in **Table 2**. The Se (around 94%) was similar whatever the test and the model used. In contrary, the Sp of the iELISA (around 97-98%) was significantly higher (distinct or few overlap CrI) than the cELISA (around 94-95%).

Table 2. True prevalence, Sensitivity (Se) and specificity (Sp) estimates for the competitive (cELISA) and indirect (iELISA) enzyme-linked immunosorbent assays using a Bayesian approach

Test	Parameter	Prior	Posterior estimation in percent (CrI)
Model 1 (all animals)			
	True prevalence	Uniform [0, 0.1]	1.50 (0.36-2.47)
cELISA	Se	Beta [96, 6]	94.02 (88.56-97.78)
	Sp	Uniform [0.95, 1]	95.85 (95.05-96.76)
iELISA	Se	Beta [96, 6]	94.05 (88.73-97.77)
	Sp	Uniform [0.95, 1]	98.10 (97.03-98.92)
Sensitivity analysis regarding the model 1			
	True prevalence	Uniform [0, 0.2]	3.45 (0.24-7.01)
cELISA	Se	Beta [51,51]	50.27 (40.67-59.85)
	Sp	Uniform [0, 1]	96.08 (94.23-98 .05)
iELISA	Se	Beta [51,51]	49.78 (40.20-59.40)
	Sp	Uniform [0, 1]	98.36 (96.70-99.99)
Model 2 (unvaccinated animals)			
	True prevalence	Uniform [0, 0.1]	1.63 (0.56-2.54)
cELISA	Se	Beta [96, 6]	94.02 (88.60-97.73)
	Sp	Uniform [0.95, 1]	95.64 (95.03-96.49)
iELISA	Se	Beta [96, 6]	94.04 (88.61-97.78)
	Sp	Uniform [0.95, 1]	98.26 (97.29-98.99)
Sensitivity analysis regarding the model 2			
	True prevalence	Uniform [0, 0.2]	2.54 (0.17-5.34)
cELISA	Se	Beta [51,51]	71.18 (51.14-97.92)
	Sp	Uniform [0, 1]	95.66 (93.89-97.69)
iELISA	Se	Beta [51,51]	73.03 (50.91-98.54)
	Sp	Uniform [0, 1]	96.44 (96.73-99.90)
Model 3 (vaccinated animals with Buck 19)			
	True prevalence	Uniform [0, 0.1]	0.97 (0.005-2.54)
cELISA	Se	Beta [96, 6]	94.04 (88.7-97.76)
	Sp	Uniform [0.95, 1]	97.4 (95.57-98.88)
iELISA	Se	Beta [96, 6]3-	94.08 (88.7-97.79)
	Sp	Uniform [0.95, 1]	98.28 (96.56-99.5)
Sensitivity analysis regarding the model 3			
	True prevalence	Uniform [0, 0.2]	2.55 (0.16-6.16)
cELISA	Se	Beta [51,51]	50.06 (40.41-59.73)
	Sp	Uniform [0, 1]	97.87 (95.58-99.7)
iELISA	Se	Beta [51,51]	49.89 (40.39-59.57)
	Sp	Uniform [0, 1]	98.64 (96.68-99.91)

Model 4 (vaccinated animals with RB51)			
	True prevalence	Uniform [0, 0.1]	2.75 (0.50-5.32)
cELISA	Se	Beta [96, 6]	94.01 (88.5-97.77)
	Sp	Uniform [0.95, 1]	96.38 (95.08-98.09)
iELISA	Se	Beta [96, 6]	94.06 (88.69-94.34)
	Sp	Uniform [0.95, 1]	97.33 (95.39-99.02)
Sensitivity analysis regarding the model 4			
	True prevalence	Uniform [0, 0.2]	4.21 (0.20-13.02)
cELISA	Se	Beta [51,51]	54.89 (5.38-97.16)
	Sp	Uniform [0, 1]	95.9 (92.38-99.48)
iELISA	Se	Beta [51,51]	50.69 (4.03-96.63)
	Sp	Uniform [0, 1]	96.68 (93.47-99.7)

Legend: CrI, credibility interval; Se, sensitivity; Sp, specificity.

As an outcome (model 1), the model also estimated an overall true prevalence of brucellosis to be 1.50% (95%; CrI, 0.36–2.47). Using the models 2 to 4, the estimation of the true prevalence was estimated in three different setting. Indeed, the true prevalence was estimated as 1.63% (CrI: 0.56-2.54) in non-vaccinated animals, as 0.97 (CrI: 0.005-2.54) in Buck-19-vaccinated animals and as 2.75 (CrI: 0.5-5.32) in RB51-vaccinated animals.

3.5. Sensitivity analysis

Using non-informative priors for the prevalence, the Se and the Sp, the corresponding posterior estimates were summarized in **Table 2.** The validity criteria were followed (i.e. BGR, bayesp, DIC, pD and density distribution) and presented in **Table 3**. The results indicated that using the above non-informative priors, (i) the estimated prevalence increases for around 1.5 to 2.5 times in function of the setting (all, non-vaccinated, Buck 19 vaccinated and RB51 vaccinated animals); (ii) the estimated Se decreases drastically until around 50% for all animals (M1-SA) and vaccinated group of animals (M3-SA and M4-SA) and to 70% for non-vaccinated group of animals (M2-SA); and (iii) the estimated Sp for the models M1-SA to M4-SA are around the same as for the models M1 to M4, with informative priors (**Table 2**). The use of non-informative priors for all parameters led to models with good mixing of the Markov chains (BGR), values of the bayesp and DIC close to the optimal values and close to the values obtained with the models M1 to M4. However, for models M1-SA to M4-SA, the DIC was systematically higher than the models M1 to M4. In addition, the density distribution of models M1-SA to M4-SA were less identifiable than the density distribution of the models M1 to M4 (**Appendix A3**). Finally, we selected the models M2 to M4 as the models of interest and M2 to M4 for the best estimation in function of the setting (non-vaccinated, Buck 19 vaccinated and RB51 vaccinated).

Table 3. Validity criteria for the four models (M1 to M4) with informative priors and four additional models used for the sensitivity analysis with non-informative priors (see Table 2 for the details of priors)

Model	Prior	BGR (mixing chains)	bayesp	DIC	pD	Density distribution identifiable
M1	Informative	Yes	0.4937	23.923	2.884	More
M1-SA	Non-informative	Yes	0.4926	23.986	2.907	Less
M2	Informative	Yes	0.4918	23.097	2.821	More
M2-SA	Non-informative	Yes	0.5047	23.387	2.965	Less
M3	Informative	Yes	0.5182	16.111	2.583	More
M3-SA	Non-informative	Yes	0.5241	16.282	2.642	Less
M4	Informative	Yes	0.4681	17.675	2.620	More
M4-SA	Non-informative	Yes	0.4909	18.048	2.770	Less

Legend: M1 to M4, models 1 to 4; M1-SA to M4-SA, the same model as before but with non-informative priors for the prevalence, sensitivity and specificity of both tests; BGR, the Brooks–Gelman–Rubin statistic (analysis of convergence, i.e. degree of mixing of Markov chains); bayesp, Bayesian P-value; DIC, Deviance Information Criterion; pD, the effective number of estimated parameter.

4. Discussion

The aims of this study were to estimate the true prevalence and the diagnostic characteristics of two ELISA tests, one competitive (cELISA) and one indirect with a new synthetic oligosaccharide antigen representing the capping M epitope elements of *Brucella* O-polysaccharides (iELISA) in non-vaccinated, S19 vaccinated and RB51 vaccinated settings [Bundle & McGiven, 2017]. This study was based on 3299 bovines older than 24 months

coming form 223 farms, originating from all 23 provinces of continental Ecuador, which is endemic of bovine brucellosis. The randomized large sampling induces representativeness and accuracy of the estimates. The iELISA with the synthetic antigen given two advantages. By its nature, this iELISA is easier standardizable and give less false positive serological reaction [Bundle & McGiven, 2017].

The estimation of the Se and Sp of a diagnostic test requires knowledge of the true disease status of the animals on which this assay is applied [Sanogo et al., 2014]. In absence of perfect test, a Bayesian approach is helpful to estimate test Se, Sp and prevalence of brucellosis (e.g. [Fosgate et al., 2002]) and the WOAH recognize this approach to estimate the diagnostic Se and the diagnostic Sp of tests [World Organization for Animal Health, 2018]. We used literature references for the estimation of the priors as true prevalence [Paucar et al., 2021; Lucero et al., 2008], Se and Sp of both cELISA and iELISA [Saegerman et al., 2010; Godfroid et al., 2010]. Using these priors (see **Table 2**) and using Bayesian modelling, we calculated posterior estimations of the true prevalence and the Se and Sp of the two tests in different settings. Depending of the setting (model M1 to M4), we found different prevalence, around the same Se (94%) and a higher Sp with the iELISA versus cELISA (around 2% more). In general, the Sp is better with a cELISA [Saegerman et al. 2010; Godfroid et al., 2010] but here it is different and attributed to the nature of the synthetic antigen used [Bundle & McGiven, 2017].

After the sensitivity analysis using non-informative priors, the M2 to M4 were selected as optimal. Their interest is related to cover three important settings (non-vaccinated, Buck 19 vaccinated and RB51 vaccinated animals). In Ecuador, the brucellosis control program is managed by the Agencia de Regulación y Control Fito y Zoosanitario (AGROCALIDAD). It is noted that in Ecuador, vaccination of females (S19 and RB51) is not mandatory but under the responsibility of the farmers (AGROCALIDAD, 2020). AGROCALIDAD have standard operating procedures for its use. The commercialized vaccines are registered in AGROCALIDAD based on the documentation provided by the companies. At this moment, there are no control of quality of the vaccines (e.g., counting of viable cells, determination of the phase smooth or rough of the bacteria as recommended by WOAH) and also no investigation on the level of compliance of vaccination procedures (effectiveness). The results of this study indicate a true prevalence of 1.63% in non-vaccinated animals (CrI: 0.56-2.54). This estimate is close of a previous estimation of Paucar et al. (2021) that consider all of the population without distinction of the setting and with a true prevalence of 1.6% (CrI: 1.0-2.4). The model M3 (S19 vaccinated animals) and M4 (RB51 vaccinated animals) estimate a true prevalence in these settings of 0.97% (CrI: 0.005-2.54) and 2.75% (0.5-5.32), respectively. According to a recent meta-analysis, a dose of 10^9 CFU for S19 and 10^{10} CFU for RB51 are the most suitable for the prevention of abortion and infection caused by *B. abortus* (Martins de Oliveira et al., 2022). The results of the present study suggest a decreasing of the true prevalence using the S19 vaccination. Alves et al. (2015) have also demonstrated

that the vaccination with S19 vaccine in 90% of the replacement heifers of 3–8 months of age provides excellent economic returns for the farmers. The results suggest also an increasing of true prevalence animals vaccinated by RB51. Eradication of bovine brucellosis in the Azores (Portugal) based on both test-and-slaughter and mass RB51 vaccine coverage for a sufficiently long period of time was effective to control bovine brucellosis (Martins et al., 2009). Indeed, the most plausible hypothesis in relation to the observed results in Ecuador is the non-mandatory vaccination, absence of quality control of vaccine commercialized and absence of guidelines to estimate the compliance of vaccination procedure. This preliminary finding strongly suggests more investigation on the quality of the vaccination process and a refining of the current national bovine brucellosis control program.

The level of agreement between the two diagnostic tests was measured through the Kappa coefficient and both the positive (Ppos) and the negative (Pneg) agreement. According to the Kappa coefficient, our results suggest a global moderate agreement between the two diagnostic tests. The value of the negative agreement (97.46%) indicated a good level of agreement when negative results for both tests considered. However, the positive agreement for the two diagnostic tests was only 44.44%. It is due to the fact that for similar Se between the two diagnostic tests, the Sp of the iELISA with the synthetic antigen is higher. Given these characteristics, the iELISA based on innovative synthetic antigen can be proposed as screening test for the national bovine brucellosis control program in Ecuador.

5. Conclusion

In comparison with the cELISA, the results of this study indicate that with similar sensitivity and higher specificity, the iELISA based on innovative synthetic antigen, which is more standardizable should be proposed as a screening test for bovine brucellosis in Ecuador. In addition, the approach proposed gives insights on the quality of the vaccination campaign and claims for refining of the Ecuadorian national brucellosis control program.

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Institutional Review Board Statement: A specific ethical clearance was unnecessary for this study because veterinary practitioners have a legal sanitary mandate granted by the Agrocalidad (Tumbaco, Ecuador) to carry out bB surveillance according to national predefined and validated standard operating procedures.

Informed Consent Statement: Not applicable. This study was performed in the framework of the mandatory surveillance of brucellosis in Ecuador by Agrocalidad.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

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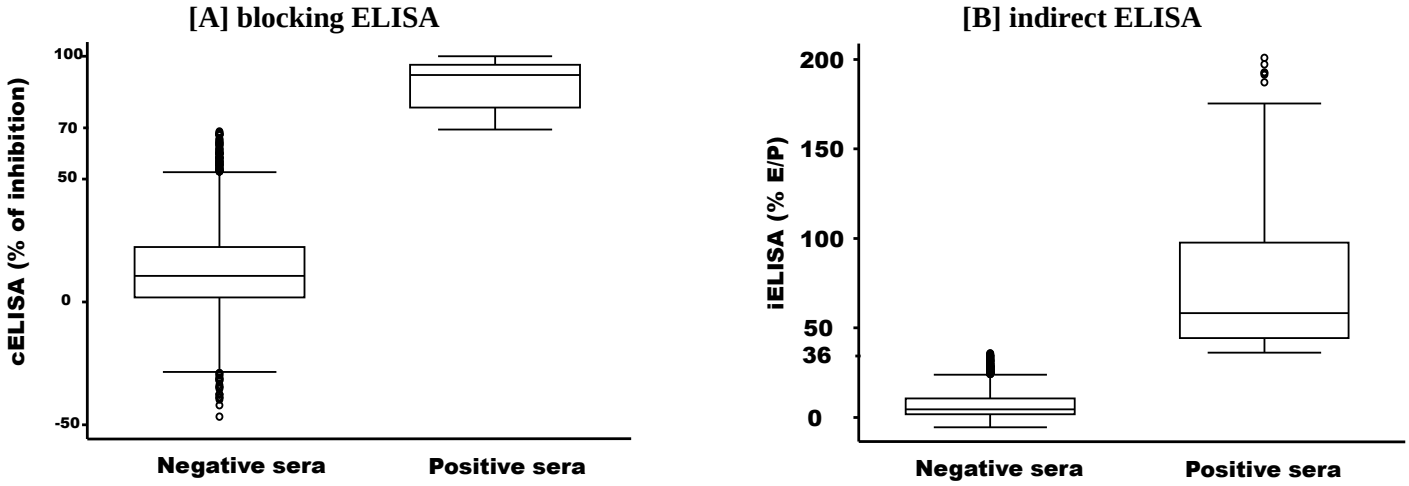
Appendix A1. The cut-off of the blocking ELISA [A] and the indirect ELISA with synthetic antigen [B] calculated to be in line with the official reference serum provided by the Belgian federal reference laboratory Sciensano (determined as 70% inhibition and 39% sample/positive ratio, respectively)



Appendix A2. Codification of the models (with Elisa as cELISA and SYN as iELISA)

M1 – Model 1 for all animals

```
{
r[1:4] ~ dmulti(p[1:4], n)
p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)
p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)
p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)
p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)
ls <- (SeElisa-1)*(1-SeSYN)
us <- min(SeElisa,SeSYN) - SeElisa*SeSYN
lc <- (SpElisa-1)*(1-SpSYN)
uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN
pi ~ dunif(0, 0.1)
SeElisa ~ dbeta(96,6)
SpElisa ~ dunif(0.95,1)
SeSYN ~ dbeta(96,6)
SpSYN ~dunif(0.95,1)
covDn ~ dunif(lc, uc)
covDp ~ dunif(ls, us)
```

```
rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(64,117,43,3075), n=3299)
```

M1-SA - Model 1 for the sensitivity analysis (non-informative priors)

```
{

r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN
```

```
lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN

pi ~ dunif(0,0.2)

SeElisa ~ dbeta(51,51)

SpElisa ~ dunif(0.5,1)

SeSYN ~ dbeta(51,51)

SpSYN ~ dunif(0.5,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(64,117,43,3075), n=3299)
```

M2 – Model 2 for non-vaccinated animals

```
{

r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN

lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN

pi ~ dunif(0, 0.1)

SeElisa ~ dbeta(96,6)

SpElisa ~ dunif(0.95,1)

SeSYN ~ dbeta(96,6)

SpSYN ~dunif(0.95,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)
```

```
rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(48,99,32,2327), n=2506)
```

M2-SA - Model 2 for the sensitivity analysis (non-informative priors)

```
r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN
```

```
lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN

pi ~ dunif(0, 0.2)

SeElisa ~ dbeta(51,51)

SpElisa ~ dunif(0.5,1)

SeSYN ~ dbeta(51,51)

SpSYN ~dunif(0.5,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(48,99,32,2327), n=2506)
```

M3 – Model 3 for S19-vaccinated animals

```
{

r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN

lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN

pi ~ dunif(0,0.1)

SeElisa ~ dbeta(96,6)

SpElisa ~ dunif(0.95,1)

SeSYN ~ dbeta(96,6)

SpSYN ~dunif(0.95,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)
```

```
{
d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))
d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))
}
bayesp <- step(sum(d[]) - sum(d2[]))
}
list(r=c(4,7,4,368), n=383)
```

M3-SA – Model 3 for the sensitivity analysis (non-informative priors)

```
{
r[1:4] ~ dmulti(p[1:4], n)
p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)
p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)
p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)
p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)
ls <- (SeElisa-1)*(1-SeSYN)
us <- min(SeElisa,SeSYN) - SeElisa*SeSYN
lc <- (SpElisa-1)*(1-SpSYN)
uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN
pi ~ dunif(0,0.2)
SeElisa ~ dbeta(51,51)
```

```
SpElisa ~ dunif(0.5,1)

SeSYN ~ dbeta(51,51)

SpSYN ~dunif(0.5,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(4,7,4,368), n=383)
```

M4 – Model 4 for RB51-vaccinated animals

```
{

r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN

lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN

pi ~ dunif(0,0.1)

SeElisa ~ dbeta(96,6)

SpElisa ~ dunif(0.95,1)

SeSYN ~ dbeta(96,6)

SpSYN ~dunif(0.95,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))
```

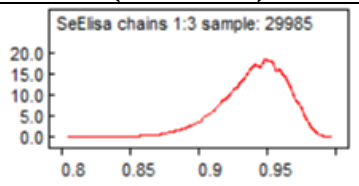
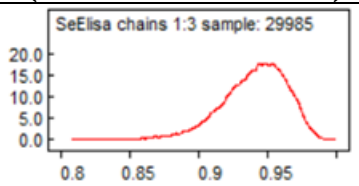
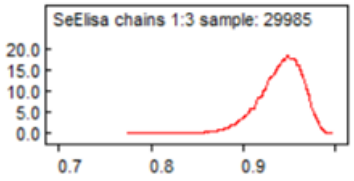
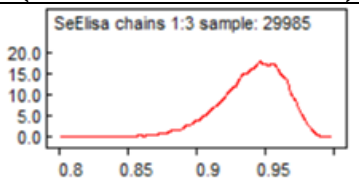
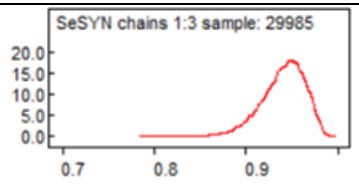
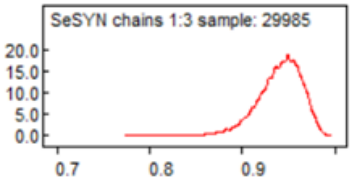
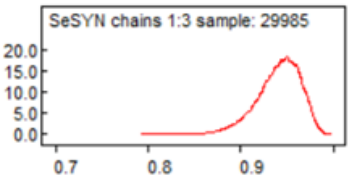
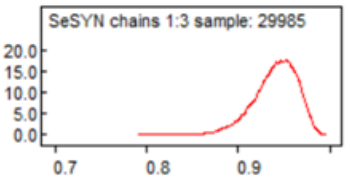
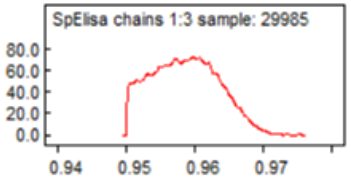
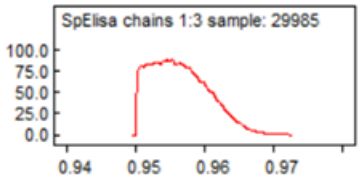
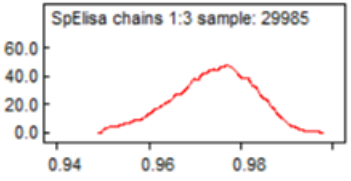
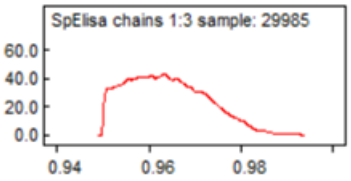
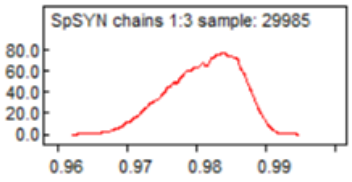
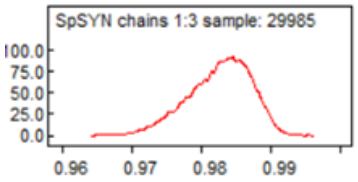
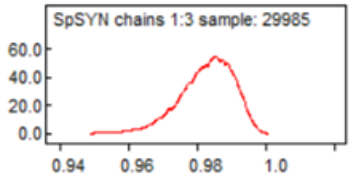
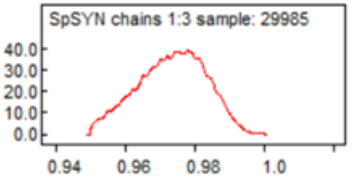
```
r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(12,11,7,368), n=392)
```

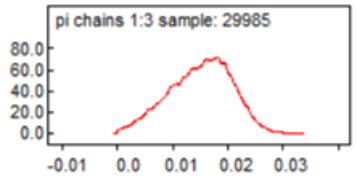
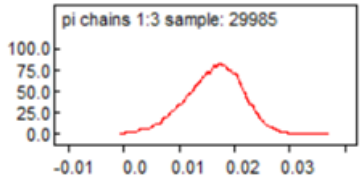
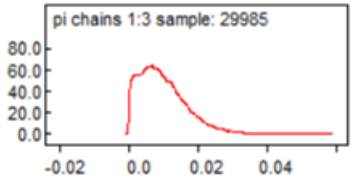
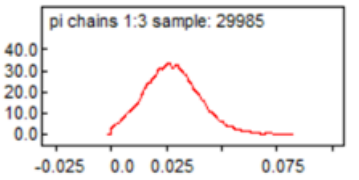
M4-SA – Model 4 for the sensitivity analysis (non-informative priors)

```
{

r[1:4] ~ dmulti(p[1:4], n)

p[1] <- pi*(SeElisa*SeSYN+covDp) + (1-pi)*((1-SpElisa)*(1-SpSYN)+covDn)

p[2] <- pi*(SeElisa*(1-SeSYN)-covDp) + (1-pi)*((1-SpElisa)*SpSYN-covDn)

p[3] <- pi*((1-SeElisa)*SeSYN-covDp) + (1-pi)*(SpElisa*(1-SpSYN)-covDn)

p[4] <- pi*((1-SeElisa)*(1-SeSYN)+covDp) + (1-pi)*(SpElisa*SpSYN+covDn)

ls <- (SeElisa-1)*(1-SeSYN)

us <- min(SeElisa,SeSYN) - SeElisa*SeSYN

lc <- (SpElisa-1)*(1-SpSYN)

uc <- min(SpElisa,SpSYN) - SpElisa*SpSYN
```

```
pi ~ dunif(0,0.2)

SeElisa ~ dbeta(51,51)

SpElisa ~ dunif(0.5,1)

SeSYN ~ dbeta(51,51)

SpSYN ~dunif(0.5,1)

covDn ~ dunif(lc, uc)

covDp ~ dunif(ls, us)

rhoD <- covDp / sqrt(SeElisa*(1-SeElisa)*SeSYN*(1-SeSYN))

rhoDc <- covDn / sqrt(SpElisa*(1-SpElisa)*SpSYN*(1-SpSYN))

r2[1:4] ~ dmulti(p[1:4],n)

for ( i in 1:4)

{

d[i] <- r[i]*log(max(r[i],1)/(p[i]*n))

d2[i] <- r2[i]*log(max(r2[i],1)/(p[i]*n))

}

bayesp <- step(sum(d[]) - sum(d2[]))

}

list(r=c(12,11,7,368), n=392)
```

Appendix A3. Distribution of parameters for models M1 to M4

Parameter	M1 (all animals)	M2 (non-vaccinated animals)	M3 (S19-vaccinated animals)	M4 (RB51 vaccinated animals)
Sensitivity of the cELISA				
Sensitivity of the iELISA				
Specificity of the cELISA				
Specificity of the iELISA				

True prevalence				

Legend: Elisa as cELISA; SYN as iELISA; Se as sensitivity; Sp as specificity; pi as true prevalence.

Supplemental material

The following are the first pages of the diagnostic protocols implemented at the Agency with the support of the BruTryp Project and which are currently in place to provide services to internal and external customers

AGROCALIDAD	PROCEDIMIENTO ESPECIFICO DE ENSAYO	PEE/BM/89
		Rev. 1
	TIPIFICACIÓN DE ESPECIES DE *BRUCELLA*	Hoja 1 de 14

AGROCALIDAD
AGENCIA DE REGULACIÓN Y CONTROL FITO Y ZOOSANITARIO

LABORATORIO DE BIOLOGIA MOLECULAR

PROCEDIMIENTO PEE/BM/89

TIPIFICACIÓN DE ESPECIES DE *BRUCELLA*

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LABORATORIO BIOLOGIA MOLECULAR

PROCEDIMIENTO PEE/BM/88

IDENTIFICACIÓN DE *Brucella abortus*

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