RAFAELLA SILVA ANDRADE

EVALUATION OF TESTS AND DIAGNOSTIC STRATEGIES IN BOVINE BRUCELLOSIS CONTROL AND ERADICATION PROGRAMS

LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Sanidade Animal e Saúde Coletiva, para a obtenção do título de Doutor.

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RAFAELLA SILVA ANDRADE

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AVALIAÇÃO DE TESTES E ESTRATÉGIAS DIAGNÓSTICAS EM PROGRAMAS DE CONTROLE E ERRADICAÇÃO DA BRUCELOSE BOVINA

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Sanidade Animal e Saúde Coletiva, para a obtenção do título de Doutor.

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RESUMO

Devido ao impacto da infecção por *Brucella abortus* na pecuária e na saúde pública, o controle e erradicação da brucelose bovina é uma meta importante de vários países onde a doença é endêmica. O diagnóstico de *Brucella* spp. nos programas de controle e erradicação da brucelose é geralmente baseado em testes bacteriológicos e sorológicos. Embora importantes para o diagnóstico da doença, os métodos de tipagem fenotípica geralmente têm menor poder discriminatório em comparação com os métodos genotípicos e, portanto, dificultam o rastreamento de surtos e o controle da disseminação da doença. Neste sentido, o objetivo do presente trabalho foi avaliar em um contexto geral as diretrizes que influenciam e estratégias em um programa de controle e erradicação da brucelose bovina, tendo como objetivos específicos: estimar os valores de sensibilidade diagnóstica (DSe) e especificidade diagnóstica (DSp) dos principais testes sorológicos utilizados no diagnóstico da brucelose bovina, bem como a estimação da dependência condicional e traçar estratégias para a redução e erradicação da brucelose bovina aplicados a programas de controle. Além de avaliar os riscos potenciais da utilização de oócitos de animais positivos para brucelose em biotecnologias reprodutivas e realizar uma ampla caracterização fenotípica e genotípica de duas cepas de *B. abortus* biovar 4 descritas pela primeira vez em bovinos do Brasil, fornecendo dados epidemiológicos de alta resolução sobre isolados raros de *B. abortus* entre bovinos no país. Os principais resultados que podemos destacar oriundos dos estudos que compõem a presente tese são: os testes sorológicos mais utilizados para o diagnóstico da brucelose bovina em todo o mundo apresentaram superestimação das estimativas de Dse e DSp; todos os testes sorológicos, exceto iELISA_SOD, que foram avaliados apresentaram dependência condicional; o iELISA_SOD pode ser utilizado para o diagnóstico de animais infectados, aumentando a gama de testes sorológicos disponíveis para o diagnóstico de brucelose bovina, com a vantagem de ser livre de S-LPS. Em contraste, o iELISA_MDH mostrou baixa utilidade como teste diagnóstico para brucelose bovina, bem como para diferenciar infecção de vacinação; mesmo no fluido folicular ovariano em vacas soropositivas não há presença de *Brucella* spp., o que oferece maior segurança para as biotecnologias reprodutivas realizadas a partir do aspirado folicular desses animais; e a tipagem de cepas de *B. abortus* isoladas de bovinos no Brasil confirmou a ocorrência de *B. abortus* biovar 4 no país, dando subsídios para a vigilância do patógeno dentro do programa de controle e erradicação da brucelose bovina no país.

PALAVRAS-CHAVE: Sensibilidade diagnóstica. Especificidade Diagnóstica. Acurácia. Testes sorológicos.

ABSTRACT

Due to the impact of *Brucella abortus* infection on livestock and public health, the control and eradication of bovine brucellosis is an important goal in several countries where the disease is endemic. The diagnosis of *Brucella* spp. in brucellosis control and eradication programs is usually based on bacteriological and serological tests. Although important for disease diagnosis, phenotypic typing methods generally have lower discriminatory power compared to genotypic methods and therefore make it difficult to track outbreaks and control disease spread. In this sense, the objective of the present study was to evaluate, in a general context, the guidelines that influence and strategies in a bovine brucellosis control and eradication program, having as specific objectives: to estimate the values of diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the main serological tests used in the diagnosis of bovine brucellosis, as well as the estimation of conditional dependence and outline strategies for the reduction and eradication of bovine brucellosis applied to control programs. In addition to assessing the potential risks of using oocytes from animals positive for brucellosis in reproductive biotechnologies and performing a broad phenotypic and genotypic characterization of two strains of *B. abortus* biovar 4 described for the first time in cattle in Brazil, providing high-resolution epidemiological data on rare isolates of *B. abortus* among cattle in the country. The main results that we can highlight from the studies that make up this thesis are: the serological tests most used for the diagnosis of bovine brucellosis worldwide showed overestimation of Dse and DSp estimates; all serological tests, except iELISA_SOD, that were evaluated showed conditional dependence; iELISA_SOD can be used for the diagnosis of infected animals, increasing the range of serological tests available for the diagnosis of bovine brucellosis, with the advantage of being free of S-LPS. In contrast, iELISA_MDH showed low utility as a diagnostic test for bovine brucellosis, as well as for differentiating infection from vaccination; even in the ovarian follicular fluid of seropositive cows there is no presence of *Brucella* spp., which offers greater safety for the reproductive biotechnologies performed from the follicular aspirate of these animals; and the typing of *B. abortus* strains isolated from cattle in Brazil confirmed the occurrence of *B. abortus* biovar 4 in the country, providing subsidies for the surveillance of the pathogen within the program for the control and eradication of bovine brucellosis in the country.

KEYWORDS: Diagnostic sensitivity. Diagnostic specificity. Accuracy. Serological tests.

SUMMARY

FIRST PART

INTRODUCTION

Brucellosis is a zoonotic disease of worldwide importance caused by bacteria of the genus *Brucella*, which infects a wide variety of wild and domestic animals, as well as humans (WOAH, 2022). In cattle, the infection is mainly caused by *Brucella abortus* and the economic losses associated with bovine brucellosis are mainly related to reproductive problems, such as abortion, stillbirth, birth of weak calves, retained placenta, temporary or permanent infertility and disposal of positive animals (MCDERMOTT *et al.*, 2013; OLSEN *et al.*, 2010). The clinical diagnosis of brucellosis in animals based on abortion is, however, misleading, since many pathogens can induce abortion. Laboratory tests are therefore essential (GALL *et al.*, 2004).

The diagnosis of brucellosis in control and eradication programs is generally based on bacteriological and serological tests (GALL *et al.*, 2004). Diagnostic tests can be applied for different purposes: confirmatory diagnosis, screening or prevalence studies, certification and, in countries where brucellosis has been eradicated, surveillance to prevent the reintroduction of brucellosis through the importation of infected animals or animal products (GODFROID *et al.*, 2010).

Diagnostic methods include direct tests, involving microbiological analysis or DNA detection by methods based on polymerase chain reaction (PCR) and indirect tests, which are applied *in vitro* (mainly to milk or blood) or *in vivo* (allergic testing) (MCGIVEN, 2013; NIELSEN *et al.*, 2010). Biotyping provides valuable epidemiological information that allows tracing infections back to their sources in countries where multiple biotypes are co-circulating. However, when a given biovar is predominant, classical typing techniques are useless because they do not allow the differentiation of isolates belonging to the same biovar of a given biotype species (OLIVEIRA *et al.*, 2017). In this context, fingerprinting methods such as *Multiple Locus Variable Number of Tandem Repeat (VNTR) Analysis* - MLVA which measures the number of tandem repeats at a given locus, and *Multiple Locus Sequence Typing* (MLST), can differentiate isolates within a given biovar (HIGGINS *et al.*, 2012).

The tests conventionally used in the diagnosis of bovine brucellosis are serological tests due to their low cost, sensitivity and availability (MCGIVEN, 2013; NIELSEN *et al.*, 2010) however, most use crude protein extracts, whole bacterial cells or smooth lipopolysaccharide (S-LPS) fractions as antigens (POESTER *et al.*, 2010), which is not able to differentiate vaccinated animals from infected animals (FARIA *et al.*, 2020). These tests have different diagnostic sensitivity (DSe) and diagnostic specificity (DSp) depending on numerous variables, to different study designs, serological protocols, compositions of the antigen panel and the

adopted cutoff points (and consequently the interpretation of the test) (GODFROID *et al.*, 2010).

Given this context, this thesis aimed to evaluate in general guidelines that can influence and seek diagnostic strategies for programs to control and eradicate bovine brucellosis, being divided into five chapters: 1) Accuracy of serological tests for bovine brucellosis: a systematic review and meta-analysis; 2) Accuracy and covariance of routine serological tests for the diagnosis of bovine brucellosis; 3) Use of recombinant malate dehydrogenase (MDH) and superoxide dismutase (SOD) [Cu-Zn] as antigens in indirect ELISA for diagnosis of bovine brucellosis; 4) Absence of *Brucella* spp. in ovaries of seropositive cattle; 5) Phenotypic and genotypic characterization of *Brucella abortus* biovar 4 isolates from cattle in Brazil.

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SECOND PART - ARTICLES *

**Prepared in accordance with journal standards*

Article 1

Prepared and formatted according to the guidelines of the journal *Preventive Veterinary Medicine*

Accuracy of serological tests for bovine brucellosis: a systematic review and meta-analysis

Abstract

This systematic review and meta-analysis aimed to recalculate the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of serological tests used in diagnosis of bovine brucellosis. CABI, Cochrane Library, PubMed/MEDLINE, SciELO, Scopus and Web of Science databases were used to select articles. The search resulted in 5,308 studies, of which 71 were selected for systematic review using quality assessment tools and 65 studies were included in the meta-analysis. For the meta-analysis, 178 assays and 11 different serological tests were considered. To calculate DSe and DSp of the tests, studies were divided according to animal selection for the studies: (1) studies that carried out a random or consecutive selection of participants (noncase–control studies) and (2) all studies (including case–control studies). Comparison of the DSe and DSp obtained from these two groups showed a variation, being values of DSe and DSp for all tests (n = 11) overestimated when case–control studies were included in the analyses. Considering only the noncase–control studies, the DSe ranged from 85.02 to 96.52%, with iELISA (indirect enzyme-linked immunosorbent assay -bacterial suspension as antigen) (96.52%, 95% CI: 94.14-97.95%) and 2ME (2-mercaptoethanol test) (85.02%, 95% CI: 79.62-89.19) being the tests with the highest and lowest sensitivity, respectively. For DSp, taking into account only the noncase–control group, the tests that presented better and worse performance were FPA (fluorescence polarization assay) (99.70%, 95% CI: 99.51-99.82%) and PCFIA

(protein concentration fluorescence immunoassay) (78.53%, 95% CI: 70.03-85.13'%), respectively. Overall, our results showed overestimation in the DSe and DSp of the eleven serological tests assessed when case–control studies were included in the meta-analysis, which is a concern considering that several of these tests are routinely used in the control and eradication of bovine brucellosis. Furthermore, the tests that exhibited the best DSe and DSp were iELISA (BS) and FPA, respectively, which are relatively easy to perform and interpret, in addition FPA showed the best accuracy, a DSp significantly different from all other tests, except for 2ME.

Keywords: sensitivity; specificity; *Brucella abortus*; diagnosis.

1. Introduction

Brucellosis is a highly contagious disease caused by intracellular Gram-negative, nonspore-forming, nonmotile and facultative bacteria belonging to the genus *Brucella* (Corbel, 2006; WOAH, 2022). Consistent with its ranking among the most economically important zoonoses, brucellosis causes significant losses to livestock, affecting the economies of several countries (McDermott et al., 2013). In cattle, the infection is mainly caused by *Brucella abortus*, and the economic losses associated with bovine brucellosis are mainly related to reproductive problems, such as abortion in the final trimester of pregnancy, retained placenta, temporary or permanent infertility and discard of positive animals (WOAH, 2022). For the control, eradication and surveillance of bovine brucellosis, the correct diagnosis of the disease, which is performed strictly using laboratory tests due the lack of specific clinical signs, is crucial (Cardenas et al., 2019).

The diagnosis of brucellosis in control and eradication programs is generally based on bacteriological and serological tests (Gall & Nielsen, 2004). Isolation and identification of *Brucella* spp. are considered the gold standard for confirmatory and accurate diagnosis of brucellosis (Nielsen, 2002; WOAH, 2022). However, the probability of obtaining a positive culture from a live infected animal is very low when samples are not collected from an abortion

(McGiven, 2013). In addition, culture is unfeasible for use on a large scale, considering the costs and biosafety risks involved, since *B. abortus* is a biosafety level 3 pathogen (Poester et al., 2010). Indeed, this procedure is associated with high risk of infection to laboratory personnel (Pereira et al., 2020). Other direct diagnostic techniques, such as PCR (polymerase chain reaction) tests using serum/blood, swabs and milk, which are relatively easily accessible materials, are used as alternative tools for the diagnosis of brucellosis; however, the accuracy of such tests has not been well established, and the existing information is conflicting (McGiven, 2013; Nielsen & Yu, 2010).

In this scenario, serological tests are the basis for the diagnosis of bovine brucellosis, as the clinical sample used is readily available and the tests are relatively easy to perform and inexpensive (Nielsen & Yu, 2010). However, although there are several serological tests used for the diagnosis of brucellosis, no test is considered 100% accurate, and there is no concept of an ideal test when used alone due to variations in its diagnostic sensitivity (DSe) and diagnostic specificity (DSp), which may also be related not only to the test but also to the epidemiology of the disease (Greiner & Gardner, 2000; WOAH, 2022). Traditionally, the serological diagnosis of bovine brucellosis is performed using a series strategy employing screening and confirmatory tests. Screening tests are highly sensitive, inexpensive and rapid, e.g Rose Bengal test (RBT), the buffered plate agglutination test (BPAT) and indirect enzyme-linked immunosorbent assay (iELISA); however, positive results in these tests should be subjected to confirmatory tests (Gall & Nielsen, 2004). On the other hand, confirmatory tests have good sensitivity and high specificity but generally require elaborate equipment and skillful interpretation of results, e.g., the complement fixation test (CF) and competitive enzyme-linked immunosorbent assay (cELISA) (Poester et al., 2010).

A common observation is that DSe (the ability of the test to correctly identify an animal with the disease) and DSp (the ability of the test to correctly identify animals without the

disease) for the same tests can vary substantially considering different published validation studies. These variations attributed to the DSe and DSp estimates for the same tests may be due to different study designs, serological protocols, compositions of the antigen panel and the adopted cutoff points (and consequently the interpretation of the test). An absence of consistent DSe and DSp estimates for bovine brucellosis serological tests preclude the understanding the real scenario of the disease and the optimization of the diagnosis strategy for their use in brucellosis control and eradication programs. Therefore, herein we performed a systematic review and meta-analysis to recalculate the accuracy (DSe and DSp) of the most used serological tests for bovine brucellosis diagnosis worldwide to determine which tests are more reliable for the diagnosis of this disease.

2. Material and methods

The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Page et al., 2021) were formally adopted in this review and are detailed in Supplementary Table S1.

2.1 Search strategy

The search was carried out on May 27, 2020. The selected keywords were investigated in all sections of the articles (title, abstract and full text) in the following databases: CABI, the Cochrane Library, PubMed/MEDLINE, SciELO, Scopus and Web of Science. Briefly, the PICO (population, intervention, comparison and outcome) used for the research were cattle, cows, heifers and calves (population), brucellosis diagnosis (intervention), serological tests (comparison) and validation, sensitivity, specificity, optimization, and evaluation (outcome), without restrictions regarding when and where the studies were published. The search terms used in each database are shown in Supplementary Table S2.

2.2 Selection of the studies

In the first stage, the studies were selected based on their titles (RSA and MMO). Then, two reviewers (RSA and MMO) independently evaluated the abstracts of the studies selected by

their titles. Subsequently, the full texts of abstract-selected papers were evaluated in terms of their relevance according to the inclusion/exclusion criteria. If the two reviewers disagreed in any step, a third reviewer (EMSD) was responsible for the final decision.

An unpublished study (Andrade et al.) of the authors was also included. The inclusion and exclusion criteria, as well the quality parameters for inclusion in the systematic review and meta-analysis followed the same criteria established for published papers.

2.3 Inclusion and exclusion criteria

Articles that performed the assessment of DSe and DSp of an index test (a diagnostic test that is being evaluated in a study of test accuracy) designed for the serological diagnosis of brucellosis in cattle were included in the review. Articles that did not assess DSe and DSp (i); articles that focused on evaluation of response to vaccination (ii); articles that assessed other nonserological tests (iii); articles that investigated diseases other than brucellosis (iv); or articles that evaluated the diagnosis of brucellosis in animal species than cattle (v) were excluded. In addition, papers written in languages other than English, Spanish, French or Portuguese were ineligible. The complete inclusion and exclusion criteria are shown in Supplementary Table S3.

2.4 Types of studies

Original articles with a cohort, case–control or cross-sectional study design were included in the review. Case series reports, case reports, conference proceedings, reviews, book chapters and books were excluded.

2.5 Quality assessment

The studies included in the qualitative synthesis were divided based on the statistical inference framework (frequentist or Bayesian) adopted to evaluate the accuracy (DSe and DSp) of serological test(s). Studies within frequentist framework were evaluated for quality according to the revised Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) (Whiting et al., 2011) using the RevMan 5.4 software (Review Manager 5.4, Cochrane). The

QUADAS-2 tool is used to assess the risk of bias and applicability of results across four domains: participant selection, index test, reference standard, and flow and timing. We custom-tailored QUADAS-2 criteria to the needs of our review as can be seen in Supplementary Table S4. Briefly, it was assessed if selected papers used a consecutive or random sampling of the animals; how the results of the index test were interpreted; and if the reference and index test were assessed using the same serum samples. Furthermore, to define patients it was accepted culture/isolation of the agent or two or more serological tests, whereas, for the definition of non-sick patients, two or more serological tests or animals from brucellosis free herds or areas should be used. Negative culture was accepted for definition of non-cases when it was also used for definition of patients.

Studies that used Bayesian approach were evaluated for quality following the Standards for the Reporting Studies of Diagnostic Accuracy that use Bayesian Latent Class Models (STARD-BLCM), which aims to evaluate the quality of reporting on the design, conduct, and outcomes of studies that conducted Bayesian assessment of diagnosis tests using latent class models (Kostoulas et al., 2017).

2.6 Data extraction

Data were extracted from the articles by one of the reviewers (RSA) and later checked for accuracy by another reviewer (EMSD). Disagreements regarding data extraction among reviewers were resolved by consensus. For all selected articles, the data extracted included: first author; geographic location where the study was performed; study period; index test(s); total number of animals used; study design; antigen(s); antigen type(s); strain(s) used in antigen preparation; cutoff point(s) adopted; statistical model used (frequentist approach or Bayesian model). For those articles that adopted the frequentist methodology data on negative and positive reference standard (gold standard), number of true positives (TPs), true negatives (TNs), false positives (FPs) and false negatives (FNs), and DSe and DSp values were also

extracted. For papers that used a Bayesian model, the DSe and DSp estimates and their respective credibility intervals, as well as the priors used for DSe and Dsp, were extracted. Those data were used to infer actual numbers (TPs, TNs, FPs and FNs) recorded by the researcher. This backward estimation was done using conjugacy properties of the beta distribution to combine prior and posterior information and equating resulting expressions with reported summaries of the posterior.

2.7 Meta-analysis

Serological tests using the same antigen(s) that were evaluated by more than one study were included in the meta-analysis. DSe and DSp were estimated in the meta-analysis considering the values of TPs, TNs, FPs and FNs for each individual index test, considering the possible different antigens used (Dohoo et al., 2012).

To assess the possibility of introducing biases considering to the selection of the animals for the studies, the studies were divided as follows: (1) group of studies that performed a randomized or consecutive selection of participants (noncase–control studies) and (2) group of studies that selected participants from an at-risk population (case-control studies) (Supplementary Table S4) according to the Cochrane Methodological Quality Assessment Manual for Systematic Reviews (Reitsma et al., 2009). Studies that include a consecutive or random series of patients who meet all selection criteria are considered an ideal diagnostic accuracy study, and studies that select two separate groups to sample patients with the target condition and patients without the target condition produce estimate accuracy biases (Reitsma et al., 2009).

DSe was defined by $TP/(TP + FN)$, DSp by $TN/(TN + FP)$ and accuracy by $(Dse + DSp)/2$ (Dohoo et al., 2012). The model used in the meta-analysis of DSe and DSp was a generalized linear mixed model (binomial with logit link) (Chu & Cole, 2006). The linear predictor was a function of the fixed effects for the study design (case–control or not) and the

random effects for the article (author) and index tests. All analyses were performed using R statistical software (Team, 2021).

3. Results

3.1 Selected studies

The search strategy adopted identified a total of 5,308 articles (1,040 duplicates were excluded), of which 3,579 articles were excluded by title, 66 articles were excluded based on abstract screening; therefore, 623 full texts were sought for retrieval and 35 articles were not retrieved. Subsequently, 588 were assessed for eligibility, of which 439 were excluded with a reason and an unpublished study was included (Andrade et al., unpublished data). Following, 150 studies were assessed by quality level according to the statistic approach adopted (QUADAS-2/frequentist; STARD-BLCM/Bayesian), with 71 articles included in the qualitative synthesis (systematic review) and 65 in the quantitative synthesis (meta-analysis) after a full review (Figure 1). The main reasons for exclusion of the 79 papers for quality [QUADAS-2 (n = 76)/STARD-BLCM (n = 3)] are detailed in Supplementary Table S5 and Supplementary Figure S1. As a study can assess multiple index tests, an entire manuscript was referred to as a ‘study’, whereas each single serological test (index test) assessed in a study was referred to as a ‘assay’. A total of 71 studies and 232 assays were evaluated.

The 71 articles selected in the systematic review were published from 1969 to 2020, with 1.41% (1/71) published between 1960-1969 (1 frequentist), 5.63% (4/71) between 1970-1979 (4 frequentist), 7.04% (5/71) between 1980-1989 (5 frequentist), 19.72% (14/71) between 1990-1999 (14 frequentist), 30.99% (22/71) between 2000-2009 (19 frequentist and 3 Bayesian), 32.39% (23/71) published between 2010-2019 (16 frequentist and 7 Bayesian) and 1.41% (1/71) in 2020 (frequentist) and 1.41% (1/71) unpublished data (Andrade et al.) (frequentist) (Figure 2A). The main characteristics [index test(s), reference standard adopted, country and year where and when the study was conducted, and number of animals tested] of the papers included in the systematic review are summarized in Supplementary Table S6.

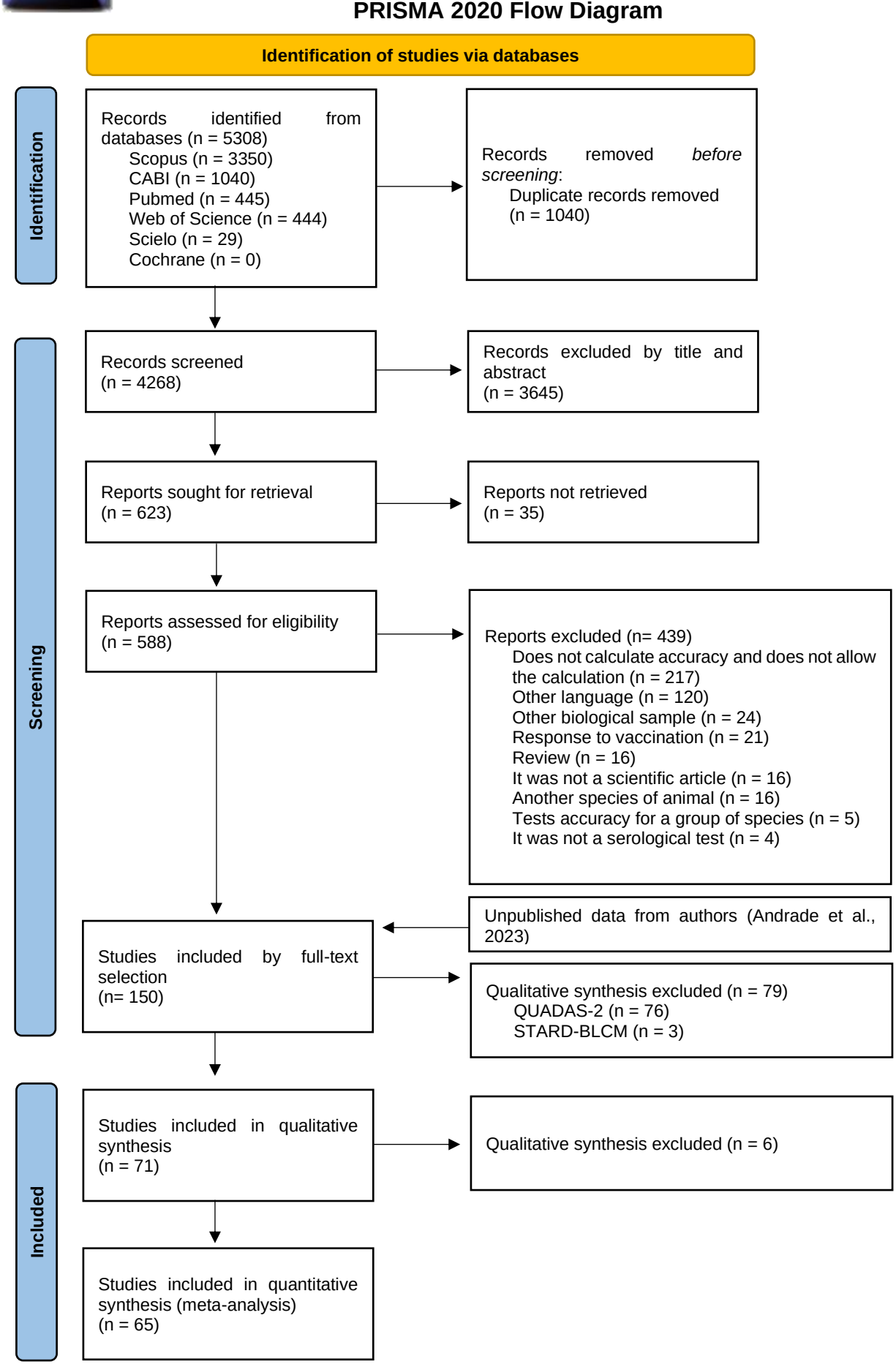

Figure 1: PRISMA flowchart used in the selection of the studies for this systematic review and meta-analysis

3.2 Evaluated tests and antigens

The 232 assays evaluated included 31 different serological tests for the diagnosis of bovine brucellosis: 28.02% (65/232) iELISA (indirect enzyme-linked immunosorbent assay), 13.36% (31/232) cELISA (competitive enzyme-linked immunosorbent assay), 12.93% (30/232) RBT (Rose Bengal test), 10.78% (25/232) CF (complement fixation test), 6.03% (14/232) FPA (fluorescence polarization assay), 6.03% (14/232) SAT (serum agglutination test), 3.88% (9/232) BPAT (buffered plate antigen test), 2.59% (6/232) LFIA (lateral flow immunochromatographic assay), 2.16% (5/232) RID (radial immunodiffusion), 1.29% (3/232) 2ME (2-mercaptoethanol test), 1.29% (3/232) AGID (agar gel immunodiffusion), 1.29% (3/232) ERIFA (enzymatic rapid immunofiltration assay), 1.29% (3/232) PFCIA (particle concentration fluorescence immunoassay), 0.86% (2/232) EDTA (ethylene diamine tetra-acetic acid), 0.86% (2/232) ELISA-EDTA (indirect enzyme-linked immunosorbent assay-ethylene diamine tetra-acetic acid), 0.86% (2/232) SPAT (standard plate agglutination test), 0.43% (1/232) ABGT-R (antibovine globulin test, rapid method), 0.43% (1/232) ABGT-T (antibovine globulin test, tube method), 0.43% (1/232) AlphaLISA, 0.43% (1/232) Chaotropic_ELISA_1M_KSCN, 0.43% (1/232) Chaotropic_ELISA_2M_KSCN, 0.43% (1/232) Chaotropic_ELISA_3M_KSCN, 0.43% (1/232) counterimmunoelectrophoresis, 0.43% (1/232) DID (double gel immunodiffusion), 0.43% (1/232) dot blot (dot blot test), 0.43% (1/232) FPA-blood (fluorescence polarization assay performed from blood), 0.43% (1/232) HIGT (hemolysis-in-gel test), 0.43% (1/232) IHLT (indirect hemolysis test), 0.43% (1/232) qRBT (quantitative Rose Bengal test), 0.43% (1/232) RBT-automated (Rose Bengal test-automated) and 0.43% (1/232) RIV (rivanol test) (Figure 2B).

Seven serological tests assessed more than one antigen: iELISA (17 different antigens), 64.6% (42/65) smooth lipopolysaccharides (S-LPS), 10.8% (7/65) bacterial suspension (BS), 3.1% (2/65) rough lipopolysaccharides (R-LPSs), 1.5% (1/65) S-LPSs/R-LPSs, 1.5% (1/65)

BP26, 1.5% (1/65) *Brucella* antigen IFFA-Merieux 1.5% (1/65) cytosolic proteins, 1.5% (1/65) native hapten, 1.5% (1/65) polysaccharides, 1.5% (1/65) disaccharide 1, 1.5% (1/65) nonasaccharide 6, 1.5% (1/65) pentasaccharide 5, 1.5% (1/65) tetrasaccharide 4, 1.5% (1/65) trisaccharide 2 terminal α(1→ 2), 1.5% (1/65) trisaccharide 3 terminal α(1→ 3), 1.5% (1/65) the antigen used in the kit IDEXX Brucellosis Serum X2 Ab Test (undisclosed) and 1.5% (1/65) the antigen used in the kit USDA (NADI) (undisclosed); AGID (3 different antigens), 33.3% (1/3) O-chain, 33.3% (1/3) native hapten, 33.3% (1/3) S-LPSs; ERIFA (3 different antigens), 33.3% (1/3) chromatographic fractions of cell lysate, 33.3% (1/3) O-polysaccharide and 33.3% (1/3) S-LPSs; RID test (3 different antigens), 40% (2/5) native hapten, 40% (2/5) S-LPSs, 20% (1/5) cytosolic proteins; LFIA (2 different antigens), 83.3% (5/6) S-LPSs and 16.7% (1/6) the antigen used in the kit BIONOTE (undisclosed); CF (2 different antigens), 96% (24/25) bacterial suspension and 4% (1/25) S99/RB51; and cELISA (2 different antigens), 96.8% (30/31) S-LPSs and 3.2% (1/31) the antigen used by Kalleshamurthy et al. (2020) (undisclosed).

All tests referred in the studies as the card test were grouped together with the RBT (Fosgate, 2002; Greenlee, 1994; O'Reilly, 1971; Stemshorn, 1985), since the RBT and the card test are very similar (Alton et al., 1988; Sayour et al., 2017).

3.3 Study design

The average number of assays tested per study was 3.27 ± 2.8, ranging from 1 to 19 assays per study (Supplementary Table S6). Among the 71 studies, 85.9% (61/71) used frequentist statistic and 14.1% (10/71) used Bayesian approach (Figure 2A). Regarding the 232 assays, 85.8% (199/232) adopted frequentist and 14.2% (33/232) Bayesian statistic.

The positive and negative reference standards of the 199 assays that used frequentist statistics were classified according to the quality assessment tool QUADAS-2 (Supplementary Table S4). As positive reference standard, positive culture was adopted by 50.3% (100/199) of the assays, positive serological tests (at least two different tests) by 18.6% (37/199) of the

assays, positive culture and positive serological test(s) by 17.1% (34/199) of the assays, suggestive epidemiology + two or more positive serological tests by 9.5% (19/199) of the assays, positive culture or serological tests (at least two different tests) by 2.5% (5/199) of the assays, and experimental challenge and positive culture by 1.5% (3/199) of the assays, whereas positive serological tests (at least two different tests) plus positive PCR were adopted by 0.5% (1/199) of the assays (Figure 3A). According to the 34 studies that used serological tests to define the positive reference standard, the main serological tests used to classify the animals as positive were the CF [67.6% (23/34)], RBT [61.8% (21/34)], 2ME [35.3% (12/34)], SAT [32.4% (11/34)] and iELISA [14.7% (5/34)] (Supplementary Table S6).

As negative reference standard, sera of animals from brucellosis free areas were used by 59.8% (119/199) of the assays, 21.6% (43/199) of the assays used animals with negative serological test(s) from brucellosis free areas, 12.1% (24/199) of the assays used animals with negative serological tests (at least two different tests), 6% (12/199) of the assays used animals with negative culture and 0.5% (1/199) of the assays used animals with negative serological test(s) from brucellosis free areas plus negative PCR (Figure 3B).

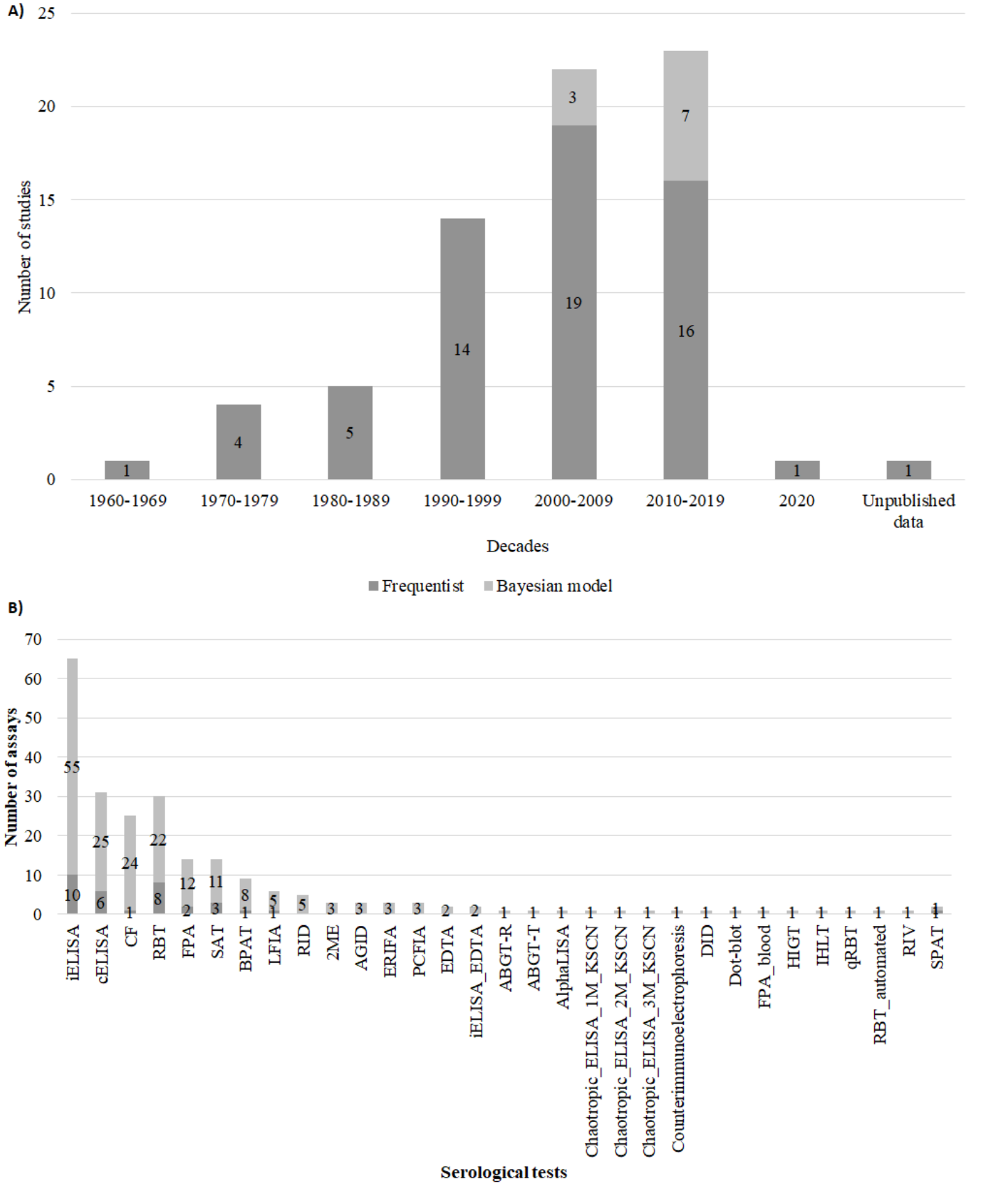

Figure 2. Characteristics of the 71 studies and 232 assays included in the systematic review. A) Distribution of the selected studies according to statistical methodology employed and decade when they were published. B) Distribution of 232 assays among the 31 different serological tests for the diagnosis of bovine brucellosis included in the systematic review.

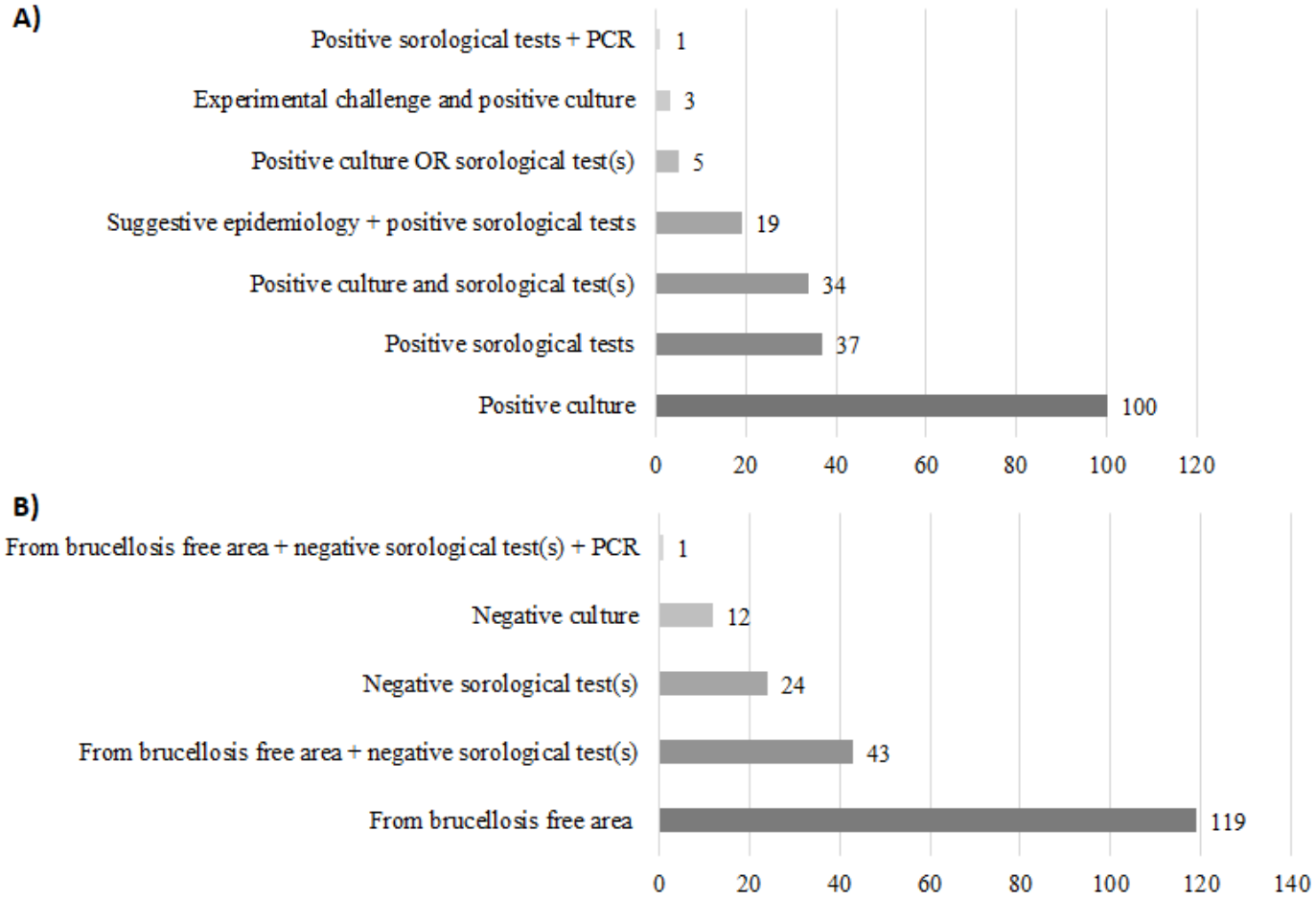

Figure 3. Classification of the 199 assays with a frequentist methodology for defining a positive and negative reference standard used in the meta-analysis. A) Classification of the positive group according to the reference standard with frequentist methodology in the studies included in the meta-analysis; B) Classification of the negative group according to the reference standard with frequentist methodology in the studies included in the meta-analysis.

3.4 Cutoff points used

Of the 31 serological tests included in this systematic review 67.74% (21/31) were quantitative tests and 32.26% (10/31) qualitative tests (Supplementary Table S7). The cutoff points described for the quantitative tests showed a difference within the same test. iELISA was the test that exhibited the greatest diversity of cutoff points (50), followed by cELISA (12), CF (7), SAT (7) and FPA (6) (Supplementary Table S7). The qualitative tests had their positive results defined for the presence of agglutination (BPAT, RBT-automated and RBT), precipitation lines (AGID, counterimmunoelectrophoresis, DID, LFIA and RID) and color development in the cassette (dot blot, ERIFA).

3.5 Meta-analysis

To estimate the DSe and DSp of bovine brucellosis serological tests, 65 studies and 178 assays were included in the meta-analysis. Six studies (Bastos, 2018; Byrd & Hidalgo, 1979; Cho, 2010; Daffner, 1999; Elshemey, 2014; Gall et al., 2006) and 54 assays were excluded because there was just one assay in each combination test/antigen assessed (Supplementary Table S8). Among the 65 studies included in the meta-analysis 84.62% (55/65) employed frequentist statistics, and 15.38% (10/65) employed a Bayesian model.

DSe and DSp were estimated for 11 serological tests, being iELISA the only in which 2 different antigens (bacterial suspension and S-LPSs) were assessed (Table 1 and Figure 4). To estimate the level of bias introduced by case-control studies, two estimates of DSe and DSp were performed: one with 100% (65/65, 178 assays) of the studies included in the meta-analysis and the other including only the studies that used randomized/consecutive sampling (noncase–control studies) [53.85% (35/65) of the studies and 41.01% (73/178) of the assays]. All studies that used the Bayesian model were classified as noncase–control studies since they performed a random or consecutive selection of the participants.

DSe for brucellosis serological tests considering all studies ranged from 92.23 to 98.31%,

with iELISA (bacterial suspension) (98.31%; 95% CI: 97.11-99.72%), iELISA (S-LPSs) (98.05%; 95% CI: 97.40-98.54%), cELISA (97.64%; 95% CI: 96.86-98.22%) and BPAT (97.54%; 95% CI: 96.60-98.23%) showing the highest DSe, whereas 2ME showed the lowest DSe (92.23%; 95% CI: 89.09-94.52%) (Table 1 and Figure 4). DSp ranged from 97.41 to 99.97% considering all studies. FPA was the test that exhibited the highest DSp (99.97%; 95% CI: 99.95-99.98%), followed by 2ME 99.92% (95% CI: 99.79-99.97%), BPAT (99.87%; 95% CI: 99.79-99.92%) and cELISA (99.83%; 95% CI: 99.73-99.89%), while PCFIA was the test with the lowest value of DSp (97.41%; 95% CI: 96.01-98.33%) (Table 1 and Figure 4).

Taking into account only the group of studies that did not have case–control study designs, the values for both DSe and DSp had greater variation compared with the estimates that included all studies. The DSe values varied from 85.02 to 96.52%, with iELISA (BS) (96.52%; 95% CI: 94.14-97.95%) and 2ME (85.02%; 95% CI: 79.62-89.19) being the tests with the highest and lowest DSe, respectively (Table 1 and Figure 4A). Regarding DSp, the estimated values ranged from 78.53 to 99.70%, and better and worse performance was observed, as in the estimates that included all studies, for FPA (99.70%; 95% CI: 99.51-99.82%) and PCFIA (78.53%; 95% CI: 70.03-85.13%) (Table 1 and Figure 4B). Meta-analysis estimates of DSe and DSp for each test considering all studies or just the studies that used randomized/consecutive sampling (noncase–control studies) are summarized in Table 1.

Regarding the accuracy of the tests in the two scenarios, all studies and only the noncase–control studies, the test that exhibited the highest and lowest performance were FPA and PCFIA, respectively. Taking into account only the values of noncase-control (randomized) studies, the FPA showed an accuracy of 98.65% (95% CI: 97.97-99.10%), while PCFIA of 82.47% (95% CI 75.61-87.71%).

Considering as significantly different only the estimates in which the confidence did not overlap, the DSp values obtained for the noncase-control studies (randomized), showed more

significant statistical differences than the values obtained for DSe, comparing the 11 assessed tests. In fact, the FPA exhibited a DSp significantly superior to those obtained by all the other tests except for 2ME, whereas for the DSe estimates, the iELISA (BS) showed a significantly higher performance compared with RBT, CF, PCFIA and 2ME (Table 1 and Supplementary Table S9). Comparisons of the confidence intervals among all tests obtained for the DSe and DSp in the noncase-control group (randomized) is detailed in Supplementary Table S9.

Table 1: Diagnosis sensitivity (DSe) and diagnosis specificity (DSp) meta-analysis estimates for 11 serological tests used for the diagnosis of bovine brucellosis, considering all studies (including case-control studies) and only the studies that carried out the random / consecutive selection of participants (non-case-control studies).

Tests	All studies (Including case control)									Randomized								
	DSe[a]	95% CI[b]		DSp[c]	95% CI		Accuracy	95% CI		Dse	95% CI		DSp	95% CI		Accuracy	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
2ME[d]	0.9223	0.8909	0.9452	0.9992	0.9979	0.9997	0.9916	0.9841	0.9956	0.8502	0.7962	0.8919	0.9914	0.9784	0.9966	0.9624	0.9301	0.9801
BPAT[e]	0.9754	0.9660	0.9823	0.9987	0.9979	0.9992	0.9942	0.9915	0.9961	0.9499	0.9315	0.9636	0.9864	0.9787	0.9913	0.9738	0.9616	0.9821
cELISA[f]	0.9764	0.9686	0.9822	0.9983	0.9973	0.9989	0.9936	0.9907	0.9956	0.9518	0.9366	0.9635	0.9825	0.9728	0.9888	0.9708	0.9583	0.9797
CF[g]	0.9582	0.9455	0.9681	0.9946	0.9916	0.9965	0.9849	0.9784	0.9894	0.9164	0.8924	0.9355	0.9471	0.9197	0.9655	0.9334	0.9069	0.9527
FPA[h]	0.9707	0.9597	0.9788	0.9997	0.9995	0.9998	0.9971	0.9956	0.9981	0.9407	0.9192	0.9568	0.9970	0.9951	0.9982	0.9865	0.9797	0.9910
iELISA (BS)[i]	0.9831	0.9711	0.9901	0.9972	0.9951	0.9984	0.9931	0.9880	0.9960	0.9652	0.9414	0.9795	0.9717	0.9513	0.9836	0.9686	0.9466	0.9817
iELISA (S-LPS)[j]	0.9805	0.9740	0.9854	0.9969	0.9951	0.9980	0.9922	0.9887	0.9946	0.9601	0.9472	0.9700	0.9686	0.9516	0.9797	0.9646	0.9494	0.9753
LFIA[k]	0.9660	0.9267	0.9846	0.9850	0.9666	0.9933	0.9774	0.9503	0.9899	0.9314	0.8581	0.9683	0.8644	0.7374	0.9354	0.9030	0.8047	0.9546
PCFIA[l]	0.9268	0.8959	0.9490	0.9741	0.9601	0.9833	0.9562	0.9350	0.9707	0.8582	0.8045	0.8990	0.7853	0.7003	0.8513	0.8247	0.7561	0.8771
RBT[m]	0.9614	0.9498	0.9704	0.9893	0.9834	0.9932	0.9796	0.9709	0.9857	0.9225	0.9004	0.9400	0.9000	0.8516	0.9338	0.9119	0.8781	0.9370
SAT[n]	0.9642	0.9526	0.9731	0.9925	0.9881	0.9953	0.9835	0.9761	0.9887	0.9280	0.9057	0.9453	0.9276	0.8894	0.9533	0.9278	0.8978	0.9495

[a] DSe: Diagnosis sensitivity; [b] 95% CI: confidence intervals of 95%; [c] DSp: Diagnosis specificity; [d] 2-mercaptoethanol; [e] BPAT: buffered plate antigen test; [f] cELISA: competitive enzyme-linked immunosorbent assay; [g] CF: complement fixation test; [h] FPA: fluorescence polarization assay; [i] iELISA (BS): indirect enzyme-linked immunosorbent assay (bacterial suspension); [j] iELISA (S-LPS): indirect enzyme-linked immunosorbent assay (smooth lipopolysaccharide); [k] LFIA: lateral flow immunochromatographic assay; [l] PCFIA: particle concentration fluorescence immunoassay; [m] RBT: Rose Bengal test; [n] SAT: serum agglutination test.

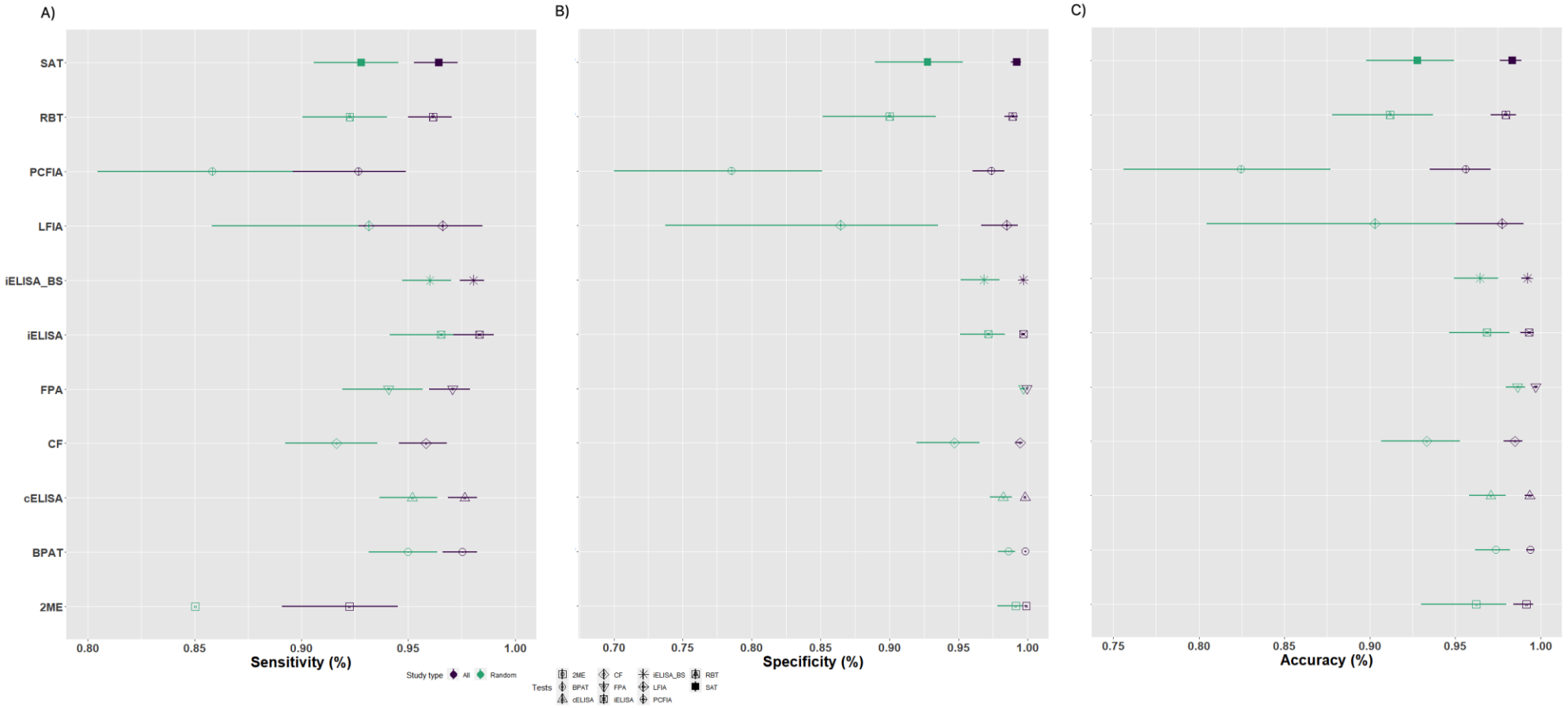

Figure 4. Forest plot of diagnosis sensitivity (DSe) (A) and diagnosis specificity (DSp) (B) accuracy (C) estimates for 11 serological tests used in the diagnosis of bovine brucellosis included in the meta-analysis. The geometric shapes in purple represent the estimates for all studies selected for the meta-analysis (All) and in green shapes represent the estimated values considering only the randomized studies (Random) (those that avoided a case-control study design). The geometric shapes are the DSe and DSp media estimated for each test and the lines are the 95% confidence interval.

4. Discussion

Systematic reviews and well-executed meta-analyses play an important role in the summarization of knowledge for clinical and epidemiological decision-making, as they condense the results of a number of studies (Sargeant & O'Connor, 2020). The current accuracy estimates of diagnostic tests for bovine brucellosis are diverse and highly variable, which makes their assertive use difficult in clinical practice and in control and eradication programs. Given this gap, the present study aimed to systematically review the literature and to recalculate the accuracy (DSe and DSp) of the main serological tests used for the diagnosis of bovine brucellosis worldwide. Our results highlighted an important overestimation of DSe and DSp, especially DSe, in the meta-analysis estimates that considered all studies included in the quantitative synthesis compared to those obtained only from the randomized studies (noncase–control studies). Moreover, the data also showed iELISA (BS) as antigen and FPA, as the tests with better DSe and DSp, respectively, considering both scenarios assessed (all studies and noncase–control studies).

To date, despite the vast literature available on serological diagnostic tests for bovine brucellosis, no meta-analysis has been performed to determine which tests have the best accuracy (DSe and DSp) to support their use in control and eradication strategies. In 2006, at the request of the European Commission, the European Food Safety Authority (EFSA) carried out a meta-analysis on the suitability of current tests used by the European Union (EU) and some new tests [FPA, cELISA, radial immunodiffusion test with native hapten (RIDNH)] for the diagnosis of brucellosis in cattle in intracommunity trade (EFSA, 2006). Later, the EFSA (2006) meta-analysis data were used by Greiner et al. (2009) to perform a new meta-analysis that aimed to refine the statistical model used before and to allow the comparison of multiple situations (a candidate test versus a set of comparative tests). However, it is important to mention that the objectives of both meta-analyses were different from the present one (different tests), and the information source used was also different, as the previous meta-analysis

included only the Cochrane Library and data from EU reference laboratories, data requested from suppliers and unpublished data from the working group.

Similarly, Ducrotoy et al. (2018) also conducted a systematic review on immunological tests used for the diagnosis of brucellosis in cattle; however, they did not perform a meta-analysis and only described the DSe and DSp of serological and delayed type hypersensitivity (DTH) tests and their applications in different contexts. Their justification in not performing a meta-analysis was the difficulty of determining the dependence or conditional independence of the tests considering different tests (antigen, isotype, etc.) and populations (different epidemiological scenarios, stages of the disease, etc.). Indeed, these are important points to be considered when conducting a meta-analysis; therefore, we rigorously analyzed biases based on QUADAS-2 (Whiting et al., 2011) and STARD-BLCM (Kostoulas et al., 2017), conducted two separate meta-analyses considering the selection of the participants in the studies (all studies and noncase–control studies) and adopted a robust statistical model that used the study design as a fixed effect (including case–control studies or not) and the studies (author) and tests/antigens as random effects, which reduced the heterogeneity described above.

The results of our meta-analysis highlighted a relevant overestimation of the accuracy of serological tests for bovine brucellosis diagnosis for all assessed serological tests from the comparison of the estimates obtained for the studies that used a randomized sampling with those that considered all selected studies. This overestimation can be explained by the study design of the articles used, since the meta-analysis that included all studies mainly included studies with case–control designs, in which the selection of participants was based on an at-risk population and compared patients (cases) *versus* nondiseased subjects (controls) (Sainani & Popat, 2011). In contrast, in the randomized/consecutive selection of participants (e.g., sectional studies), health events are randomly distributed in the population (Dohoo et al., 2012). The implication of using case–control studies is that the strategy of selecting the extremes of the

population introduces biases, such as selection bias, which leads to a trend in the estimates (DSe and DSp) and consequently to conclusions that are systematically different from the truth (Dohoo et al., 2012).

The discrepancy in the DSe values was more notable than that in the DSp values when comparing the meta-analysis with all studies with the meta-analysis that included only noncase–control studies (Table 1). As the DSe is the probability of an infected animal being classified as positive by the diagnostic, an overestimated DSe means a higher proportion of false-negative results (Dohoo et al., 2012). This may have consequences for the diagnosis of a disease, given that an animal with a false-negative result is not retested for a period of time, and thereby the spread of the disease continues to occur in the herd or region. These overestimated DSe values for serological tests have an impact on bovine brucellosis control and eradication programs, especially in endemic areas (Africa, Asia and Latin America) (Zhang et al., 2018), where disease control can be delayed, increasing the time for reducing the prevalence and the program cost, as the assessed tests are used in the diagnostic strategy by most of these programs around the world. False-negative results can occur for a variety of reasons, such as improper timing of the test, which can result in the test failing to detect infection; nonspecific inhibitors or blocking antibodies and contaminated or hemolyzed samples, which prevent antigen/antibody reactions; and finally, a serological test that is too insensitive to detect antibodies (Thrusfield, 2018).

Even with a lower impact compared to DSe, DSp was also overestimated in the meta-analysis results that included all studies (including the case–control studies) in comparison with the noncase–control studies (Table 1), which has a direct relation with the proportion of false-positive results of a test. In a brucellosis control and eradication programs based on vaccination and test-and-slaughter policies, false-positive results are more harmful in confirmatory tests (Hui & Walter, 1980), as they can result in the definitive disposal of nondiseased animals. This situation may result in financial losses for the producers and the mistaken disposal of animals,

impacting the cost and time to eliminate the disease (WOAH, 2022). Serological false-positive results for bovine brucellosis can be caused by infection with several other Gram-negative bacteria that can induce cross-reactive antibody responses, mainly *Yersinia enterocolitica* O:9 (McGiven, 2013; Munoz et al., 2005; WOAH, 2022). In addition, vaccination with S19 also induces antibodies that can be detected in routine serological tests for brucellosis, primarily if the age recommendations for vaccination and testing are not strictly followed (Dorneles et al., 2015). However, it is important to mention that the real impact of the overestimated DSe and DSp observed on bovine brucellosis control and eradication programs should be estimated by specific studies comparing the cost and the economic viability of eventual changes in the diagnosis protocols.

Albeit the case–control design adopted by most of the selected studies have resulted in the overestimation of DSe and DSp obtained in the meta-analysis; nevertheless, this is an issue difficult to circumvent when performing validation of serological tests for bovine brucellosis. In fact, sera from culture-positive animals are considered ideal (gold standard) to determine the DSe of serological tests for bovine brucellosis and were used for most of the selected assays (57.9%; 103/178 assays included in the meta-analysis) (Gall & Nielsen, 2004; Nielsen & Yu, 2010); however, as the DSe of culture and isolation of *Brucella* spp. is usually low (20 to 50%) (Debeaumont et al., 2005; Poester et al., 2010), the selection of participants in validation assays using this method results in biased DSe estimates with low external validity. Although it is important to emphasize that a positive culture and isolation of the agent means that the animal is indisputably positive for bovine brucellosis. On the other hand, the reverse is not true; the negative test result of an animal does not mean the absence of infection with *Brucella* spp. Therefore, the estimation of the DSp of serological tests for bovine brucellosis is usually performed with negative reference standard sera from animals known to be disease-free (Ducrotoy et al., 2018; EFSA, 2006; Gall & Nielsen, 2004; Greiner & Gardner, 2000). This

differential verification of the reference standards (when some patients are verified by one type of reference standard and other patients by a different standard) typically leads to an overestimation of test accuracy if the reference standards differ in accuracy (Reitsma et al., 2009), which is usually the case in validation studies of brucellosis serological tests. However, taking into account the ethical issues involving the use of an invasive reference test (e.g., tissue biopsy) in animals that have a very low risk of disease (except for abortion, postpartum or post-abortion vaginal swabs the probability of obtaining a positive culture from infected live animals from any tissues is very low) (Xavier et al., 2009), we defined the eligibility criteria for article selection considering both possibilities, the use of the same test to define the positive and negative reference standard (random/consecutive selection of participants) or the selection of the reference standards from different populations (case–control studies) (Supplementary Table S4). Among the frequentist assays included in the systematic review, 57.9% (103/178) defined the positive reference standard based on positive culture, and 69% (123/178) used animals from brucellosis-free areas as the negative reference standard, showing the challenge of performing validation studies using different approaches.

An alternative to overcome the overestimation of DSe and DSp introduced by the case-control study design could be the use of different diagnostic strategies, such as the application of the tests in parallel or in series to increase the DSe and DSp (Dohoo et al., 2012). The combination of tests is a common practice in many animal disease control and eradication programs; normally, the tests are considered conditionally independent, and the theoretical sensitivities and specificities of the combined tests are calculated directly from the values of the individual tests. However, for tests that measure similar physiological/biological processes, test results for a given animal are likely to be correlated (Branscum et al., 2005; Ducrotoy et al., 2018). Therefore, there is a need to consider the possibility of conditional dependence of the tests (covariance), as the dependence of the test substantially alters the theoretical values of

DSe and DSp of the combined tests from those obtained when conditional independence is pointed out (Gardner et al., 2000). Therefore, the determination of the dependence of serological tests for the diagnosis of bovine brucellosis would help to establish the best strategy for using them for the diagnosis of the disease, which could overcome the accuracy overestimation observed in the present study.

The results extracted from our systematic review and meta-analysis showed that the tests that exhibited the best performance were iELISA (BS) for DSe and FPA for DSp and accuracy (Table 1), regardless of study design considered in the meta-analysis. Although a variety of *Brucella* antigens have been tested as alternatives for their use in indirect ELISA, our results demonstrated that the BS antigen was the one with better performance, as previously demonstrated by Abalos (2000) and Pinochet et al. (1990). An important advantage of this antigen is that is easier to prepare, can detect antibodies at low concentrations, being also inexpensive. Likewise, the FPA has been standardized to be one of the main confirmatory tests for the diagnosis of brucellosis in cattle in several endemic countries (WOAH, 2022). The FPA findings are corroborated by previous studies that suggest that this test has superior DSp (Mathias, 2010; McGiven et al., 2003; Nielsen & Yu, 2010). In addition, the FPA could be considered the test choice if a single test is to be used in the diagnosis of bovine brucellosis, since it exhibited the test that showed the best accuracy (98.65%; 95 CI: 97.97-99.10%), together with its characteristics of being a simple and fast (Nielsen & Yu, 2010).

On the contrary, despite CF is the reference test recommended by the WOAH (World Organization for Animal Health) for the international transport of animals and is widely used as a confirmatory test, being considered a highly specific test (WOAH, 2022), our results showed that FPA, 2ME, BPAT and cELISA exhibited a significantly superior DSp compared to CF (Supplementary Table S9). Furthermore, it is also worth to mention that CF was the test most used as reference standards in validation of frequentist studies that used serological tests

as gold standard [67.6% (23/34)], probably because it is one of the most useful conventional tests in the differentiation of post-vaccinal titers (Crawford et al., 1988; Nicolleti, 1976; Nicolleti, 1979).

A limitation of the present work that should be mentioned, it is the bias introduced in the estimates of DSe and DSp of all assessed ELISA tests by the lack of standardization in the cutoff points among the selected papers. This issue should be taken into account when these estimations are analyzed, although it does not make the inferences drawn from the data invalid.

Overall, the use of serological tests for the diagnosis of bovine brucellosis is the fastest, cheapest, and most feasible strategy that can be carried out on a large scale for the control of the disease. Knowledge of the characteristics, applicability, performance, and benefits of serological tests should be improved. This systematic review and meta-analysis compiled the results of the most used tests for bovine brucellosis diagnosis worldwide and estimated their accuracy (DSe and DSp), which bring reliable and useful data to control and eradication programs.

5. Conclusion

Among the most commonly used serological tests for the diagnosis of bovine brucellosis worldwide, those that exhibited better DSe and DSp were iELISA (BS) and FPA, respectively. The DSp and particularly the DSe were overestimated due to the case–control study design used in most of the studies that involved the validation of diagnostic tests for bovine brucellosis, which should be taking into account in their applications in disease control and eradication programs.

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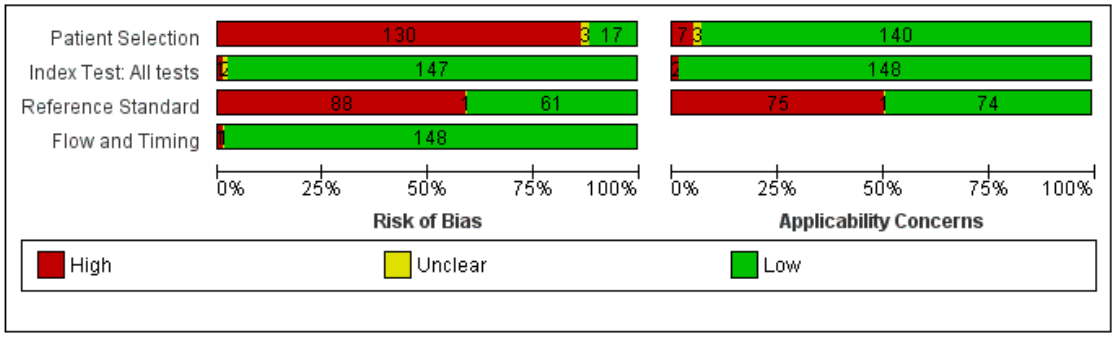
Supplementary figure S1.. Risk of bias and applicability concerns graph: review authors' judgements about each domain presented as percentages across included studies – QUADAS-2



Supplementary tables

Supplementary Table S1 – Guidelines of PRISMA statement

Section and Topic	Item #	Checklist item	Paragraph where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	§1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	§1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	§1, 2, 3
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	§4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	§4 Tab[1]. S2
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	§1 Tab. S3
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	§4
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	§1,2,3
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	§2
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	§5-6 Tab. S2
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	§6-7
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	§6
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	§7

Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	§7
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	§7
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	§8
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	§8 Tab. S4
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	§8,9
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	§8,9
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	§9
Section and Topic	**Item #**	**Checklist item**	**Location where item is reported**
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	§9-10
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	§1 Fig[2].1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	§1 Tab. S5
Study characteristics	17	Cite each included study and present its characteristics.	§2 Tab. S6
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	§1 Tab. S4
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	§4-13
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	§6-7
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	§9-12
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	§9
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	§11
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	-

Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	§9-13
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	§1
	23b	Discuss any limitations of the evidence included in the review.	§4-5
	23c	Discuss any limitations of the review processes used.	§4-5
	23d	Discuss implications of the results for practice, policy, and future research.	§11
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	-
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	-
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	-
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	§1
Competing interests	26	Declare any competing interests of review authors.	§2
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	-

[1]Tab.: Table;[2]Fig.: Figure

Supplementary Table S2 – Search terms used in CABI, Cochrane, Pubmed, Scielo, Scopus and Web of Science databases, based on the PICOTS terms.

PICOTS	Search terms
Population	bovine* OR cattle OR heifer* OR cow* OR calf OR calve*
Intervention	(diagnostic* OR method* OR test OR serolog*) AND (brucel*)
Comparison	rose bengal OR RBT OR rose bengal plate test OR card test OR buffered acidified plate antigen OR buffered Brucella antigen tests OR buffered plate agglutination test OR BPAT OR complement fixation test OR CFT OR indirect ELISA OR indirect enzyme linked immunosorbent assay OR I-ELISA OR competitive ELISA OR competitive enzyme linked immunosorbent assay OR cELISA OR serum tube agglutination test OR serum agglutination in tube OR STAT OR SAT-mercaptoethanol OR SAT OR wright OR 2-mercaptoethanol OR 2-Mercapto Ethanol OR 2-ME OR rivanol test OR RIV OR fluorescence polarization test OR fluorescence polarization assay OR FPA OR heat inactivation test OR coombs antiglobulin test OR coombs OR wright OR immunodiffusion OR RID OR gel immunodiffusion OR DGD OR SRD OR AGID OR PCFIA OR lateral flow immunochromatography OR LFiC
Outcomes	sensitiv* OR specific* OR diagnostic* OR validation OR optimization OR assess* OR evaluation
Time	-
Setting	Systematic review and meta-analysis

Supplementary Table S3 – Inclusion and exclusion criteria for selection of articles in this systematic review.

Inclusion criteria	Exclusion criteria
All countries	Evaluation of the response to vaccination
Bovine brucellosis	Did not perform the accuracy assessment
Cattle	It was not a serological test
Serological test	It was not a diagnostic test
Diagnostic test	Another animal species
Assessment of diagnostic sensitivity and specificity	Another microorganism Other biological sample Tests accuracy for a group of species Written in languages other than English, Spanish, French or Portuguese Full text not available Not a research article

Supplementary Table S4: Criteria for assessment of risk of bias and applicability concerns according to QUADAS-2 used to classify articles in this systematic review.

Domain 1: participant selection		
A. Risk of bias	Was a consecutive or random sample of patients enrolled?	Yes: if a consecutive or random sample of animals was selected. No: if sampling was non-consecutive or non-random, convenience sampling. Unclear: if sufficient information is not available to make a judgment on the spectrum or the sampling method.
	Was a case-control design avoided?	Yes: groups with and without brucellosis were recruited from the same population. No: groups with and without brucellosis were recruited separately. Unclear: if there is not enough information available to make a judgment about the spectrum or the sampling method.
	Did the study avoid inappropriate exclusions?	Yes: if there were no participants excluded from the analysis, or if the exclusions were adequately described. No: if there is an unexplained exclusion of participants. Unclear: if insufficient information was provided to assess whether any participants were excluded from the analysis.
	Could the selection of patients have introduced bias?	Low risk of bias: if 'yes' classification for all of the above 3 items. High risk of bias: if 'no' classification for any of the above 3 items. Unclear risk of bias: if 'unclear' classification for any of the above 3 items without a 'no' classification.
B. Concerns regarding applicability	Are there concerns that the included patients and setting do not match the review question?	Low concerns: if animals are the unit of investigation, and if the population characteristics are representative for those who would receive the test in practice. High concerns: if the conditions stated above are not met. Unclear concern: if insufficient information was provided.
Domain 2: index test		
A. Risk of Bias	Were the index test results interpreted without knowledge of the results of the reference standard?	Yes: if the results of the index test were interpreted blind to the results of reference standard. No: if it is clear that the results of the index test were interpreted with knowledge of the reference standard. Unclear: if it is unclear whether blinded assessments were performed.
	If a threshold was used, was it pre-specified?	Yes: if the threshold values were prespecified before start of the study. No: if the threshold values were selected based on the collected data. Unclear: if there is insufficient information to make a judgement.
	Could the conduct or interpretation of the index test have introduced bias?	Low risk of bias: if all or at least one is “yes”. High risk of bias: if 'no' classification for all of the above 2 items. Unclear risk of bias: if 'unclear' classification for any of the above 2 items without a 'no' classification.
B. Concerns regarding applicability	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Low concerns: if the conduct or interpretation of the index test and comparison tests differed from how they were likely to be used in clinical practice. High concerns: if the conduct or interpretation of the index test or comparison tests differed from how they were likely to be used. Unclear concern: if insufficient information was provided.
Domain 3: reference standard		
A. Risk of Bias	Is the reference standards likely to correctly classify the target condition?	Yes: to define patients: it was used culture/isolation or two or more serological tests. For the definition of non-sick: two or more serological tests or animals from brucellosis free herds or areas. Negative culture was accepted for definition of non-cases when it was also used for definition of patients. No: if the reference standard was other than the described above.

		Unclear: if it is unclear what reference standard was used.
	Were the reference standard results interpreted without knowledge of the results of the index tests?	Yes: if the reference standard was interpreted without the knowledge of the results of the index test. No: if the reference standard was interpreted with the knowledge of the results of the index test. Unclear: it was not clear if the reference standard was interpreted without the knowledge of the results of the index test
	Could the reference standard, its conduct, or its interpretation have introduced bias?	Low risk of bias: if 'yes' classification for all of the above 2 items. High risk of bias: if 'no' classification for any of the above 2 items. Unclear risk of bias: if 'unclear' classification for any of the above 2 items without a 'no' classification.
B. Concerns regarding applicability	Are there concerns that the target condition as defined by the reference standard does not match the question?	Low concerns: if the classification of the target condition was defined as in the above criteria. High concerns: if the classification of the target condition has not been defined or was partially defined as in the above criteria. Unclear concern: if insufficient information was provided on the classification of the target condition.
Domain 4: flow and timing		
A. Risk of Bias	Was there an appropriate interval between index test and reference standard?	Yes: if there is no interval between the index test and the reference standard. No: if there is interval between the index test and the reference standard. Unclear: if information on timing between tests was not provided.
	Did all patients receive the same reference standard?	Yes: if it is clear that all animals who received the index test also received the reference standard. No: if not all animals who received the index test also received the reference standard. Unclear: if this information is not reported.
	Were all patients included in the analysis?	Yes: if the number of participants in the two-by-two table matched the number of participants recruited into the study or if sufficient explanation was provided for any discrepancy. No: number of participants in the two-by-two table did not match the number of participants recruited into the study and insufficient explanation was provided for any discrepancy. Unclear: if insufficient information was given to permit judgement.
	Could the patient flow have introduced bias?	Low risk of bias: if the “yes” classification is for all the above items or “no” for at most two of the criteria. High risk of bias: if 'no' classification for all 3 items above. Unclear risk of bias: if 'unclear' classification for any of the above 3 items without a 'no' classification.

Supplementary Table S5. Characteristics and reason of the 79 studies excluded from the systematic review based on quality assessment

First Author	Title	Model	Main reason
Agarwal (1999)	Comparison of an inhibition enzyme linked immunosorbent assay with other serological tests for detection of antibodies to *Brucella*.	Frequentist	Standard inappropriate reference based on QUADAS-2
Aggad (2006)	Prevalence of bovine and human brucellosis in western Algeria: comparison of screening tests.	Frequentist	Standard inappropriate reference based on QUADAS-2
Aher (2012)	Comparison of serological methods for the detection of *Brucella abortus* antibodies in sera from infected bovines.	Frequentist	Standard inappropriate reference based on QUADAS-2
Barshevskaya (2019)	Triple Immunochromatographic System for Simultaneous Serodiagnosis of Bovine Brucellosis, Tuberculosis, and Leukemia.	Frequentist	Standard inappropriate reference based on QUADAS-2
Bassiony (2011)	Indirect and competitive ELISA as a tool for diagnosis of brucellosis in vaccinated and infected cattle.	Frequentist	Standard inappropriate reference based on QUADAS-2
Batra (1998)	Development of a new dot-ELISA kit for detection of antibodies in bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Bhonsle (2008)	Diagnosis of brucellosis: a comparative study.	Frequentist	Standard inappropriate reference based on QUADAS-2
Chaudhuri (2010)	Recombinant OMP28 antigen-based indirect ELISA for serodiagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Chothe (2014)	Innovative modifications to Rose Bengal plate test enhance its specificity, sensitivity and predictive value in the diagnosis of brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Dajer (1999)	Evaluation of a fluorescence-polarization assay for the diagnosis of bovine brucellosis in Mexico.	Frequentist	Standard inappropriate reference based on QUADAS-2
Díaz Herrera (2015)	Development and performance evaluation of a fast immunochromatographic test for brucellosis diagnosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
D'Pool (2004)	Prevalence of bovine brucellosis using the competitive ELISA test in La Cañada de Urdaneta municipality, Zulia state, Venezuela.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ekgatat (2009)	The accuracy of an indirect ELISA for diagnosis of *Brucella* spp. infection in cattle and goats.	Frequentist	Standard inappropriate reference based on QUADAS-2
El-Eragi (2014)	Evaluation of immunochromatographic assay for serodiagnosis of bovine brucellosis in Gezira State, Sudan.	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author	Title	Model	Main reason
Emmerzaal (2002)	The Dutch *Brucella abortus* monitoring programme for cattle: the impact of false-positive serological reactions and comparison of serological tests.	Frequentist	Standard inappropriate reference based on QUADAS-2
Etman (2014)	Evaluation of efficacy of some serological tests used for diagnosis of brucellosis in cattle in Egypt using latent class analysis.	Bayesian model	Inappropriate criteria based on STARD-BLCM
Fosgate (2006)	Likelihood ratio estimation without a gold standard: A case study evaluating a brucellosis c-ELISA in cattle and water buffalo of Trinidad.	Bayesian model	Inappropriate criteria based on STARD-BLCM
Ganesan (2012)	Standard tube agglutination test and C-elisa in the diagnosis of *Brucella abortus* infection.	Frequentist	Standard inappropriate reference based on QUADAS-2
Genç (2010)	Development of qualitative and quantitative ELISA models for bovine brucellosis diagnosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ghazy (2016)	Efficiency of different preparations of rapid slide agglutination antigens for the diagnosis of bovine and ovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ghodasara (2010)	Comparison of Rose Bengal Plate Agglutination, standard Tube Agglutination and indirect ELISA tests for detection of *Brucella* antibodies in cows and buffaloes.	Frequentist	Standard inappropriate reference based on QUADAS-2
Greve (2007)	Comparative study on the sensitivity and specificity of the buffered acidified antigen and 2-mercaptoethanol tests in the diagnostic of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Hall (1984)	Detection of serum antibody to *Brucella abortus* in cattle by use of a quantitative fluorometric immunoassay.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups
Hall (1987)	Comparison of TRACK XI fluorometric immunoassay system with other serologic tests for detection of serum antibody to *Brucella abortus* in cattle.	Frequentist	Standard inappropriate reference based on QUADAS-2
Hop (2016)	An evaluation of ELISA using recombinant *Brucella abortus* bacterioferritin (Bfr) for bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Huber (1986)	Comparison of the results of card, rivanol, complement-fixation, and milk ring tests with the isolation rate of *Brucella abortus* from cattle.	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author	Title	Model	Main reason
Jones (1980)	Evaluation of a radial immunodiffusion test with polysaccharide B antigen for diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Kerby (1997)	Field evaluation of an indirect ELISA for detection of brucellosis in lowland Bolivia.	Frequentist	Standard inappropriate reference based on QUADAS-2
Khodabakhsh (2019)	A new ELISA kit based on antigenic epitopes for diagnosing *Brucella abortus*.	Frequentist	Standard inappropriate reference based on QUADAS-2
Lim (2012)	Evaluation of recombinant 28 kDa outer membrane protein of *Brucella abortus* for the clinical diagnosis of bovine brucellosis in Korea.	Frequentist	Standard inappropriate reference based on QUADAS-3
Londhe (2011)	Serodetection of bovine brucellosis by RBPT and AB-ELISA.	Frequentist	Standard inappropriate reference based on QUADAS-2
López M (1995)	Comparison of an immunoenzyme technique with the Rose Bengal and Rivanol tests for the detection of antibodies to *Brucella abortus*.	Frequentist	Standard inappropriate reference based on QUADAS-2
Madiajagan (2017)	An evaluation of ELISA using recombinant P17 antigen for cattle brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Mahajan (2017)	Comparison of diagnostic tests for the detection of bovine brucellosis in the natural cases of abortion.	Frequentist	Standard inappropriate reference based on QUADAS-2
Manasa (2019)	Protein-G-based lateral flow assay for rapid serodiagnosis of brucellosis in domesticated animals.	Frequentist	Standard inappropriate reference based on QUADAS-2
Matovi? (2008)	Examination of sensitivity and specificity of some serological tests in diagnostics of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Mejía Martínez (2012)	Comparing the rosa de bengala and rivanol in the elisa test for diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Muktaderul (2011)	Evaluation of four serological tests for the detection of brucellosis in goats and cattle under the field condition of Bangladesh.	Frequentist	Standard inappropriate reference based on QUADAS-2
Mythili (2011)	Development and Comparative Evaluation of a Competitive ELISA with Rose Bengal Test and a Commercial Indirect ELISA for Serological Diagnosis of Brucellosis.	Bayesian model	Inappropriate criteria based on STARD-BLCM
Neha (2017)	Comparative efficacy of serological diagnostic methods and evaluation of polymerase chain reaction for diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Nicoletti (1981)	Indirect hemolysis test in the serodiagnosis of bovine brucellosis.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups

First Author	Title	Model	Main reason
Nicoletti (1993)	Comparison of enzyme-labeled immunosorbent assay and particle concentration fluorescence immunoassay with standard serologic methods and bacteriologic culture for detection of *Brucella* sp-infected cows in herds with brucellosis.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups
Nielsen (A) (2004)	Rough lipopolysaccharide of *Brucella abortus* RB51 as a common antigen for serological detection of *B-ovis*, *B-canis*, and *B-abortus* RB51 exposure using indirect enzyme immunoassay and fluorescence polarization assay.	Frequentist	Standard inappropriate reference based on QUADAS-2
Nielsen (2002)	Field trial of the brucellosis fluorescence polarization assay.	Frequentist	Standard inappropriate reference based on QUADAS-2
Nielsen (2005)	Towards single screening tests for brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ortiz (2002)	Serological study of bovine brucellosis using DAVIH BRU2 ELISA system.	Frequentist	Standard inappropriate reference based on QUADAS-2
Paulin (2002)	Comparative study of 2-Mercaptoethanol and Complement Fixation Test in brucellosis diagnosis in bovine serum.	Frequentist	Standard inappropriate reference based on QUADAS-2
Perrett (2010)	Evaluation of Competitive ELISA for Detection of Antibodies to *Brucella* Infection in Domestic Animals.	Frequentist	Standard inappropriate reference based on QUADAS-2
Priyadarshini (2012)	Diagnostic tests for seroprevalence of brucellosis in cattle.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ramadan (2019)	Effect of serum treatment with chloroform on increasing specificity of Rose Bengal test for diagnosis of brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Renukaradhya (2001)	Development and field validation of an avidin-biotin enzyme-linked immunosorbent assay kit for bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Rojas (1995)	ELISAS for the diagnosis and epidemiology of *Brucella abortus* infection in cattle in Chile.	Frequentist	Standard inappropriate reference based on QUADAS-2
Ruppanner (1980)	Comparison of the enzyme-linked immunosorbent assay with other tests for brucellosis, using sera from experimentally infected heifers.	Frequentist	Standard inappropriate reference based on QUADAS-2
Saadat (2017)	Diagnosis of Cattle brucellosis by PCR and serological methods: comparison of diagnostic tests.	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author	Title	Model	Main reason
Saegerman (2004)	Evaluation of three serum i-ELISAs using monoclonal antibodies and protein G as peroxidase conjugate for the diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Salem (1987)	Sensitivity of some diagnostic procedures for brucellosis in cattle.	Frequentist	Standard inappropriate reference based on QUADAS-2
Salman (2012)	Evaluation of four serological tests to detect prevalence of bovine brucellosis in Khartoum State.	Frequentist	Standard inappropriate reference based on QUADAS-2
Samartino (A) (1999)	Fluorescence polarization assay: Application to the diagnosis of bovine brucellosis in Argentina.	Frequentist	Standard inappropriate reference based on QUADAS-2
Sanganagouda (2014)	Comparative serological studies in milk and serum and evaluation of efficacy of *Brucella abortus* S99.	Frequentist	Standard inappropriate reference based on QUADAS-2
Saravi (1995)	Comparative performance of the enzyme-linked immunosorbent assay (ELISA) and conventional assays in the diagnosis of bovine brucellosis in Argentina.	Frequentist	Standard inappropriate reference based on QUADAS-2
Sarumathi (2003)	Comparison of Avidin - Biotin ELISA with RBPT and STAT for screening of antibodies to bovine brucellosis.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups
Sayour (2017)	Validation of different versions of the card or Rose-Bengal test for the diagnosis of *Brucella* melitensis infection in ruminants.	Frequentist	Standard inappropriate reference based on QUADAS-2
Shi (2020)	A novel, rapid and simple method for detecting brucellosis based on rapid vertical flow technology.	Frequentist	Standard inappropriate reference based on QUADAS-2
Shome (2018)	Lateral flow assay for brucellosis testing in multiple livestock species.	Frequentist	Standard inappropriate reference based on QUADAS-2
Shringi (2002)	Comparative study of conventional serological test for the diagnosis of brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Shrivastava (1991)	A comparison of dot-enzyme-linked immunosorbent assay (dot-ELISA) with other conventional tests for the serodiagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Simborio (2015)	Evaluation of the combined use of the recombinant *Brucella abortus* Omp10, Omp19 and Omp28 proteins for the clinical diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Sotnikov (2019)	Immunochromatographic serodiagnosis of brucellosis in cattle using gold nanoparticles and quantum dots.	Frequentist	Standard inappropriate reference based on QUADAS-2
Sulima (2010)	Evaluation of tests for diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2

First Author	Title	Model	Main reason
Tan (2012)	Recombinant VirB5 protein as a potential serological marker for the diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Tittarelli (2008)	Use of chemiluminescence for the serological diagnosis of bovine and ovine brucellosis with indirect and competitive enzyme-linked immunosorbent assays.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups
Tiwari (2011)	Evaluation of the Recombinant 10-Kilodalton Immunodominant Region of the BP26 Protein of *Brucella abortus* for Specific Diagnosis of Bovine Brucellosis.	Frequentist	It is not possible to separate vaccinated from unvaccinated groups
Trangadia (2012)	Evaluation of fluorescence polarization assay for the diagnosis of brucellosis in cattle and buffaloes in India.	Frequentist	Standard inappropriate reference based on QUADAS-2
Trap (1976)	[Serologic diagnosis of bovine and ovine brucellosis by a buffered antigen test].	Frequentist	Standard inappropriate reference based on QUADAS-2
Uzal (1995)	Evaluation of an indirect ELISA kit for the diagnosis of bovine brucellosis in Latin America.	Frequentist	Standard inappropriate reference based on QUADAS-2
Vanzini (1998)	Evaluation of an indirect ELISA for the diagnosis of bovine brucellosis in milk and serum samples in dairy cattle in Argentina.	Frequentist	Standard inappropriate reference based on QUADAS-2
Vanzini (2003)	Determination of the quality control limits and adjustment of the cut off point for an indirect ELISA applied to the diagnosis of bovine brucellosis milk samples.	Frequentist	Standard inappropriate reference based on QUADAS-2
Wang (2015)	Development of an improved competitive ELISA based on a monoclonal antibody against lipopolysaccharide for the detection of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2
Zakaria (2018)	Comparative assessment of sensitivity and specificity of Rose Bengal Test and modified in-house ELISA by using IS711 Taqman real time PCR assay as a gold standard for the diagnosis of bovine brucellosis.	Frequentist	Standard inappropriate reference based on QUADAS-2

Supplementary Table S6. Main characteristics of the 71 studies included in the systematic review**.**

First author, Year	Country	Year	Model	Negative reference standard	Negative_Tests	N Neg	Positive reference standard	Positive_Tests	N Pos	Total animals	Index tests (N of assays)	N index tests	Metanalysis
Abalos_2000	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF, RBT, SAT	130	Positive culture and sorological test(s)	Culture, CF, RBT, SAT	75	205	iELISA(3)	3	1
Abdoel_2008	Portugal	NI	Frequentist	From brucellosis free area	NA	19	Positive culture	Culture	11	30	LFIA	1	1
Abernethy_2012	Northern Irland	2003-2004	Frequentist	From brucellosis free area	NA	2663	Positive culture	Culture	162	2825	CF, cELISA, EDTA(2), iELISA, RBT, SAT(2)	8	1
Adams_1991	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF, PCFIA	2729	Positive culture	Culture	84	2813	cELISA	1	1
Adone_2002	NI	NI	Frequentist	From brucellosis free area	NA	10	Positive culture	Culture	35	45	CF(2)	2	1
Ahasan_2017	Bangladesh	2009	Bayesian model	NA	NA	NA	NA	NA	NA	160	cELISA, iELISA, RBT, SAT	4	1
Akhtar_2010	NI	NI	Frequentist	Negative culture	Culture	74	Positive culture	Culture	26	100	RBT	1	1
Andrade_2022 (Unpublished data)	NI	NI	Frequentist	From brucellosis free area	NA	32	Positive culture	Culture	51 - 53	83 - 85	2ME, FPA, RBT	3	1
Arif_2018	Pakistan	2015	Bayesian model	NA	NA	NA	NA	NA	NA	441	cELISA, iELISA, RBT	3	1
Bastos_2018	NI	NI	Frequentist	Negative sorological test(s)	2ME, CF	1087	Suggestive epidemiology + positive sorological tests	2ME, CF	136	1223	Dot-blot	1	0
Bronsvoort_2009	Sub-Saharan Africa	2000	Bayesian model	NA	NA	NA	NA	NA	NA	1375	cELISA, LFIA	2	1
Byrd_1979	Frequentist	NI	Frequentist	Negative sorological test(s)	Card Test, CF, RIV, SAT	100	Suggestive epidemiology + positive sorological tests	Card Test, CF, RIV, SAT	100	200	iELISA	1	0
Cargill_1985	NI	1981-1982	Frequentist	Negative culture	Culture	110	Positive culture	Culture	41	151	CF, iELISA, IHLT, RBT	4	1
Chisi_2017	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF or MRT	186	Positive culture	Culture	46	232	cELISA, CF, iELISA, RBT, SAT	5	1
Cho_2010	Korea	2005-2006	Frequentist	From brucellosis free area	NA	734	Positive culture	Culture	128	862	qRBT	1	0
Daffner_1999	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF, RBT, SAT	130	Positive culture and sorological test(s)	Culture, CF, iELISA, RBT, SAT	76	206	AGID(3), RID(3)	6	0
Dájer_1998	NI	NI	Frequentist	From brucellosis free area	NA	248	Suggestive epidemiology + positive sorological tests	CF, RBT	83	331	iELISA, RIV	2	1
Dohoo_1986	Canada	NI	Frequentist	From brucellosis free area + negative sorological test(s)	BPAT, CF, SAT	1128	Positive culture	Culture	174	1302	BPAT, CF, iELISA(4), HIGT, SAT(2)	9	1

First author, Year	Country	Year	Model	Negative reference standard	Negative_Tests	N Neg	Positive reference standard	Positive_Tests	N Pos	Total animals	Index tests (N of assays)	N index tests	Metanalysis
Elshemey_2014	NI	NI	Frequentist	Negative sorological test(s)	iELISA, RBT	2	Suggestive epidemiology + positive sorological tests	iELISA, RBT	72	74	LFIA	1	0
Fosgate_2002	Trinidad and Tobago	1999	Bayesian model	NA	NA	NA	NA	NA	NA	391	BPAT, Card Test, SAT, SPAT,	4	1
Gall_2001	Canada	NI	Frequentist	From brucellosis free area	NA	4437	Positive culture	Culture	561	4998	FPA	1	1
Gall_2006	Canada	NI	Frequentist	From brucellosis free area	NA	480	Positive culture	Culture	51	531	iELISA	1	0
Genç_2011	NI	NI	Frequentist	From brucellosis free area	NA	120	Positive culture	Culture	18	138	CF, iELISA(2), ERIFA(3), RBT	7	1
Getachew_2016	Ethiopia	NI	Bayesian model	NA	NA	NA	NA	NA	NA	278	CF, iELISA, RBT	3	1
Gower_1974	Surrey	1974	Frequentist	Negative sorological test(s)	CF, SAT	3589 - 16557	Positive sorological tests	CF, SAT	109 - 508	3698 - 17065	RBT, RBT_automated	2	1
Greenlee_1994	NI	1990-1991	Frequentist	Negative culture	Culture	574 - 639	Positive culture	Culture	162	736 - 801	Card Test, CF(2), PCFIA(2)	5	1
Gusi_2019	NI	NI	Frequentist	From brucellosis free area	NA	83 - 84	Positive culture	Culture	87 - 88	170 - 172	iELISA, LFIA, RBT	3	1
Hobbs_1985	New Zealand	NI	Frequentist	Negative culture	Culture	1940	Positive culture	Culture	19	1959	CF, iELISA	2	1
Kalleshamurthy_2018	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s) + PCR	iELISA, PCR, RBT	200	Positive sorological tests + PCR	iELISA, PCR, RBT	100	300	FPA	1	1
Kalleshamurthy_2020	NI	NI	Frequentist	Negative sorological test(s)	cELISA, iELISA, RBT	364	Positive sorological tests	cELISA, iELISA, RBT	271	635	cELISA, FPA	2	1
Mathias_2010	NI	NI	Frequentist	Negative sorological test(s)	2ME, CF, RBT	603	Positive sorological tests	2ME, CF, RBT	336	939	FPA	1	1
Matope_2011	Zimbabwe	2004-2005	Bayesian model	NA	NA	NA	NA	NA	NA	1440	cELISA, FPA, RBT	3	1
McGiven_2003	Germany	2001	Frequentist	From brucellosis free area	NA	995 - 6957	Positive culture	Culture	146	1141 - 6973	cELISA, CF, iELISA, FPA, SAT	5	1
McGiven_2008	NI	NI	Frequentist	From brucellosis free area	NA	230	Positive culture OR sorological test(s)	Culture, CF, 2ME	27	257	AlphaLISA, cELISA, iELISA, FPA	4	1
McGiven_2015	NI	NI	Frequentist	From brucellosis free area	NA	68	Positive culture	Culture	45	113	cELISA, CF, iELISA(7), SAT	10	1
Meirelles-Bartoli_2010	NI	NI	Frequentist	Negative sorological test(s)	2ME, SAT	455	Positive sorological tests	2ME, SAT	606	1061	CF, RBT	2	1
Morgan_1969	NI	NI	Frequentist	Negative sorological test(s)	CF, SAT	5833	Positive sorological tests	CF, SAT	591	6424	RBT	1	1
Muma_2007	Zambia	2003-2005	Bayesian model	NA	NA	NA	NA	NA	NA	189	cELISA, FPA, RBT	3	1
Munoz_2005	NI	NI	Frequentist	From brucellosis free area	NA	41 - 112	Positive culture and sorological test(s)	Culture, RBT, CF	43 - 114	84 - 226	Chatropic_ELISA(3), cELISA, CF,	19	1

First author, Year	Country	Year	Model	Negative reference standard	Negative_Tests	N Neg	Positive reference standard	Positive_Tests	N Pos	Total animals	Index tests (N of assays)	N index tests	Metanalysis
Mylrea_1976	NI	NI	Frequentist	Negative sorological test(s)	CF, RBT	206	Positive sorological tests	CF, RBT	133	339	Counterimmuno-electrophoresis, DID, iELISA (9), RID (2), RBT 2ME, ABGT-R, ABGT-T, SAT	4	1
Naves_2011	NI	NI	Frequentist	Negative sorological test(s)	2ME, RBT	46	Positive sorological tests	2ME, RBT	46	92	iELISA(2)	2	1
Nielsen_1995	Canada	NI	Frequentist	From brucellosis free area	NA	1446	Positive culture	Culture	636	2082	BPAT, cELISA, CF, iELISA	4	1
Nielsen_1996	Canada	NI	Frequentist	From brucellosis free area	NA	763 - 15715	Positive culture	Culture	424 - 654	1187 - 16369	BPAT, cELISA(2), CF, iELISA(2)	8	1
Nielsen_1996a	Canada	NI	Frequentist	From brucellosis free area	NA	8669	Positive culture	Culture	561	9230	FPA	1	1
Nielsen_1998	Canada	NI	Frequentist	From brucellosis free area	NA	354 - 10137	Positive culture and sorological test(s)	Culture, BPAT, CF	150 - 811	504 - 10948	BPAT, cELISA, CF, FPA, PCFIA, RBT	6	1
Nielsen_2001	NI	NI	Frequentist	Negative sorological test(s)	BPAT, cELISA	472	Positive sorological tests	BPAT, cELISA	219	691	CF, FPA, FPA_blood	3	1
Nielsen_2004	NI	NI	Frequentist	From brucellosis free area	NA	176	Positive sorological tests	CF, RBT	67	243	iELISA(2)	2	1
Nielsen_2007	Canada	NI	Frequentist	From brucellosis free area	NA	1033	Positive culture	Culture	238	1271	cELISA(2), iELISA, FPA	4	1
Nielsen_2008	Canada	NI	Frequentist	From brucellosis free area	NA	2014	Positive culture	Culture	410	2424	cELISA(2), iELISA	3	1
O'Reilly_1971	NI	NI	Frequentist	Negative sorological test(s)	CF, SAT	528	Positive sorological tests	CF, SAT	674	1202	Card Test	1	1
Pajuaba_2010	Brazil	NI	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, RBT, SAT	278	Suggestive epidemiology + positive sorological tests	2ME, RBT, SAT	41	319	iELISA(2)	2	1
Paulin_2009	Brazil	2002	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, CF	111	Suggestive epidemiology + positive sorological tests	2ME, CF	53	164	iELISA, RBT	2	1
Paweska_2002	NI	NI	Frequentist	From brucellosis free area	NA	834	Positive culture	Culture	72	906	CF, iELISA	2	1
Percy_du_Sert_1998	NI	NI	Frequentist	Negative sorological test(s)	CF, RBT	211	Positive sorological tests	CF, RBT	33	244	iELISA	1	1
Portanti_2006	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF, RBT	748	Positive culture OR sorological test(s)	Culture, CF, RBT	265	1013	cELISA	1	1
Praud_2016	France	2011	Frequentist	From brucellosis free area	NA	4430	Positive sorological tests	CF, iELISA, RBT, SAT	309	4739	cELISA(3), CF, iELISA, FPA, RBT, SAT	8	1
Putini_2008	NI	NI	Frequentist	Negative sorological test(s)	2ME, RBT	46	Positive sorological tests	2ME, RBT	45	91	iELISA	1	1
Quintero_2018	Cuba	NI	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, CF, RBT, SAT	338	Suggestive epidemiology + positive sorological tests	2ME, CF, RBT, SAT	111	449	LFIA(2)	2	1

First author, Year	Country	Year	Model	Negative reference standard	Negative_Tests	N Neg	Positive reference standard	Positive_Tests	N Pos	Total animals	Index tests (N of assays)	N index tests	Metanalysis
Rahman_2019	Bangladesh	2007-2008	Bayesian model	NA	NA	NA	NA	NA	NA	1020	iELISA, RBT, SAT	3	1
Samartino_1999	Argentina	NI	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, CF	500	Positive sorological tests	2ME, CF	1000	1500	BPAT, cELISA, iELISA, RBT	4	1
Sanchez-Villalobos_2009	Venezuela	NI	Frequentist	From brucellosis free area	NA	438	Suggestive epidemiology + positive sorological tests	BPAT, FPA	338	776	cELISA, RBT	2	1
Sanogo_2013	Ivory Coast	2005	Bayesian model	NA	NA	NA	NA	NA	NA	995	iELISA, RBT	2	1
Silva_2006	NI	NI	Frequentist	Negative sorological test(s)	CF, RBT	1093	Positive sorological tests	CF, RBT	85	1178	iELISA	1	1
Stack_1999	NI	NI	Frequentist	From brucellosis free area	NA	640	Positive culture	Culture	147	787	cELISA, CF	2	1
Stemshorn_1985	Canada	NI	Frequentist	From brucellosis free area	NA	730	Positive culture	Culture	167	897	2ME, BPAT, Card test, CF, RBT, SAT, SPAT	7	1
Torioni de Echaide_2002	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	cELISA, CF	628	Positive sorological tests	cELISA, CF	181	809	BPAT(2)	2	1
Trangadia_2015	India	2007-2008	Bayesian model	NA	NA	NA	NA	NA	NA	404	cELISA, iELISA(5)	6	1
Uzal_1996	Argentina	NI	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, RBT	235	Suggestive epidemiology + positive sorological tests	2ME, RBT	185	420	cELISA	1	1
Uzal_1996a	Argentina	NI	Frequentist	From brucellosis free area + negative sorological test(s)	2ME, RBT	243	Suggestive epidemiology + positive sorological tests	2ME, RBT	184	427	iELISA	1	1
Van-Aert_1984	NI	NI	Frequentist	From brucellosis free area + negative sorological test(s)	CF, RBT, SAT	556 - 602	Suggestive epidemiology + positive sorological tests	CF, RBT, SAT	183 - 229	739 - 831	CF, iELISA, RBT, SAT	4	1
Weynants_1996	NI	NI	Frequentist	From brucellosis free area	NA	936	Experimental challenge and positive culture	Culture	18	954	cELISA, CF, RBT	3	1

NI: Not informed
NA: Not applied

Supplementary Table S7. Description of the cutoff points considered by the quantitative tests and the results regarding the presence and absence of qualitative tests included in the systematic review

Index test	Cutoffs/Positive definition	N assays	Assays total	Studies
2ME	Positive >1:20	1	3	Mylrea (1976)
	Positive >1:25	2		Andrade (2022); Stemshorn (1985)
ABGT-R	Positive ≥ 160 IU/mL	1	1	Mylrea (1976)
ABGT-T	Positive ≥ 160 IU/mL	1	1	Mylrea (1976)
AGID	Presence the precipitation lines	3	3	Daffner (1999)
AlphaLISA	Positive >21.5 PI	1	1	McGiven (2008)
BPAT	Positive in the presence of partial or complete agglutination.	9	9	Dohoo (1986); Fosgate (2002); Nielsen (1995); Nielsen (1996); Nielsen (1998); Samartino (1999); Stemshorn (1985); Torioni de Echaide (2002)
cELISA	Interpreted as directed by the manufacturers' instructions - Brucella-Ab C-ELISA (Svanova)	1	31	Abernethy (2012)
	Positive >35.73 %P	1		Munoz (2005)
	Positive ≥80 %I	1		Adams (1991)
	Positive ≥30 %I	14		Ahasan (2017); Arif (2018); Chisi (2017); Kalleshamurthy (2020); Matope (2011); Muma (2007); Nielsen (1995); Nielsen (1996); Nielsen (1998); Nielsen (2007); Praud (2016); Sanchez-Villalobos (2009); Trangadia (2015)
	Positive >50 CU/ml	1		Weynants (1996)
	Positive >70 %I	3		Bronsvoort (2009); McGiven (2003); McGiven (2008)
	Positive >60 %I	3		McGiven (2015); Praud (2016); Stack (1999)
	Positive >20 %P	1		Nielsen (2008)
	Positive >11 %I	1		Nielsen (2007)
	Positive >27-40 %I	1		Nielsen (2008)

	Positive >67.5 %I	1		Portanti (2006)
	Positive ≥40 %I	3		Praud (2016); Samartino (1999); Uzal (1996)
CF	Positive >16 IU/mL	2	25	Abernethy (2012); Cargill (1985)
	Positive >25 IU/mL	1		Van-Aert (1984)
	Positive >60 IU/mL	1		Chisi (2017)
	Positive ≥10	1		Greenlee (1994)
	Positive ≥20 IU/ml	14		Adone (2002); Genç (2011); Hobbs (1985); McGiven (2003); McGiven (2015); Meirelles-Bartoli (2010); Munoz (2005); Nielsen (1996); Nielsen (1998); Paweska (2002); Praud (2016); Stack (1999); Weynants (1996)
	Positive ≥40	1		Greenlee (1994)
	Positive >1:5	5		Dohoo (1986); Getachew (2016); Nielsen (1995); Nielsen (2001); Stemshorn (1985)
Chaotropic_ELISA_1M_KSCN	Positive >12.62 %P	1	1	Munoz (2005)
Chaotropic_ELISA_2M_KSCN	Positive >25.79 %P	1	1	Munoz (2005)
Chaotropic_ELISA_3M_KSCN	Positive >21.22 %P	1	1	Munoz (2005)
Counterimmuno-electrophoresis	Positive in the presence the bands	1	1	Munoz (2005)
DID	Presence the precipitation lines	1	1	Munoz (2005)
Dot-blot	Positive samples contain a strong or weak purplish circle, surrounded by a circle without staining	1	**1**	Bastos (2018)
EDTA	Positive >31 IU	1	**2**	Abernethy (2012)
	Positive >51 IU	1		Abernethy (2012)
ERIFA	Positive was considered the development of colors in cassettes	3	3	Genç (2011)
FPA	Positive >15.5 mP	2	14	McGiven (2003); McGiven (2008)

	Positive >90 mP	6		Matope (2011); Muma (2007); Nielsen (2001); Nielsen (2007); Nielsen (1996[a]); Nielsen (1998)
	Positive >93,6 mP	1		Mathias (2010)
	Positive ≥11 was derived by screening known negative and positive panel samples	2		Kalleshamurthy (2018); Kalleshamurthy (2020)
	Positive >20 mP	2		Andrade (2022); Praud (2016)
	Positive >91 mP	1		Gall (2001)
FPA_blood	Positive >95 mP	1	1	Nielsen (2001)
HIGT	A zone of hemolysis of 6 mm was considered to be the minimum seropositive threshold.	1	1	Dohoo (1986)
iELISA	Interpreted as directed by the manufacturers' instructions - iELISA, Pourquier	1	65	Abernethy (2012)
	Interpreted as directed by the manufacturers' instructions - Svanova Biotech AB, Sweden	1		Ahasan (2017)
	NI	1		Percy du Sert (1998)
	Positive >0.122 OD	1		Putini (2008)
	Positive >13.2 %P	1		Gall (2006)
	Positive >15.87 %P	1		Munoz (2005)
	Positive >27.07 %P	1		Munoz (2005)
	Positive >31.12 %P	1		Munoz (2005)
	Positive >37.45 %P	1		Munoz (2005)
	Positive >38.47 %P	1		Munoz (2005)
	Positive >46% P	2		Nielsen (1995); Nielsen (1996)
	Positive >5 IU/mL	1		Rahman (2019)
	Positive >50% OD ***	1		Gusi (2019)

Positive >62% P	1	Nielsen (1996)
Positive >62.42 %P	1	Munoz (2005)
Positive >64.58 %P	1	Munoz (2005)
Positive >75.04 %P	1	Munoz (2005)
Positive 1% RI	1	Paweska (2002)
Positive 1:32 dilution	1	Hobbs (1985)
Positive 2 IU/mL	1	Sanogo (2013)
Positive 22 RI	1	Pajuaba (2010)
Positive 49 RI	1	Pajuaba (2010)
Samples were considered reactive when their optical density was below the threshold value (0.5 x mean of negative controls).	1	Silva (2006)
Sera yielding a fluorescence intensity at least 3 times higher than the blanks were considered positive.	1	Van-Aert (1984)
Positive >14 %P	3	Abalos (2000); Nielsen (2004)
Positive >8 %P	1	Abalos (2000)
Positive >34 %P	1	Abalos (2000)
Positive ≥80 %P	1	Arif (2018)
Positive ≥4 EV	1	Byrd (1979)
Positive >10 %P	5	Cargill (1985); Getachew (2016); McGiven (2003); McGiven (2008); Trangadia (2015)
Positive >120 %P	2	Chisi (2017); Praud (2016)
Positive >28 %P	1	Dájer (1998)
Positive >0.220 OD	1	Dohoo (1986)
Positive >0.260 OD	1	Dohoo (1986)

	Positive >0.300 OD	1		Dohoo (1986)
	Positive >0.340 OD	1		Dohoo (1986)
	Positive >0.600 %P	2		Genç (2011)
	Positive >22.45 %P	6		McGiven (2015)
	Positive >37.1 %P	1		McGiven (2015)
	Positive >35.29 %P	1		Munoz (2005)
	Positive >0.612 OD	1		Naves (2011)
	Positive >0.715 OD	1		Naves (2011)
	Positive >20 %P	2		Nielsen (2007); Nielsen (2008)
	Positive ≥45 %P	1		Paulin (2009)
	Positive ≥40 %P	1		Samartino (1999)
	Positive >40% OD	1		Trangadia (2015)
	Positive >80 %P	1		Trangadia (2015)
	Positive >45 %P	1		Trangadia (2015)
	Positive >25 %P	1		Trangadia (2015)
	Positive ≥31 %P	1		Uzal (1996a)
iELISA_EDTA	Positive >0.460 OD	1	2	Nielsen (1996)
	Positive >0.607 OD	1		Nielsen (1996)
IHLT	Positive >25 % haemolysis at a serum dilution of 1/16	1	1	Cargill (1985)
LFIA	Positive was considered the development of colors in cassettes	6	6	Abdoel (2008); Bronsvoort (2009); Elshemey (2014); Gusi (2019); Quintero (2018)
PCFIA	PCFIA ≤0.250	1	3	Nielsen (1998)
	PCFIA ≤0.300	1		Greenlee (1994)
	PCFIA ≤0.600	1		Greenlee (1994)
qRBT	Agglutination intensity - sensitivity 10 and averaging 3	1	1	Cho (2010)

RBT	Positive - any degree of agglutination	30	30	Abernethy (2012); Ahasan (2017); Akhtar (2010); Andrade (2022); Arif (2018); Cargill (1985); Chisi (2017); Fosgate (2002); Genç (2011); Getachew (2016); Gower (1974); Greenlee (1994); Gusi (2019); Matope (2011); Meirelles-Bartoli (2010); Morgan (1969); Muma (2007); Munoz (2005); Nielsen (1998); O'Reilly (1971); Paulin (2009); Praud (2016); Rahman (2019); Samartino (1999); Sanchez-Villalobos (2009); Sanogo (2013); Stemshorn (1985); Van-Aert (1984); Weynants (1996)
RBT_automated	Positive - any degree of agglutination	1	1	Gower (1974)
RID	Presence the precipitation lines	5	5	Daffner (1999); Munoz (2005)
RIV	Positive ≥1:25	1	1	Dájer (1998)
SAT	Positive >25 IU/mL	2	14	Ahasan (2017); Fosgate (2002)
	Positive >30 IU/mL	6		Dohoo (1986); McGiven (2003); McGiven (2015); Praud (2016); Rahman (2019); Van-Aert (1984)
	Positive >31 IU/mL	1		Abernethy (2012)
	Positive >51 IU/mL	1		Abernethy (2012)
	Positive >60 IU/mL	2		Dohoo (1986); Stemshorn (1985)
	Positive >161 IU/mL	1		Chisi (2017)
	Positive >160 IU/mL	1		Mylrea (1976)
SPAT	Positive ≥3+ at 1/50	2	2	Fosgate (2002); Stemshorn (1985)

IU/mL = International units per millilitre
% P = percent positivity
% I = percent inhibition
EV = extinction value
CU/mL - competitive units per millilitre
mP - millipolarization units
* CF result listed as the reciprocal of the dilution with the last digit representing the degree of fixation (e.g. CF of 11 = 1+ at 1:10)
** Reactivity index (RI) = (mean OD sample/mean OD positive control) x100.
*** % OD = 100 × mean OD of duplicated sample/mean OD of duplicate positive control

Supplementary Table S8. The characteristics of studies (6) and assays (54) that were excluded from the meta-analysis for not meeting the defined criteria.

First_author_Year	Model	Index_tests	Antigen type	Antigen	Antigen strain
Abalos_2000	Frequentist	iELISA	Native hapten	Native hapten	*B. melitensis* 16M
Abernethy_2012	Frequentist	EDTA	NI	EDTA	NI
Abernethy_2012	Frequentist	EDTA	NI	EDTA	NI
Adone_2002	Frequentist	CF	S99/RB51	S99/RB51	*B. abortus* RB51
Arif_2018	Bayesian	iELISA	IDEXX	IDEXX	NI
Bastos_2018	Frequentist	Dot-blot	Bacterial suspesion	Bacterial suspesion	*B. abortus*
Byrd_1979	Frequentist	iELISA	USDA, NADI	USDA, NADI	NI
Cargill_1985	Frequentist	IHLT	NI	IHLT	NI
Cho_2010	Frequentist	qRBT	NI	RBT	*B. abortus* 1119-3
Daffner_1999	Frequentist	RID	O-chain	S-LPS	*B. abortus* 1119-3
Daffner_1999	Frequentist	AGID	O-chain	O-chain	*B. abortus* 1119-3
Daffner_1999	Frequentist	AGID	Native hapten	Native hapten	*B. melitensis* 16M
Daffner_1999	Frequentist	AGID	Polysaccharide	S-LPS	*B. abortus* 1119-3
Daffner_1999	Frequentist	RID	Native hapten	Native hapten	*B. melitensis* 16M
Daffner_1999	Frequentist	RID	Polysaccharide	S-LPS	*B. abortus* 1119-3
Dájer_1998	Frequentist	RIV	NI	RIV	NI
Dohoo_1986	Frequentist	HIGT	NI	HIGT	*B. abortus* 413
Elshemey_2014	Frequentist	LFIA	BIONOTE	BIONOTE	*B. abortus*
Fosgate_2002	Bayesian	SPAT	NI	SPAT	*B. abortus* 1119-3
Gall_2006	Frequentist	iELISA	S-LPS/R-LPS	S-LPS/R-LPS	*B. abortus*
Genç_2011	Frequentist	ERIFA	Chromatographic fractions of cell lysate	Chromatographic fractions of cell lysate	*B. abortus* S19
Genç_2011	Frequentist	ERIFA	O-polysaccharide	O-polysaccharide	*B. abortus* 1119-3
Genç_2011	Frequentist	ERIFA	S-LPS	S-LPS	*B. abortus* 2308
Gower_1974	Frequentist	RBT_automated	NI	RBT	NI
Kalleshamurthy_2020	Frequentist	cELISA	NI	NI	NI

McGiven_2008	Frequentist	AlphaLISA	S-LPS	S-LPS	*B. melitensis* 16M
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Disaccharide 1	*B. abortus* S99
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Nonasaccharide 6	*B. abortus* S99
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Pentasaccharide 5	*B. abortus* S99
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Tetrasaccharide 4	*B. abortus* S99
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Trisaccharide 2 terminal 1 2	*B. abortus* S99
McGiven_2015	Frequentist	iELISA	Synthetic oligosaccharides	Trisaccharide 3 terminal 1 4	*B. abortus* S99
Munoz_2005	Frequentist	iELISA	NH	NH	*B. melitensis*
Munoz_2005	Frequentist	Chaotropic_ELISA_1M_KSCN	S-LPS	S-LPS	*B. melitensis*
Munoz_2005	Frequentist	Chaotropic_ELISA_2M_KSCN	S-LPS	S-LPS	*B. melitensis*
Munoz_2005	Frequentist	Chaotropic_ELISA_3M_KSCN	S-LPS	S-LPS	*B. melitensis*
Munoz_2005	Frequentist	DID	S-LPS	S-LPS	*B. melitensis*
Munoz_2005	Frequentist	RID	NH	NH	*B. melitensis*
Munoz_2005	Frequentist	iELISA	R-LPS	R-LPS	*B. ovis*
Munoz_2005	Frequentist	iELISA	R-LPS	R-LPS	*B. abortus*
Munoz_2005	Frequentist	iELISA	BP26	BP26	*B. abortus*
Munoz_2005	Frequentist	iELISA	Cytosolic proteins	Cytosolic proteins	*B. melitensis* 115
Munoz_2005	Frequentist	Counterimmuno-electrophoresis	Cytosolic proteins	Cytosolic proteins	*B. melitensis* 115
Munoz_2005	Frequentist	RID	Cytosolic proteins	Cytosolic proteins	*B. melitensis* 115
Mylrea_1976	Frequentist	ABGT-R	Bacterial suspesion	Bacterial suspesion	*Brucella* spp.
Mylrea_1976	Frequentist	ABGT-T	Bacterial suspesion	Bacterial suspesion	*Brucella* spp
Nielsen_1996	Frequentist	iELISA_EDTA	S-LPS	S-LPS	*B. abortus*
Nielsen_1996	Frequentist	iELISA_EDTA	S-LPS	S-LPS	*B. abortus*

Nielsen_2001	Frequentist	FPA_blood	O-polysaccharide	O-polysaccharide	*B. abortus*
Stemshorn_1985	Frequentist	SPAT	NI	SPAT	*B. abortus* 413
Trangadia_2015	Bayesian	iELISA	S-LPS	S-LPS	*Brucella* spp.
Trangadia_2015	Bayesian	iELISA	S-LPS	S-LPS	*Brucella* spp.
Trangadia_2015	Bayesian	iELISA	S-LPS	S-LPS	*Brucella* spp.
Van-Aert_1984	Frequentist	iELISA	Brucella antigen IFFA-Meriuex	Brucella antigen IFFA-Meriuex	*B. abortus*

Supplementary Table S9. Comparison of the confidence intervals of DSe, DSp and accuracy of the 11 tests assessed in the meta-analysis considering the noncase-control studies (randomized). Different letters indicate no overlapping in the confidence intervals.

Test	**DSe[1]**	**DSp[2]**	**Accuracy**
iELISA (BS)[3]	a	b,c,d,e	a,b,c,d
iELISA (S-LPS)[4]	a,b	b,c,d,e,f	b,c,d,e
cELISA[5]	a,b,c	b,c,d	a,b,c
BPAT[6]	a,b,c,d	b,c	a,b
FPA[7]	a,b,c,d,e	a	a
LFIA[8]	a,b,c,d,e,f	g,h,i,j	d,e,f,g,h,i,j
SAT[9]	a,c,d,e,f,g	e,f,g,h	d,e,f,g,h
RBT[10]	c,d,e,f,g,h	g,h,i	f,g,h,i,
CF[11]	d,e,f,g,h,i	e,f,g	d,e,f,g
PCFIA[12]	f,i,j	i,j	j
2ME[13]	f,j	a,b	a,b,c,d,e,f

[1] DSe: Diagnosis sensitivity; [2] DSp: Diagnosis specificity; [3] iELISA (BS): indirect enzyme-linked immunosorbent assay (bacterial suspension); [4] iELISA (S-LPS): indirect enzyme-linked immunosorbent assay (smooth lipopolysaccharide); [5] cELISA: competitive enzyme-linked immunosorbent assay; [6] BPAT: buffered plate antigen test; [7] FPA: fluorescence polarization assay; [8] LFIA: lateral flow immunochromatographic assay; [9] SAT: serum agglutination test; [10] RBT: Rose Bengal test; [11] CF: complement fixation test; [12] PCFIA: particle concentration fluorescence immunoassay; [13] 2-mercaptoethanol;

Article 2

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Accuracy and covariance of routine serological tests for the diagnosis of bovine brucellosis

Abstract

Conditional dependence and diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were estimated using a Bayesian approach for eight serological tests used in the diagnosis of bovine brucellosis. Serum samples divided into five groups: group 1 - 52 serum samples with positive culture; group 2 - 32 sera samples from animals in a brucellosis-free area; group 3 - 114 sera from animals vaccinated with S19, collected on different days post vaccination; group 4 - 60 serum samples from animals vaccinated with RB51, 28 and 56 days after vaccination; and group 5 - 42 serum samples from animals inoculated with *Yersinia enterocolitica* group O:9 were tested in parallel by RBT (Rose Bengal test), SAL (serum agglutination test), 2ME (2-mercaptoethanol test), FPA (fluorescence polarization assay), BPAT (Buffered plate antigen test), iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), iELISA_SOD (Superoxide dismutase [Cu-Zn]) and CF (complement fixation test). Conditional dependence was exhibited in 75% (21/28) of the pairwise combinations between tests for both Se (C^+) and Sp (C^-), with FPA and iELISA_IDEXX tests (0.2126) and BPAT and RBT (0.1205) having the highest covariance in DSe and DSp, respectively. The test that performed best in DSe was BPAT 68.46% (95% credibility interval (95% CI): 63.08 – 75.00%) and in DSp FPA 84.49% (95% CI: 80.25 - 88.98%). All the serological tests, except iELISA_SOD, evaluated in the present study showed conditional dependence, emphasizing the importance of considering the covariance of the tests in their validation and in the proposal of their use in effective diagnostic strategies. The tests that showed better DSe and DSp were BPAT and FPA, respectively.

1. Introduction

Bovine brucellosis is an infectious disease constantly considered among the most economically important zoonoses worldwide affecting the health of human, livestock and wildlife populations (WHO, 2015). Economic losses in cattle, caused by *Brucella abortus* infection, are mainly due to reproductive problems, such as abortion and infertility, besides reduction milk and meat production (McDermott et al., 2013).

A common strategy among countries where bovine brucellosis is endemic is the implementation of control and eradication programs based on vaccination, test and slaughter and surveillance (Olsen & Stoffregen, 2005; Zhang et al., 2018), with the diagnosis methods having a central place in these programs. In this context, bacterial culture is the gold standard, albeit it can produce false negative results or being impractical for large herds or for large numbers of animals (Chisi et al., 2017; WOAH, 2022). Given that, serological tests offer a more practical means for the diagnostic of bovine brucellosis, being several already described and used worldwide (Nielsen & Yu, 2010).

In control and eradication programs, as no test offer a complete certain on a disease status, a combination of serological tests is usually employed in series strategy, to improve the diagnostic specificity (reducing the false positive results) (Poester et al., 2010). Diagnosis tests can also be used in parallel strategy to increase the diagnostic sensitivity, reducing the false negative results (Dohoo et al., 2012). Nonetheless, in these strategies usually the tests are erroneously considered independent, even when they measure similar biological processes. Indeed, when the conditionally dependency of the tests is neglected, this substantially alters the theoretical values of diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of the combined tests, consequently this assumption of test independence will result in an overestimation of the values of those obtained considering conditional dependence (Gardner et al., 2000; Thibodeau, 1981).

Therefore, determining the covariance (conditional dependence) of the tests commonly used for the diagnosis of bovine brucellosis is essential to improve their use in control and eradication programs, allowing a realistic assessment of the time and resources needed for the program to achieve

its objectives. In view of this, our objective was to estimate the DSe and DSp of the conventional serological tests routinely used in the diagnosis of bovine brucellosis and especially their covariance, in order to support their correct use in control and eradication programs.

2. Material and methods

2.1 Bovine sera

According to standards established by the World Organization for Animal Health (WOAH), a minimum of 20 sera samples are suggested for validation of serological tests (WOAH, 2018). Serum samples from five groups of cattle were used. The groups were composed by: group 1 – naturally infected animals (52 serum samples), with positive culture, belonging to serum bank from Laboratório Federal de Defesa Agropecuária (LFDA - Pedro Leopoldo, Minas Gerais, Brazil); group 2 – animals negative for brucellosis (32 serum samples), selected from brucellosis-free herds in the state of Santa Catarina, Brazil (Dorneles et al., 2014), kindly provided by CIDASC (Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina, Santa Catarina, Brazil); group 3 – calves vaccinated with S19 (114 serum samples), between 3 and 8 months, 28, 56 and an average of 688 (±406.4) days after vaccination (CETEA 139/2010 and CEUA 069/2018); group 4 – calves vaccinated with RB51 (60 serum samples), between 3 and 8 months, 28 and 56 days post-vaccination (CEUA 069/2018); group 5 – heifers upper 24 months of age inoculated with *Yersinia enterocolitica* group O:9 (Standard Sample YE 383 – strain 52212, 3 x 10^{10} colony forming units/animal inactivated with β-propiolactone) (42 serum samples), 7, 14, 21, 28, 35, 42 and 49 days after inoculation (Corbel & Cullen, 1970) (CEUA 024/2021). All sera were stored at -20 ºC until the tests.

2.2 Serological tests

The BPAT (Buffered plate antigen test) was performed as described by Alton et al. (1988). The antigen Brucellic Acid Amortiguated was used (BIOTANDIL DIAGNÓSTICOS, Argentina). The sera that present absence of agglutination were considered negative and presence of partial or total agglutination considered positive.

The CF (complement fixation test), RBT (Rose Bengal test), 2ME (2-mercaptoethanol test) and SAL (serum agglutination test) were also performed based on the procedures described by Alton et al. (1988). The CF test was carried out at the Vet Vida Laboratory (Cuiabá, Mato Grosso, Brazil, PGF:000014.0130228/2020), authorized and accredited by the Ministério da Agricultura, Pecuária e Abastecimento-MAPA (Ministry of Agriculture, Livestock and Supply). Samples that showed a fixation level > 50% at 1:4 dilution (20 IU/mL) or higher were considered positive. In RBT, any agglutination was recorded as a positive result. For 2ME and SAL, tests were considered positive when any agglutination was observed at a dilution ≥ 1:25.

The FPA (fluorescence polarization assay) was performed as described by Nielsen et al. (1996) . The antigen used was *Brucella* S Antibody Test Kit (B1001BRA, Ellie LLC, USA) using the Ellie Sentry 201 handheld fluorescence polarized (FPA) reader (Ellie Technical Notes, single tube reader, Germantown, USA). The tests were carried out at the official laboratory LFDA, Pedro Leopoldo, Minas Gerais, Brazil and the results were expressed as delta mP values (ΔmP) of the samples and calculated as the difference between the mP value of the samples and the mean of the mP values of the negative controls. Seropositivity was set at > Δ20 mP, according to manufacture instructions.

The iELISA (indirect enzyme-linked immunosorbent assay) (iELISA_IDEXX) was performed using the IDEXX Brucellosis Serum X2 (Brucella abortus Antibody Test Kit – BAT1132T, IDEXX™ Laboratories, USA) according to the manufacturer's instructions.

The iELISA using superoxide dismutase (SOD) [Cu-Zn] (iELISA_SOD) recombinant protein as antigen was performed as previously described by Faria et al. (2020) and R. S. Andrade et al. (unpublished data). Recombinant SOD [Cu-Zn] protein was synthesized commercially by Genscript (USA). Briefly, plates (Nunc Maxisorp™, Thermo Fisher Scientific, USA) were sensitized with 0.25 µg/well of recombinant protein (SOD) in 0.06 M carbonate-bicarbonate buffer (pH 9.6) at 4° C for 16–18 hours. Binding sites were blocked with phosphate-buffered saline with 0.05% Tween™-20 (PBS-T) (0.01 M, pH 7.6) supplemented with 5% nonfat dry milk at 37 °C for 1 h. Sera samples were diluted (1:200) in PBS-T supplemented with 0.5% nonfat dry milk and incubated at 37 °C for 1 h.

Plates were washed three times with PBS-T and then incubated with anti-bovine IgG peroxidase conjugate (clone IL-A2, Bio-Rad Laboratories, USA) diluted 1:2000 in PBS-T supplemented with 0.5% nonfat dry milk at 37 °C for 1 h. After three washes with PBS-T, the reactions were developed with 3,3', 5,5'-tetramethylbenzidine peroxidase (TMB) (Sigma-Aldrich, USA), and the plates were incubated for 10 min at room temperature, in the dark. The reactions were stopped by the addition of 2 N H_2SO_4 and the plates were read at 450 nm in Agilent Biotek Epoch™ Multiskan Go Reader Microplate Spectrophotometer (BioTek Instruments, Germany). The results of the iELISA_SOD was expressed as optical density (OD) values. A ROC curve was performed to evaluate the DSe and DSp the AUC for the test was 0.8420 (95% CI: 0.7595 to 0.9244) with a cut-off value (0.3945).

2.3 Statistical analysis

DSe and DSp were estimated for 8 tests: RBT, SAL, 2ME, FPA, BPAT, iELISA_IDEXX, iELISA_SOD and CF, as well as pairwise sensitivity (Se) and specificity (Sp) covariances were calculated using the same tests. The model used to estimate the DSe, DSp and covariances was similar to that proposed by Wang et al. (2020), a hierarchical model of conditional dependency without multinomial imposition, which takes into account the potential paired dependency between tests. For all tests, non-informative priors in the form of uniform distributions between the zero and one intervals modeled using Beta (1,1) were chosen. Residual correlation analysis was applied to detect any significant covariance between multiple tests. The Just Another Gibbs Sampler (JAGS) implementation was efficiently adopted to configure the hierarchical structure of the models for implementation applied to Markov Chain Monte Carlo (MCMC). Data analysis was performed using R software version 4.2.2 (Team, 2021) with aid of the packages ‘R2jags’ (Su & Yajima, 2021) and ‘coda’ (Plummer et al., 2006).

3. Results

A total of 328 individual serum samples of five populations were tested in parallel for detection of *Brucella* spp. specific antibodies by RBT, SAL, 2ME, FPA, BPAT, iELISA_IDEXX,

iELISA_SOD and CF. Bayesian estimation was performed using dichotomized tabulated combination of results from eight different tests shown in Table 1. The possible result combinations for the eight tests are 256, but only 51 were found in the results. Among the 328 serum samples analyzed 33 were positive in all tests (10.06%), while 26.22% (86/328) tested negative in all brucellosis tests.

Bayesian DSe and DSp estimates and their respective 95% credibility interval (CI) for the whole studied population, not taking into account the group subdivision, are shown in Table 2. Among the eight tests, those that showed the best DSe were BPAT 68.46% (95% CI: 63.08 – 75.00%), SAL 68.28% (95% CI: 62.54 – 73.90%) and RBT 67.10 (95% CI: 62.19 - 72.91%), respectively. For DSp, those that exhibited better results were FPA 84.49% (95% CI: 80.25 - 88.98%), 2ME 84.19% (95% CI: 80.24 – 87.88%) and RBT 83.12% (95% CI: 79.54 – 86.78%).

The DSe and DSp estimates for the eight tests according to the five groups are shown in Table 3. In group 1, naturally infected animals, the DSe between the tests varied from 66.74% (CI 95%: 53.54 – 78.94%) to 95.43% (95% CI: 87.99 - 99.94%). The tests that exhibited the best DSe were RBT 95.43% (95% CI: 87.99 - 99.94%), BPAT 95.34% (95% CI: 89.55 - 99.94%) and FPA 95.19% (95% CI: 87.91 – 99.99%). In group 2, animals negative for brucellosis, DSp ranged from 87.30% (95% CI: 74.40 – 97.00%) to 97.48% (95% CI: 93.32 - 99.99%). The tests that showed the best performance were FPA 97.48% (95% CI: 93.32 - 99.99%) and 2ME 97.43% (CI 95%: 93.00 – 99.94%). In group 3, vaccinated with S19, the tests that showed the best DSp were 2ME 97.69% (CI 95%: 92.77 - 99.98%) and SAL 97.67% (CI 95%: 92.82 – 99.97%), while in group 4, vaccinated with RB51, iELISA_IDEXX 97.52% (CI 95%: 92.57 - 99.98%) and FPA 96.95% (CI 95%: 91.95 – 99.99%) showed better DSp. In group 5, animals inoculated with *Y. enterocolitica* group O:9, the FPA 96.75% (95% CI: 90.58 - 99.96%) and 2ME 92.60% (95% CI: 80.03 – 99.96%) tests exhibited the best DSp among the eight serological tests.

Analyzes of the pairwise covariances of Se (C^+) and Sp (C^-) are detailed in Table 4. Mean estimates of C^+ ranged from 0.2126 to -0.0964, with the combination of FPA and iELISA_IDEXX

tests (0.2126) with the highest covariance and 2ME and iELISA_IDEXX (-0.0964) with the lowest covariance. Likewise, the combination of BPAT and RBT (0.1205) was the highest and CF and iELISA_SOD (-0.0236) the lowest covariance of C^{-}.

Table 1. The 51 cross counts of 328 individual sera samples from the dichotomous result of eight tests for bovine brucellosis. Values '1' and '0' refer to test positive and test negative status for each of the eight diagnostic tests, respectively.

Test result pattern	RBT[a]	SAL[b]	2ME[c]	FPA[d]	BPAT[e]	iELISA IDEXX[f]	iELISA SOD[g]	CF[h]	Animals with test result pattern	
									Nº	%
1	0	0	0	0	0	0	0	0	86	26.22
2	0	0	0	0	0	0	0	1	53	16.16
3	1	1	1	1	1	1	1	0	36	10.98
4	1	1	1	1	1	1	1	1	33	10.06
5	0	1	1	1	1	1	1	1	10	3.05
6	1	1	1	1	1	1	0	0	8	2.44
7	0	0	1	0	0	0	0	0	7	2.13
8	0	1	1	1	1	1	1	0	7	2.13
9	1	1	1	1	0	1	0	0	6	1.83
10	1	1	1	1	0	1	0	1	6	1.83
11	0	0	0	0	0	0	1	1	5	1.52
12	0	0	0	0	0	1	0	1	5	1.52
13	1	1	1	0	1	1	1	0	5	1.52
14	0	0	0	0	0	1	0	0	4	1.22
15	1	1	1	0	1	1	1	1	4	1.22
16	1	1	1	1	1	1	0	1	4	1.22
17	1	0	1	0	0	0	1	1	3	0.91
18	1	1	0	0	1	1	1	1	3	0.91
19	1	1	1	1	0	1	1	0	3	0.91
20	0	0	0	0	0	0	1	0	2	0.61
21	0	0	1	0	0	0	0	1	2	0.61
22	0	1	0	0	0	1	1	0	2	0.61
23	0	1	1	0	0	1	0	1	2	0.61
24	0	1	1	0	1	1	1	1	2	0.61
25	0	1	1	1	0	1	1	1	2	0.61
26	1	0	1	1	1	1	1	0	2	0.61
27	1	1	1	1	0	1	1	1	2	0.61
28	0	0	1	0	0	0	1	1	1	0.30
29	0	0	1	0	0	1	0	0	1	0.30

30	0	0	1	0	0	1	0	1	1	0.30
31	0	0	1	1	0	0	0	0	1	0.30
32	0	0	1	1	0	1	0	0	1	0.30
33	0	0	1	1	0	1	0	1	1	0.30
34	0	1	0	0	0	0	0	0	1	0.30
35	0	1	1	0	0	1	0	0	1	0.30
36	0	1	1	1	0	0	0	0	1	0.30
37	0	1	1	1	0	1	0	0	1	0.30
38	0	1	1	1	0	1	1	0	1	0.30
39	0	1	1	1	1	1	0	0	1	0.30
40	1	0	0	0	0	0	0	0	1	0.30
41	1	0	0	0	0	0	1	0	1	0.30
42	1	0	0	0	0	0	1	1	1	0.30
43	1	0	0	0	0	1	0	1	1	0.30
44	1	0	1	0	0	1	1	1	1	0.30
45	1	1	0	0	0	1	0	0	1	0.30
46	1	1	0	0	1	0	0	0	1	0.30
47	1	1	0	0	1	1	1	0	1	0.30
48	1	1	1	0	0	0	0	1	1	0.30
49	1	1	1	1	0	0	0	0	1	0.30
50	1	1	1	1	0	0	1	0	1	0.30
51	1	1	1	1	1	0	1	0	1	0.30
Total	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**328**	**100**

[a] RBT (Rose Bengal test), [b] SAL (serum agglutination test), [c] 2ME (2-mercaptoethanol test), [d] FPA (fluorescence polarization), [e] BPAT (Buffered plate antigen test), [f] iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), [g] iELISA_SOD [Cu-Zn] (superoxide dismutase), [h] CF (complement fixation test). NA = not applicable.

Table 2. Estimates of diagnostic sensitivity (DSe) and specificity (DSp) for eight serological tests for the diagnosis of brucellosis using Bayesian methods for cattle applied to a population with unknown status.

Serological test	DSe[a]	95% CI[b]		DSp[c]	95% CI	
		lower	upper		lower	upper
RBT[d]	0.6710	0.6219	0.7291	0.8312	0.7954	0.8678
SAL[e]	0.6828	0.6254	0.7390	0.8006	0.7402	0.8574
2ME[f]	0.6547	0.6083	0.7140	0.8419	0.8024	0.8788
FPA[g]	0.6296	0.5734	0.6726	0.8449	0.8025	0.8898
BPAT[h]	0.6846	0.6308	0.7500	0.8003	0.7499	0.8526
iELISA_IDEXX[i]	0.6338	0.5862	0.6859	0.8288	0.7573	0.9019
iELISA_SOD[j]	0.6323	0.5613	0.7080	0.7836	0.7197	0.8436
CF[k]	0.6394	0.5719	0.7020	0.8254	0.7857	0.8657

[a] DSe (diagnostic sensitivity), [b] 95% CI (credibility interval of 95%), [c] DSp (diagnostic specificity) [d] RBT (Rose Bengal test), [e] SAL (serum agglutination test), [f] 2ME (2-mercaptoethanol test), [g] FPA (fluorescence polarization), [h] BPAT (Buffered plate antigen test), [i] iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), [j] iELISA_SOD [Cu-Zn] (superoxide dismutase), [k] CF (complement fixation test).

Table 3. Estimates of diagnostic sensitivity (DSe) and specificity (DSp) for eight routine serological tests for the diagnosis of brucellosis using Bayesian methods for cattle applied in five different populations.

Groups	RBT			SAL			2ME			FPA			BPAT			iELISA_IDEXX			iELISA_SOD			CF		
	Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
Group 1 (naturally infected)																								
DSe	0.9543	0.8799	0.9994	0.8959	0.8001	0.9753	0.7024	0.5670	0.8219	0.9519	0.8791	0.9999	0.9534	0.8955	0.9994	0.9361	0.8762	0.9971	0.6848	0.5648	0.8171	0.6674	0.5354	0.7894
DSp	0.7188	0.3365	0.9984	0.7534	0.3769	0.9963	0.7582	0.4048	0.9945	0.7161	0.3395	0.9966	0.5539	0.1145	0.9763	0.5519	0.1374	0.9982	0.6463	0.1321	0.9994	0.6515	0.1562	0.9991
Group 2 (negative)																								
DSe	0.4828	0.0031	0.9398	0.4816	0.0203	0.9579	0.4899	0.0563	0.9920	0.4611	0.0109	0.9083	0.4832	0.0105	0.9468	0.5003	0.0055	0.9426	0.4797	0.0008	0.9486	0.5076	0.0019	0.9568
DSp	0.9723	0.9215	0.9996	0.9467	0.8769	0.9998	0.9743	0.9300	0.9994	0.9748	0.9332	0.9999	0.9680	0.9117	0.9998	0.9681	0.9132	0.9996	0.8730	0.7440	0.9700	0.9638	0.9042	0.9998
Group 3 (vaccinated with S19)																								
DSe	0.9531	0.8717	0.9996	0.9399	0.8692	0.9997	0.9405	0.8600	0.9995	0.6830	0.5575	0.8023	0.8048	0.2926	0.9989	0.6293	0.3115	0.9565	0.4351	0.0003	0.7958	0.6153	0.0737	0.9232
DSp	0.9413	0.8625	0.9995	0.9767	0.9340	0.9997	0.9769	0.9277	0.9998	0.9743	0.9282	0.9996	0.6640	0.4370	0.8716	0.7049	0.5427	0.8716	0.5681	0.3988	0.7151	0.7194	0.5443	0.9881
Group 4 (vaccinated with RB51)																								
DSe	0.5801	0.1333	0.9912	0.6951	0.1269	0.9998	0.5935	0.1122	0.9861	0.2678	0.0002	0.7770	0.4432	0.0012	0.9319	0.3915	0.0001	0.9671	0.3951	0.0004	0.9111	0.4851	0.0644	0.9906
DSp	0.9522	0.8763	0.9997	0.8472	0.7290	0.9525	0.9441	0.8697	0.9957	0.9695	0.9195	0.9999	0.8670	0.7579	0.9570	0.9752	0.9257	0.9998	0.9015	0.8199	0.9999	0.9497	0.8901	1,0000
Group 5 (inoculated with *Yersinia enterocolitica* group O:9)																								
DSe	0.6675	0.1844	0.9988	0.7382	0.2689	0.9991	0.6470	0.2092	0.9985	0.1453	0.0001	0.5086	0.4253	0.0035	0.8364	0.3730	0.0034	0.8440	0.5185	0.0145	0.8986	0.4775	0.0726	0.9911
DSp	0.8463	0.6897	0.9987	0.6281	0.4426	0.8020	0.9260	0.8003	0.9996	0.9675	0.9058	0.9996	0.6110	0.4026	0.9664	0.8281	0.6404	0.9979	0.6476	0.4337	0.9934	0.6849	0.4817	0.9956

[a] RBT (Rose Bengal test), [b] SAL (serum agglutination test), [c] 2ME (2-mercaptoethanol test), [d] FPA (fluorescence polarization), [e] BPAT (Buffered plate antigen test), [f] iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), [g] iELISA_SOD [Cu-Zn] (superoxide dismutase), [h] CF (complement fixation test), [i] CI (credibility interval), [j] DSe (diagnostic sensitivity), [k] DSp (diagnostic specificity). Group 1 (naturally infected animals); Group 2 (animals negative for brucellosis); Group 3 (vaccinated with S19); Group 4 (vaccinated with RB51); Group 5 (inoculated with *Yersinia enterocolitica* group O:9).

Table 4. Conditional estimated paired covariances in the Se (C^+) and in the Sp (C^-) for data from the eight serological tests used in the diagnosis of bovine brucellosis.

Covariance	Test	RBT[a]	SAL[b]	2ME[c]	FPA[d]	BPAT[e]	iELISA_IDEXX[f]	iELISA_SOD[g]	CF[h]
C^+	RBT	-	0.1853	0.1884	0.1771	0.1934	0.1286	-0.0949	0.0512
	SAL	-	-	0.1984	-0.0064	0.1862	0.0953	-0.0422	0.1194
	2ME	-	-	-	0.1524	0.1654	-0.0964	-0.0894	0.1399
	FPA	-	-	-	-	0.1740	0.2126	0.1081	0.1369
	BPAT	-	-	-	-	-	0.1605	0.0452	0.1320
	iELISA_IDEXX	-	-	-	-	-	-	-0.0386	0.1113
	iELISA_SOD	-	-	-	-	-	-	-	-0.0536
	CF	-	-	-	-	-	-	-	-
C^-	RBT	-	0.1039	0.1192	0.1174	0.1205	0.1028	0.0820	0.1199
	SAL	-	-	0.1006	0.0482	0.0699	0.0542	-0.0101	-0.0177
	2ME	-	-	-	0.1184	0.0829	0.0660	-0.0020	0.0979
	FPA	-	-	-	-	0.0629	0.0726	-0.0083	0.1035
	BPAT	-	-	-	-	-	-0.0181	-0.0020	0.0459
	iELISA_IDEXX	-	-	-	-	-	-	0.0134	0.0816
	iELISA_SOD	-	-	-	-	-	-	-	-0.0236
	CF	-	-	-	-	-	-	-	-

[a] RBT (Rose Bengal test), [b] SAL (serum agglutination test), [c] 2ME (2-mercaptoethanol test), [d] FPA (fluorescence polarization), [e] BPAT (Buffered plate antigen test), [f] iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), [g] iELISA_SOD [Cu-Zn] (superoxide dismutase), [h] CF (complement fixation test).

4. Discussion

A critical point in the bovine brucellosis control and eradication strategy is the choice of the test to be used, which must be able to identify the true state of the animal. The use of multiple tests is a common in the diagnosis of bovine brucellosis, but carelessness in assuming conditional independence of diagnosis tests even when they are based on the same biological process can lead to biased estimates due to an underestimation of classification errors (Gardner et al., 2000; Thibodeau, 1981). Given that, the objective of this study was to estimate the covariance (conditional dependence) of eight serological tests used for the diagnosis of bovine brucellosis, as well as their DSe and DSp using a Bayesian approach. Our results confirmed the hypothesis of conditional dependence between the tests, calculated the mean pairwise covariances, which showed FPA and iELISA_IDEXX (0.2126) and RBT and BPAT (0.1205) the tests with higher conditional dependences in Se and Sp, respectively.

Regarding the characteristics of the tests, five of them (RBT, SAL, 2ME, BPAT and CF) are based on the detection anti-*Brucella* smooth lipopolysaccharide (S-LPS) antibodies (Nielsen & Yu, 2010), whereas the FPA test detects anti-*Brucella* O-chain antibodies (Nielsen et al., 1996), iELISA_SOD detects anti-*Brucella* SOD [Cu-Zn] antibodies (Faria et al., 2020) and the iELISA_IDEXX test detects antibodies against *B. abortus* although it does not inform which antigen(s) are used in the kit. Considering that the probability of each combination of test pairs for C^+ and C^- must be between 0 and 1 to be valid (Wang et al., 2020), 21 of the 28 (75%) possible combinations between pairs of eight tests were in the range of 0 and 1, showing conditional dependence. The seven combinations that exhibited conditional independence, in both C^+ and C^-, are tests that have more differences in assessed biological process (Table 4). Among these combinations, iELISA_SOD is present in five (both C^+ and C^-), suggesting that this test could give a higher gain in the DSe or DSp when used in a parallel or series for bovine brucellosis diagnosis together with other conventional tests, even though it not exhibited the higher DSe and DSp compared to the other evaluated tests. In addition, these findings also highlight the importance of developing and validating

tests free of the immunodominant antigen S-LPS, which also contribute to minimize cross-reaction with other Gram-negative bacteria (Faria et al., 2020). However, the improvement in the bovine brucellosis diagnosis potentially offered by the use independent tests must be carefully assessed also considering the epidemiological status of the disease, laboratory capacity and resources available for the disease control.

The test combinations that exhibited the highest covariances in Se (C^+) were FPA and iELISA_IDEXX (0.2126), SAL and 2ME (0.1984), RBT and BPAT (0.1934), respectively. These results were not unexpected, since, except for iELISA_IDEXX for which the antigen is unknown, all the other pair of tests use very similar or the same antigen. Indeed, SAL and 2ME tests are extremely similar, are usually used in combination and employed the same antigen. However, SAL detects the IgM isotype of the antibody efficiently, but detects IgG less efficiently, resulting in low assay specificity (Nielsen & Yu, 2010). To decrease SAL specificity problems by lowering IgM levels, chemical treatment with 2-mercaptoethanol is used to destroy the disulfide bonds in the molecule, resulting in monomeric units of the pentameric molecule, thus increasing the specificity of the assay (Poester et al., 2010). Likewise, RBT and BPAT tests are classified within the same group of tests, as both uses buffered *Brucella* antigen, and they introduced in many countries as a standard screening test. Furthermore, in these tests, the execution procedures and the logic of the tests are similar, the cell antigen of *B. abortus* S99 or S1119.3, stained with Rose Bengal (RBT) or Brilliant Green and Crystal Violet (BPAT), and suspended in a buffer in a final pH of 3.65 are used (Alton et al., 1988). The differences between RBT and BPAT are mainly the concentration of the antigens (8% RBT and 11% BPAT), the volume of serum test used (30 µL RBT and 80 µL BPAT) and the time specified for reading the results (4 minutes for RBT and 8 minutes for BPAT) (Nielsen & Yu, 2010). Given all that, it is tempting to speculate that the antigen used in iELISA_IDEXX is probably composed by S-LPS or part of S-LPS, as well as the FPA test that uses the O chain of LPS (Nielsen et al., 1996), which would justify the higher covariance observed between the tests.

For the covariances in Sp (C^-), the highest covariances in the combinations of tests were in the RBT and BPAT (0.1205), RBT and CF (0.1199) and RBT and FPA (0.1192) tests. For the RBT and BPAT, the same reasons used above for explain the covariance in the sick animals (Se), could be used to justify the high covariance among non-diseased animals (Sp). For the covariance (C^-) in the combination of RBT and CF, it could be due to both tests use as antigens *B. abortus* whole cells and are focused in detect anti-S-LPS antigens, whereas for the combination of RBT and FPA, it could be explained by the fact that both measure anti-S-LPS antibodies. It should be noted that albeit these were the highest covariances observed in non-sick animals, they were approximately half than those for Se (C^+).

A Bayesian analysis with the latent class model was used to estimate the DSe and DSp of the eight bovine brucellosis serological tests in five different populations. Considering the whole population, the DSe values obtained were significantly lower than those estimates found in a recent meta-analysis conducted by our research group, as well as the DSp values (Andrade et al., unpublished data). The underestimation in the DSe maybe due low prevalence in the entire population, since only one group was from diseased animals among the five assessed. Indeed, the underestimation in the DSp was lower for the whole population than that observed for DSe and the DSe estimates for the group 1 (naturally infected) was significantly higher compared to other groups for the majority of the tests (Table 3). Additionally, it is important to consider that the chosen for a Bayesian approach have also influenced the estimates obtained, as a frequentist analysis certainly would give higher estimates, produced by the case-control study design adopted, in which different tests are used to define diseased and non-diseased animals (Andrade et al., unpublished data; Sainani & Popat, 2011). Moreover, the frequentist approach also considers the test as conditionally independent, as our results showed they are not (Table 4). In fact, other studies using Bayesian models also achieved similar results, especially in DSe (Ahasan et al., 2017; Arif et al., 2018; Getachew et al., 2016; Muñoz et al., 2012), when using models considering tests as conditionally dependent. Finally, we used non informative priors and some populations with low number of participants, which

may also have influenced the estimates obtained. Nonetheless, it is worth to mention that the main objective of the present study was not to give precise estimates of the DSe and DSp of bovine brucellosis serological tests, as this was done more robustly by a meta-analysis recently conducted (Andrade et al., unpublished data).

Overall, BPAT was the test that showed the best performance in DSe (68.46%), followed by SAL 68.28% and RBT 67.10%. These tests are traditionally considered to have high sensitivity, used as a screening test by many countries (WOAH, 2018). However, WOAH and the European Union (EU) have decided not to recommend the use of SAL, as they consider it inferior to other standard tests (Greiner et al., 2009; WOAH, 2008). On the other hand, the test that showed the best DSp was the FPA 84.49%, used in brucellosis control and certification programs in North America and EU (Godfroid et al., 2010).

The DSe estimates in the group 1 of naturally infected animals the tests that showed better performances were RBT (95.43%), BPAT (95.34%) and FPA (95.19%), being the CF (66.74%) and 2ME (70.24%) the tests with the worst performances. Likewise, the DSp estimates for the group 2 of negative animals, also exhibited FPA (97.48%), RBT (97.23%) and BPAT (96.80%) among those with higher values, indicating that these are the tests with best accuracy and of choice if a single test should be used. Among these tests, FPA could be considered the most accurate, as in the estimates for the vaccinated groups (S19 and RB51) and for the group inoculated with *Y. enterocolitica* group O:9, FPA was the test with higher DSp, exhibiting low levels of cross-reactivity and thereby low number of false positive reactions in healthy populations (Table 4).

In general, the results of the present study showed the conditional dependence of serological tests most used for diagnosis of bovine brucellosis worldwide, besides offers robust estimation of these covariances, which are essential to propose efficient and assertive diagnostic strategies employing multiple tests (tests in parallel and/or in series). However, the most suitable combinations of tests should be assessed taking into account different prevalence scenarios, objectives proposed by the programs and resources available, having no ideal strategy appropriate for all situations.

5. Conclusion

Except for iELISA_SOD, all the serological tests evaluated in the present study showed conditional dependence, emphasizing the importance of considering the covariance of the tests in their validation and in the proposal of their use in effective diagnostic strategies. The tests that showed better DSe and DSp were BPAT and FPA, respectively, being the FPA the test with best performance considering also DSp estimates for vaccinated groups and for the group inoculated with *Y. enterocolitica*.

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Article 3

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Use of recombinant malate dehydrogenase (MDH) and superoxide dismutase (SOD) [Cu-Zn] as antigens in indirect ELISA for diagnosis of bovine brucellosis

Abstract

The objective of this work was to validate an indirect enzyme-linked immunoassay (iELISA) using the recombinant proteins, malate dehydrogenase (MDH) and superoxide dismutase (SOD) [Cu-Zn], as antigens and to evaluate its ability to discriminate antibodies produced by vaccination from those induced by infection, in addition to its usefulness in the diagnosis of bovine brucellosis. Six groups were evaluated: G1 - Positive culture from animals (52 serum samples) naturally infected; G2 - non-vaccinated animals (28 serum samples) from selected from herds with a brucellosis, positive in RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test); G3 - Animals from brucellosis-free area (32 serum samples); G4 - S19 vaccinated heifers (114 serum samples); G5 - Heifers vaccinated with RB51 (60 serum samples); G6 - Cross-reacting animals with inactivated *Yersinia enterocolitica* O:9 (42 serum samples). Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) were estimated using the frequentist approach and the confidence interval (CI) (95%) calculated by the Clopper-Pearson (exact) method. The DSe estimates for iELISA_MDH in the G1 group was 63.46% (CI 95%: 48.96 - 76.38%) and for the G2 of 35.71% (CI 95%: 18.64 - 55.93%) and for the DSp were 50.00% in the G3 (CI 95%: 31.89 - 68.11%) and G4 58.77% (95% CI: 49.17 - 67.91%). Whereas for the iELISA_SOD estimates for DSe they were 67.31% in G1 (CI 95%: 52.89 - 79.67%) and in G2 it was 71.43% (CI 95%: 51.33 - 86.78%) and for DSp in G3 87.50% (CI 95%: 71.01 - 96.49%). iELISA_SOD exhibited could be used for the diagnosis of infected animals, increasing the range of serological tests available for the diagnosis of bovine brucellosis, with the advantage of being free of

S-LPS. In contrast, the iELISA_MDH showed low usefulness as diagnostic test for bovine brucellosis, as well as for differentiate infection from vaccination.

1. Introduction

Bovine brucellosis is predominantly caused by *Brucella abortus* and responsible for substantial economic losses mainly due to last trimester abortion, mastitis and reduced milk production in females, and orchitis and epididymitis in males (McDermott et al., 2013). The disease has been controlled in many countries by implementing programs usually based on vaccination and test and slaughter policies, although both approaches complement each other for reliable diagnosis (Khurana et al., 2021; Zhang et al., 2018). For brucellosis diagnosis, serological tests are generally preferred, since they are normally simple, inexpensive, rapid, and have good diagnostic sensitive (DSe) and specificity (DSp) (Nielsen & Yu, 2010). Despite the development of numerous different serological tests, virtually all are constructed for detection of antibodies against smooth lipopolysaccharide (S-LPS) of *Brucella* spp., part of LPS, or whole cells as the antigen (Poester et al., 2010). Although, the S-LPS based tests are more sensitive, they can produce false positive results for cattle vaccinated with *B. abortus* S19 or exposed to other Gram-negative bacteria with LPS O chains similar to *Brucella* LPS (*Yersinia enterocolitica* O:9, *Salmonella* urban group N, *Vibrio cholerae* and *Escherichia coli* O:157) (Nicoletti, 1981; Perry & Bundle, 1990).

Therefore, there is an urgent need for highly specific bovine brucellosis serological tests based on other *Brucella* antigens to minimize cross-reactivity with other Gram-negative bacteria and allow the differentiation between vaccination and infection, such as outer membrane proteins (OMPs), inner membrane proteins, cytoplasmic and ribosomal proteins (Al Dahouk et al., 2003; Nielsen & Yu, 2010; Pajuaba et al., 2012). In this context, a recent study from our research group showed a potential of two recombinants proteins, malate dehydrogenase (MDH) and superoxide dismutase (SOD) [Cu-Zn], to differentiate vaccination from infection by *B. abortus* and for the diagnosis of infected animals (Faria et al., 2020). In this study, our objective was to validate an indirect enzyme-linked

immunosorbent assays (iELISA) using the recombinant proteins MDH and SOD as antigens and assess their ability to discriminate antibodies produced by vaccination from those induced by infection, besides their usefulness in bovine brucellosis diagnosis.

2. Material and methods

2.1 Bovine sera

Sera were collected from six groups of cattle: G1 - 52 serum samples from culture positive animals, naturally infected, kindly provided by the LFDA (Laboratório Federal de Defesa Agropecuária, Pedro Leopoldo, Minas Gerais, Brazil); G2 - 28 serum samples from non-vaccinated animals selected from herds with a brucellosis outbreak in the state of Santa Catarina, Brazil, that were positive for RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test), kindly provided by CIDASC (Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina, Santa Catarina, Brazil); G3 - 32 serum samples of animals from brucellosis-free area selected from brucellosis-free herds in the state of Santa Catarina, Brazil (Dorneles et al., 2014); G4 - 114 serum samples from S19 vaccinated heifers between 3 and 8 months of age, obtained 28, 56 and 688 (±406.4) days after vaccination (CETEA 139/2010 and CEUA 069/2018); G5 - 60 serum samples from RB51 vaccinated heifers between 3 and 8 months of age, 28 and 56 days post-vaccination (CEUA 069/2018); G6 - 42 serum samples from animals upper 24 months of age, inoculated with inactivated *Y. enterocolitica* O:9 antigen (Standard Sample YE 383 – strain 52212), inactivated by exposure to β-propiolactone and at a concentration of 3 x 10^{10} colony forming unit/animal, collected at 7, 14, 21, 28, 35, 42 and 49 days post inoculation (Corbel & Cullen, 1970) (CEUA 024/2021). All sera were stored at -20 ºC until the tests. A minimum of 20 samples is suggested by the World Organization for Animal Health (WOAH) for validation of serological tests (WOAH, 2022).

2.2 Recombinant protein production

The recombinant proteins MDH and SOD [Cu-Zn] were synthesized commercially by Genscript (USA) codon-optimized for *Escherichia coli* expression. They were cloned into the pET30a and

pET22b vector, respectively, which allowed a 6-residue histidine tag to be fused to the proteins (Supplementary figure S1).

Competent *E. coli* BL21(DE3) cells were transformed with the recombinant plasmid. A single colony was inoculated into Luria Bertani medium (Gibco, USA) containing kanamycin or ampicillin; cultures were incubated at 37°C at 200 rpm. Once the cell density reached OD=0.6-0.8 at 600 nm, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG - Sigma-Aldrich, USA) was introduced for induction. The best expression conditions for the proteins were 16 h at 15ºC. Then proteins were purified using an NI column, MDH was obtained from supernatant of cell lysate and SOD was obtained from periplasmic space. MDH was eluted in 50 mM Tris-HCl, 150 mM NaCl, 10% Glycerol, pH 8.0, while SOD was eluted in PBS, 10% Glycerol, pH 7.4. Purified proteins were subjected to SDS-PAGE to confirm identity. As expected, the recombinant proteins migrated close to their calculated molecular weights, bands approx. 26.4 kDa, referring to MDH and 19.8 kDa referring to SOD.

2.3 Indirect enzyme-linked immunosorbent assays (iELISA)

The iELISA using SOD [Cu-Zn] and MDH recombinant protein as antigen was performed as previously described by Faria et al. (2020). The plates (Nunc Maxisorp™, Thermo Fisher Scientific, USA) were sensitized with 0.25 μg/well of recombinant protein (SOD or MDH) in 0.06 M carbonate-bicarbonate buffer (pH 9.6) at 4° C for 16–18 hours. Binding sites were blocked with Phosphate-buffered Saline with 0.05% Tween™-20 (PBS-T) (0.01M, pH 7.6) supplemented with 5% nonfat dry milk at 37 °C for 1 h. Sera samples were diluted (1:200) in PBS-T supplemented with 0.5% nonfat dry milk and incubated at 37 °C for 1 h. Plates were washed three times with PBS-T and then incubated with anti-bovine IgG peroxidase conjugate (clone IL-A2, Bio-Rad Laboratories, USA) diluted 1:2000 in PBS-T supplemented with 0.5% nonfat dry milk at 37 °C for 1 h. After three washes with PBS-T, the reactions were developed with 3,3', 5,5'-tetramethylbenzidine peroxidase (TMB) (Sigma-Aldrich, USA), and the plates were incubated for 10 min at room temperature, in the dark. The reactions were stopped by the addition of 2 N H_2SO_4 and the plates were read at 450 nm in

Agilent Biotek Epoch™ Multiskan Go Reader Microplate Spectrophotometer (BioTek Instruments, Germany). The results of the iELISA were expressed as optical density (OD) values.

2.4 Statistical analysis

The cut-off points for both iELISAs were determined by receiver operating characteristic (ROC) analysis using GraphPad Prism version 8.0 (GraphPad Software, USA). The DSe and Dsp were estimated using the frequentist approach and the confidence interval (CI) (95%) calculated by the Clopper-Pearson (exact) method (Sergeant, 2018).

3. Results

To evaluate the performance of iELISAs using the recombinant proteins MDH and SOD [Cu-Zn] as antigen, sera from five groups of cattle were used. A ROC curve was performed to determine the cut-off point for both iELISAs, the AUC of the iELISA_MDH was 0.5478 (95% CI: 0.4192 to 0.6763) with a cut-off value of 0.8460, while for the iELISA_SOD [Cu-Zn] the AUC was 0.8420 (95% CI: 0.7595 to 0.9244) with a cut-off value of 0.3945.

The DSe and DSp estimates obtained for the iELISA_MDH and iELISA_SOD are detailed in Table 1 and Table 2. The optical density values (OD) obtained in the iELISA_MDH and iELISA_SOD for the tested populations are shown in the Figure 1.

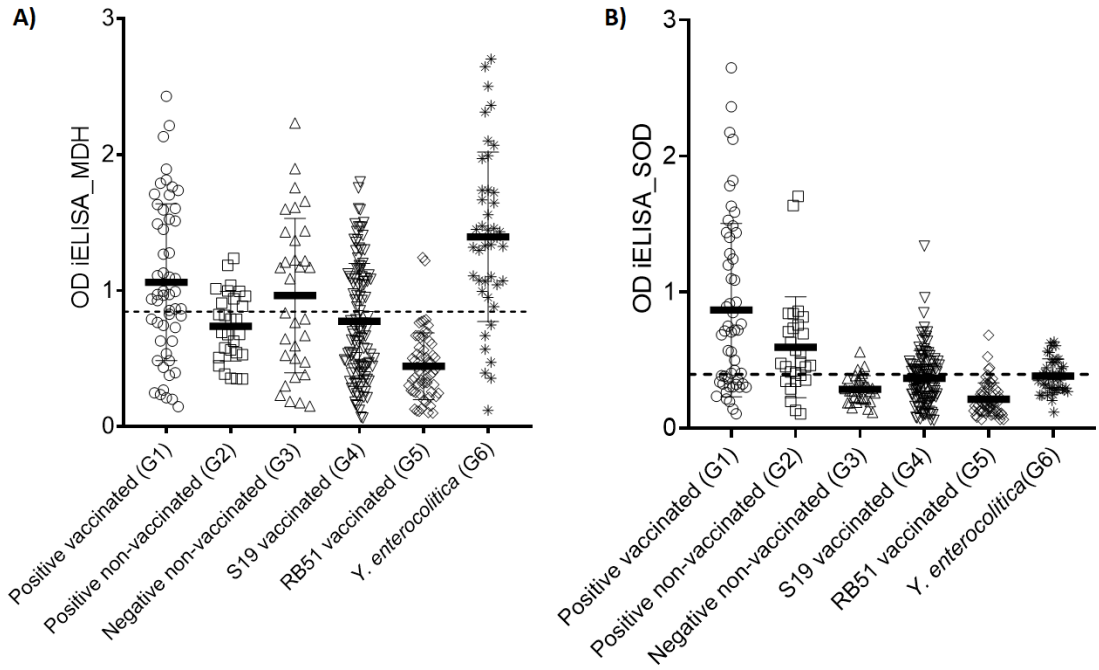

Figure 1. Indirect-ELISA (iELISA) analysis of individual bovine sera to assess reactivity against recombinant proteins. A) Malate dehydrogenase (MDH) and B) Superoxide dismutase (SOD) [Cu-Zn] were tested in iELISA against different groups of bovine sera. G1 - Positive culture from animals (52 serum samples) naturally infected; G2 - non-vaccinated animals (28 serum samples) from selected from herds with a brucellosis outbreak, positive for RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test); G3 - Animals from brucellosis-free area (32 serum samples); G4 - S19 vaccinated heifers (114 serum samples); G5 - Heifers vaccinated with RB51 (60 serum samples); G6 - Cross-reacting animals with inactivated *Yersinia enterocolitica* O:9 (42 serum samples).The lines express the mean and standard deviation of the results in optical density (OD). The dashed lines indicate the cut-off point established by the ROC curve.

Table 1. Estimation of the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of iELISA_MDH.

Groups	DSe (CI 95%)			DSp (CI 95%)		
	Mean	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
Positive vaccinated (G1) x Negative non-vaccinated (G3)	0.6346	0.4896	0.7638	0.5000	0.3189	0.6811
Positive vaccinated (G1) x S19 vaccinated (G4)	0.6346	0.4896	0.7638	0.5877	0.4917	0.6791
Positive vaccinated (G1) x RB51 vaccinated (G5)	0.6346	0.4896	0.7638	0.9667	0.8847	0.9959
Positive vaccinated (G1) x *Y. enterocolitica* (G6)	0.6346	0.4896	0.7638	0.1667	0.0697	0.3136
Positive non-vaccinated (G2) x Negative non-vaccinated (G3)	0.3571	0.1864	0.5593	0.5000	0.3189	0.6811
Positive non-vaccinated (G2) x S19 vaccinated (G4)	0.3571	0.1864	0.5593	0.5877	0.4917	0.6791
Positive non-vaccinated (G2) x RB51 vaccinated (G5)	0.3571	0.1864	0.5593	0.9667	0.8847	0.9959
Positive non-vaccinated (G2) x *Y. enterocolitica* (G6)	0.3571	0.1864	0.5593	0.1667	0.0697	0.3136
S19 vaccinated (G4) x Positive non-vaccinated (G2)	0.5877	0.4917	0.6791	0.6429	0.4407	0.8136
S19 vaccinated (G4) x Negative non-vaccinated (G3)	0.5877	0.4917	0.6791	0.5000	0.3189	0.6811
S19 vaccinated (G4) x RB51 vaccinated (G5)	0.5877	0.4917	0.6791	0.9667	0.8847	0.9959
S19 vaccinated (G4) x *Y. enterocolitica* (G6)	0.5877	0.4917	0.6791	0.1667	0.0697	0.3136
RB51 vaccinated (G5) x Positive non-vaccinated (G2)	0.0333	0.0041	0.1153	0.6429	0.4407	0.8136
RB51 vaccinated (G5) x Negative non-vaccinated (G3)	0.0333	0.0041	0.1153	0.5000	0.3189	0.6811
RB51 vaccinated (G5) x S19 vaccinated (G4)	0.0333	0.0041	0.1153	0.5877	0.4917	0.6791
RB51 vaccinated (G5) x *Y. enterocolitica* (G6)	0.0333	0.0041	0.1153	0.1667	0.0697	0.3136

G1 - Positive culture from animals (52 serum samples) naturally infected; G2 - non-vaccinated animals (28 serum samples) from selected from herds with a brucellosis outbreak, positive for RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test); G3 - Animals from brucellosis-free area (32 serum samples); G4 - S19 vaccinated heifers (114 serum samples); G5 - Heifers vaccinated with RB51 (60 serum samples); G6 - Cross-reacting animals with inactivated *Yersinia enterocolitica* O:9 (42 serum samples).

Table 2. Estimation of the diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of iELISA_SOD.

Groups	DSe (CI 95%)			DSp (CI 95%)		
	Mean	**Lower**	**Upper**	**Mean**	**Lower**	**Upper**
G1 x G3	0.6731	0.5289	0.7967	0.8750	0.7101	0.9649
G1 x G4	0.6731	0.5289	0.7967	0.5526	0.4566	0.6458
G1 x G5	0.6731	0.5289	0.7967	0.9333	0.8380	0.9815
G1 x G6	0.6731	0.5289	0.7967	0.5952	0.4328	0.7437
G2 x G3	0.7143	0.5133	0.8678	0.8750	0.7101	0.9649
G2 x G4	0.7143	0.5133	0.8678	0.5526	0.4566	0.6458
G2 x G5	0.7143	0.5133	0.8678	0.9333	0.838	0.9815
G2 x G6	0.7143	0.5133	0.8678	0.5952	0.4328	0.7437

G1 - Positive culture from animals (52 serum samples) naturally infected; G2 - non-vaccinated animals (28 serum samples) from selected from herds with a brucellosis outbreak, positive for RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test); G3 - Animals from brucellosis-free area (32 serum samples); G4 - S19 vaccinated heifers (114 serum samples); G5 - Heifers vaccinated with RB51 (60 serum samples); G6 - Cross-reacting animals with inactivated *Yersinia enterocolitica* O:9 (42 serum samples).

4. Discussion

Most conventional serological tests used in the diagnosis of bovine brucellosis detect antibodies against smooth lipopolysaccharide (S-LPS) from *Brucella* spp. Although S-LPS induces a strong antibody response in the host (Dorneles, Teixeira-Carvalho, et al., 2015), the tests routinely used are not able to differentiate antibody response triggered after vaccination from natural infection and cross-reaction with S-LPS from other Gram-negative bacteria (Nielsen & Yu, 2010). In this context, we recently observed two recombinant proteins, MDH and SOD, with potential to differentiate antibodies induced by infection from those produced by vaccination, which have their performance assessed in the present study using the iELISA platform. Our results demonstrated that iELISA_SOD was able of differentiate antibodies from infected animals from those produced by non-infected animals, while iELISA_MDH showed low usefulness as diagnostic test for bovine brucellosis, as well as for differentiate infection from vaccination.

SOD [Cu-Zn] in *Brucella* spp. plays an important role allowing them to survive the phagocytic attack of the host immune system, being considered as a virulence factor that facilitates intracellular survival (McCord et al., 1971; Sriranganathan et al., 1991). Our results demonstrated that cattle naturally infected with *B. abortus* produce antibodies against SOD [Cu-Zn], which can be identifiable by the iELISA test [DSe 67.31% (CI 95%: 52.89 - 79.67%) and DSp 87.50% (CI 95%: 71.01 - 96.49%)] (Table 1). This finding was also observed by Tabatabai and Hennager (1994) in cattle, which detected positive serologic reaction of animals naturally infected by *Brucella* spp. in a iELISA using recombinant SOD [Cu-Zn] as antigen. Nonetheless, this study did not assess the accuracy of the test, which does not allow further comparisons with the results observed in the present study.

The accuracy assessment of iELISA_SOD showed a low DSe and an acceptable DSp (G1 x G3 and G2 x G3), the low sensitivity could be explained by the intracellular localization of the SOD [Cu-Zn] (McCord et al., 1971), which probably harm the induction of specific antibodies. The DSp can be considered good especially taking into account the low cross reactivity against S19 vaccinated animals and experimentally inoculated with *Y. enterocolitica* O:9 (Figure 1 and Table 2). Indeed,

previous studies have also demonstrated a weak anti-SOD [Cu-Zn] proliferative response in animals vaccinated with S19 (Cheville et al., 1993; Stevens et al., 1994). The same occurred with animals vaccinated with RB51, which did not develop antibodies to SOD [Cu-Zn] (Cheville et al., 1993; Oñate et al., 1999), and showed in the present study OD similar to the mean of the negative group (G3) (Figure 1). SOD [Cu-Zn] has been shown to play a role in the intraphagocytic survival of *Y. enterocolitica* (Dhar & Virdi, 2014), however it induces a weak immune response in the assessed population. Overall, the iELISA_SOD results showed this test could be useful for the diagnosis bovine brucellosis, especially in low prevalence areas or in regions in process of eradication of the disease, where the predictive values of the test are low and false positive results are a huge problem, since there is the possibility of seropositivity due to vaccination or cross-reaction with antibodies induced by infection by other Gram-negative bacteria (Godfroid et al., 2010). Furthermore, it can be used in serial or parallel diagnostic strategies to increase the diagnosis accuracy (DSp and DSe, respectively), as it was previously demonstrated as having no or low conditional dependence with the tests routinely use in the serodiagnosis of bovine brucellosis, since it measure different antibodies induced by infection (Andrade et al., unpublished data) In addition, iELISA_SOD can be useful in a multiprotein diagnostic platform for the diagnosis of bovine brucellosis, which can be optimized according to the epidemiological status of the disease in the country/region. In this sense, iELISA tests have a great advantage over conventional serological tests, its flexibility and freedom of choice for the antigen used in the assay.

Malate dehydrogenase (MDH), one of the enzymes responsible for the functionality of the tricarboxylic acid (TCA) cycle, plays important metabolic roles in pathways of aerobic energy production and malate transport (Han et al., 2014). Findings in previous studies demonstrated that MDH was one of the immunogenic proteins of reactive *Brucella* in the initial periods of infection in cattle and mice (Lee et al., 2014; Reyes et al., 2016), but these studies did not evaluate vaccinated animals. In our previous study iELISA_MDH was the test that showed the best potential to separate infection from vaccination, being able to detect antibodies from vaccinated animals, regardless the

vaccine used (S19 or RB51), with OD significantly higher than those observed for positive or negative animals (Faria et al., 2020). Nevertheless, this potential was not confirmed by the present results, which different from the previous findings showed low accuracy of the iELISA_MDH, considering all the scenarios evaluated (a test to detect vaccination or infection) (Table 1). Curiously, the group with higher antibodies detected in the test was the inoculated with *Y. enterocolitica* O:9, not evaluated in the previous study, reflecting in the DSp value (16.67%). It is also worth to mention that in the previous study the number of groups and animals assessed were substantially lower than those used in the present study. Studies demonstrate that MDH is a housekeeping enzyme for *Yersinia* spp. which could explain this high production of antibodies to the MDH protein (Goullet & Picard, 1988; Mallik & Virdi, 2010). Although, Lee et al. (2014) did not notice MDH immunoreactivity in the group of mice inoculated with *Y. enterocolitica* O:9 by immunoproteomic using *B. abortus* 544 proteome; however they did not perform serological tests to evaluate the production of anti-MDH antibodies.

Another important finding, it is that regardless the antigen used (MDH and SOD), for the group vaccinated with RB51 low antibody response was observed, suggesting that RB51 vaccination does not induce significant levels of anti-MDH or anti-SOD antibodies (Figure 1 and Table 1 and 2), as well as observed for antibodies anti-S-LPS (Dorneles, Sriranganathan, et al., 2015).

Over the years, several modifications in serological tests for the diagnosis of brucellosis have been investigated to overcome the limitations resulting from the immune response against *Brucella* spp. infection. Herein, we further investigated the ability of two immunogenic recombinant proteins, MDH and SOD, in the diagnosis of infection and vaccination by *B. abortus*. One of the platforms assessed could be useful for the diagnosis of the disease when LPS-free tests are needed (iELISA_SOD), however, the iELISA_MDH proved to be low relevant either for the diagnosis of the infection and vaccination.

5. Conclusion

iELISA_SOD exhibited could be used for the diagnosis of infected animals, increasing the range of serological tests available for the diagnosis of bovine brucellosis, with the advantage of being free

of S-LPS. In contrast, the iELISA_MDH showed low usefulness as diagnostic test for bovine brucellosis, as well as for differentiate infection from vaccination.

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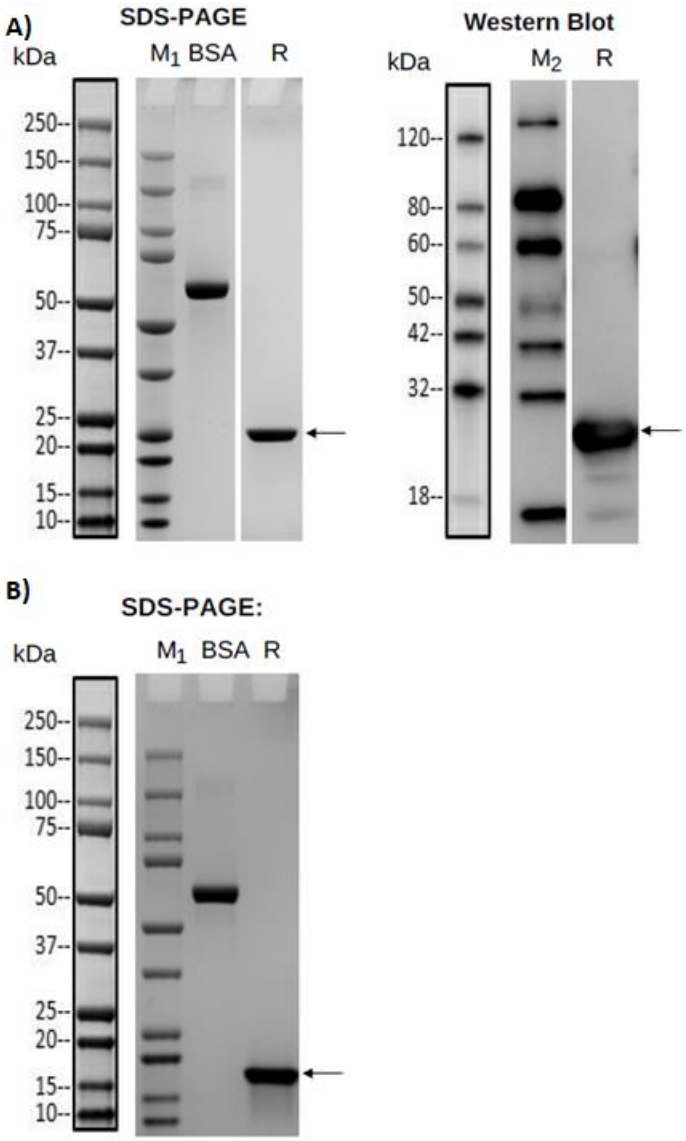

Supplementary figure S1. SDS-PAGE and Western blot analysis of *Escherichia coli* lysates producing recombinant proteins. A) Malate dehydrogenase, Lane M1: Protein Marker, Bio-rad, Cat. No. 1610374S; Lane M2: Protein Marker, GenScript, Cat. No. M00673; BSA: 2.00 µg; R: Reducing condition; Primary antibody: Mouse-anti-His mAb (GenScript, Cat. No. A00186). B) superoxide dismutase, Lane M1: Protein Marker, Bio-rad, Cat. No. 1610374S; BSA: 2.00 µg; R: Reducing condition

Article 4

Prepared and formatted according to the guidelines of the journal *Theriogenology*

Absence of *Brucella* spp. in ovaries of seropositive cattle

Abstract

Animal reproduction biotechniques are important tools for the technological advancement of livestock, as they enable the expansion and selection of the reproductive potential of superior quality females and males, accelerating genetic improvement and favoring animal selection programs. However, infectious diseases that have a predilection for the reproductive system can be an impediment and disadvantage of these technologies. Therefore, the present work aimed to detect *Brucella* spp. in the ovarian follicular fluid of brucellosis positive bovine females. A total of 47 bovine aspirates from females positive in two serological tests (RBT, 2-ME/SAL) were used for PCR. The primers used in the PCR were specific to the genus *Brucella* (B4 and B5). The expected size of the amplicon was 223 bp and the visualization of the amplified products was performed on a 1.5% agarose gel. All 47 bovine aspirates were negative for *Brucella* spp. Our results demonstrate that in the ovarian follicular fluid there is no presence of *Brucella* spp. even in seropositive cows, which offers greater safety for reproductive biotechnologies carried out from the follicular aspirate of these animals.

1. Introduction

Veterinary reproductive biotechniques enable the expansion and selection of suitable genetic material for animal breeding, in addition to allowing the control of venereal diseases and the reducing replacement costs [1]. Among the most used biotechnologies in bovine reproduction are artificial insemination (AI), fixed-time artificial insemination (FTAI), embryo transfer by superovulation (SOV) and *in vitro* embryo production (IVP) [2]. According to the report of the International Embryo Transfer Society (IETS) in 2020, more than 1.5 million bovine embryos were registered, which represents an increase of 7.0% compared to 2019 [3]. Of these 76.2% were produced *in vitro* and 43.3% in South America, from which 73.2% in Brazil [3].

In vitro embryo production techniques make it possible to use the best reproductive potential of superior quality females, accelerating genetic improvement and low-cost mass production of bovine embryos for transfer to recipient cows [4]. However, it is also necessary

to consider possible health risks and risk of disease transmission associated with IVP [5]. Infectious diseases in which the agent has a predilection for the genital tract are likely to be transmitted in embryo transfer and IVP, being a justifiable concern [6].

In this context, bovine brucellosis is an important disease, since *Brucella* spp. has preference for gravid uterus, causing 80% of abortion in the final third of pregnancy in susceptible herds [7, 8]. There are numerous irrefutable evidences (obtained by isolating the agent) of colonization of the uterus in animals infected by *Brucella* spp. but there is, so far, no evidence of the presence of the bacterium in the ovary of infected animals [9-11], although *B. melitensis* has already been isolated causing ovarian abscess in a woman [12].

Given that, the assessment of the presence of *Brucella* spp. in the ovaries of brucellosis seropositive animals is important from two point of views: first, considering the safety (disease transmission) of reproductive biotechnologies that involve the aspiration of ovaries; and second, due to the possibility of taking offspring from genetic superior animals before slaughtering them [1, 13]. These are especially important points for South America countries where brucellosis is endemic and bovine reproduction biotechnologies are widely used [3]. Therefore, aiming to assess the potential risks of using oocytes from animals positive for brucellosis in reproductive biotechnologies, the objective of this study was to investigate the presence of *Brucella* spp. in bovine ovarian follicular fluid from brucellosis seropositive cows by PCR (polymerase chain reaction).

2. Material and methods

2.1 Description of the herd

The study was conducted during a brucellosis outbreak in a cattle herd in the state of Minas Gerais, Brazil. The herd consisted of approximately 2,300 Gir, Guzerá and Girolando breeds, raised in an extensive grazing system. All young females in the herd were vaccinated with S19 between 3 and 8 months of age and the whole herd revaccinated with RB51 after detection of the first seropositive animal in November 2020. Brucellosis was serologically diagnosed in the herd in October 2020 and thereby a control and eradication program based on mass vaccination with RB51 and a test and slaughter policy was initiated [14]. All cattle older than 24 months were monthly tested for brucellosis [RBT (Bengal Rose Test) as a screening test an 2ME (2 mercaptoethanol)] as a confirmatory test) and positive animals were slaughtered [14]. The initial prevalence of the disease in the herd when the animals were selected for this study was 5.1% (CI 95%: 4.4 – 5.8%).

The experimental protocol was approved by the Ethics Committee on the Use of

Animals (CEUA) of the Universidade Federal de Lavras (CEUA/UFLA – Protocol 027/22).

2.2 Ovarian follicular fluid samples

Forty-seven samples of ovarian follicular fluid were collected from seropositive females sent for slaughter. The seropositive animals were selected considering positive results in the RBT and 2ME (titers ≥ 100) (Supplementary Table 1) [14, 15]. The aspiration technique was performed according to Pieterse et al. [16] and for each animal, 500 µL of ovarian follicular fluid was collected and subsequently stored at -20 °C until processing.

2.3 DNA extraction

DNA extraction from ovarian follicular fluid was performed using the Wizard® Genomic DNA Purification Kit (Promega Corporation, USA), following manufacture's recommendations.

2.4 Brucella *spp. PCR*

PCR assay for detection of *Brucella* spp. (genus-specific PCR) was carried out using the primers described by Baily et al. (1992) according to Richtzenhain et al. [17]. *Brucella abortus* 544 DNA (ATCC 23448) and all reagents of the PCR mix without DNA template were used as positive and negative control, respectively, in all assays. Visualization of amplified products was performed on 1.5% agarose gel in tris-borate-EDTA (TBE) buffer (Sigma-Aldrich, USA) (89 mM Tris Base, 89 mM boric acid, 2 mM EDTA, pH 8.0) stained with ethidium bromide (Sigma-Aldrich, USA) (0.5 mg/mL) and visualized under UV light. The molecular marker 100 bp DNA ladder (100 bp DNA Ladder, Promega Corporation, USA) was used in all electrophoresis.

2.5 Statistical analysis

The statistical significance of the results was assessed using a power analysis performed as described by Cohen [18]. The analysis was performed on R statistical software version 4.1.3 [19] using the *pwr* package [20].

3. Results

Brucella spp. DNA was not detected in any of the 47 tested ovarian follicular fluid from brucellosis seropositive animals. A representative gel electrophoresis of PCR products is shown in Fig. 1.

4. Discussion

IVP in cattle are a very important biotechnology to obtain a massive genetic gain in a

short time but it can have the intercurrence of reproductive infectious diseases compromising the result and safety of the method. Given that, our aim was to investigate the presence of *Brucella* spp. in ovarian follicular fluid of brucellosis-seropositive cattle using PCR, a very sensitive and specific technique. Our results showed that the health risks associated to the use of oocytes from positive animals in reproductive biotechnologies are probably neglected, pointing for the absence of *Brucella* spp. in the ovary of infected animals and providing information on biosafety for the genetic use of seropositive animals.

Indeed, albeit *B. abortus* has a predilection for the gravid uterus, being found in high concentration in this organ [21], similar to our findings previous studies also indicate the safety of the IVP performed from cattle [22], buffalo [23] and dromedary camels [24] infected by *Brucella* spp. All these studies, as well as our findings demonstrated the absence of *Brucella* spp. in the embryos obtained from brucellosis positive animals, for both embryos from IVP and SOV, suggesting the absence of ovarian colonization by *Brucella* spp. and the safety of using oocytes from infected animals.

Additionally to this evidence of absence of *Brucella* in the ovary of infected animals, it is important to consider that the embryos used in IVP and SOV, before being inoculated in the receptors, are also treated following international standards protocols to ensure that they are free from specific pathogens [25]. Indeed, previous studies testing the safety of embryo transfer technique considering the transmission of *Brucella* spp., using embryos exposed *in vitro* to *B. abortus* strain 2308, showed negative culture results after testing washing procedures, even without the addition of antibiotics [26, 27]. However, embryos with defective zona pellucida had positive culture even after washing, highlighting the importance of verifying the physical integrity of the embryo when there is concern about the transmission of infectious agents [28].

The understanding of the biosafety of IVP and SOV, it is remarkably important considering that positive animals for brucellosis have greater impacts in developing countries, such as Brazil and India, which have the two largest cattle herds and are endemic for brucellosis [29]. The possibility of using brucellosis-positive genetically superior animals for reproduction before sending them for slaughter is a way to rationalize and reduce the economic losses invariably caused by disease control measures and which may result in more incentive to the adhesion of producers to the control program.

In fact, our results showed the absence of *Brucella* spp. in the ovary of seropositive female cattle by means of PCR, a successfully technique used for the diagnosis of brucellosis from different clinical samples and which is fast and has high sensitive and specific [17]. A previous study showed that the detection limit (analytical sensitivity) using different DNA

extraction protocols for the PCR technique employed in the present study is between 2 and 20 CFU (colony forming unit)/mL of *Brucella* spp., which can be considered high [30]. Furthermore, another advantage of using PCR is that it allows the use of samples in which the microorganisms have been inactivated, making the diagnosis safer from the public health point of view [31].

Two main limitations of the present study were: the number of animals tested and that it was not possible to perform the isolation of *Brucella* spp., the unequivocal technique to confirm the infection. However, for the last statement, some arguments can help overcome the limitations; the high serology titers observed in the positive animals, the use of two different serological tests in series strategy (improving the diagnostic specificity) (Supplementary Table 1) and the historic of brucellosis infection and reproductive issues in the herd strongly indicates the active *Brucella* infection.

In conclusion, our results showed that *Brucella* spp. is not present in ovaries of brucellosis-seropositive cattle, suggesting the safety of the use reproductive biotechnologies from the follicular aspirate of these animals and for laboratory workers who will handle these oocytes. Moreover, the results also indicate that genetically superior animals diagnosed with brucellosis can be oocyte donors for IVP potentially no transmitting the disease to the recipient and to the fetus.

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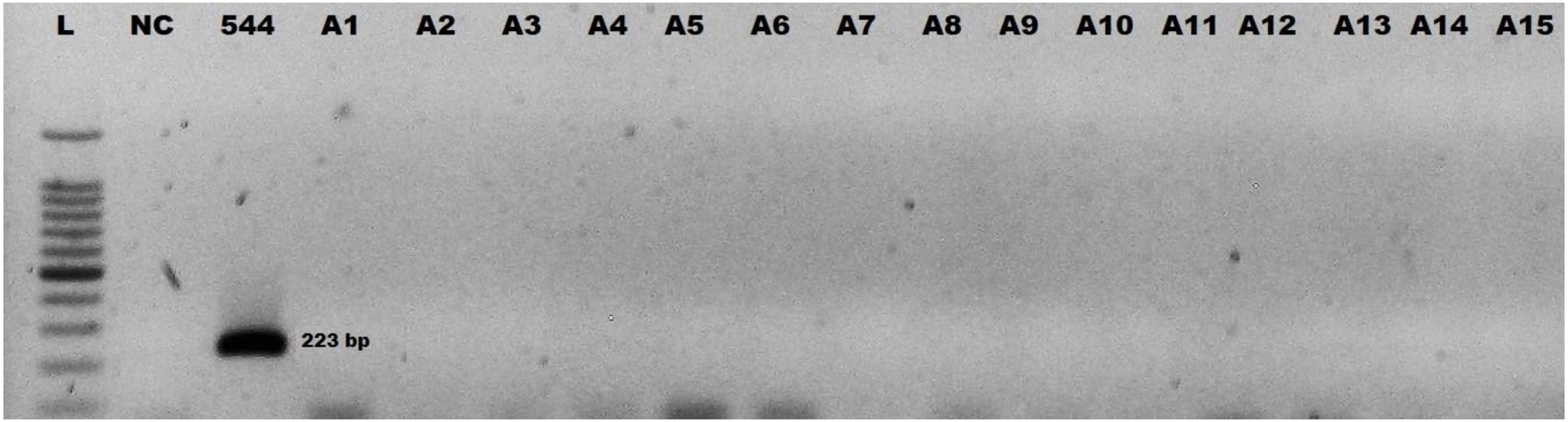

Fig. 1. Agarose gel 1.5% (w/v) stained with ethidium bromide (0.5 mg/ml) from *Brucella* spp. PCR performed on samples of bovine ovarian follicular fluid from brucellosis-positive females. *Brucella* spp. PCR: *Brucella abortus* 544 -ATCC 23448 (544) used as positive control and tested samples (A1-A15). 100 bp DNA ladder (100 bp DNA Ladder, Promega Corporation, USA) (L); Negative control (NC).

Supplementary Table 1: Individual information and the results of serological tests (RBT, 2ME) of the animals included in the study.

Animal	Breed	Date of birth	RBT	SAL	2ME	Result
A01	Guzolando	NI	Reagent	100	100	Positive
A02	Guzolando	NI	Reagent	200	200	Positive
A03	Gir	03/05/2009	Reagent	200	100	Positive
A04	Guzolando	NI	Reagent	200	200	Positive
A05	Guzolando	NI	Reagent	200	200	Positive
A06	Guzolando	NI	Reagent	200	200	Positive
A07	Guzolando	NI	Reagent	200	200	Positive
A08	Guzolando	NI	Reagent	200	200	Positive
A09	Guzolando	NI	Reagent	200	100	Positive
A10	Guzolando	NI	Reagent	100	100	Positive
A11	Guzerá	19/01/2016	Reagent	100	100	Positive
A12	Guzerá	01/10/2011	Reagent	200	200	Positive
A13	Guzerá	07/09/2013	Reagent	200	200	Positive
A14	Guzerá	18/06/2014	Reagent	200	200	Positive
A15	Gir	12/12/2013	Reagent	200	200	Positive
A16	Guzerá	27/10/2013	Reagent	200	200	Positive
A17	Guzerá	30/07/2014	Reagent	200	200	Positive
A18	Gir	01/07/2014	Reagent	200	200	Positive
A19	Gir	16/02/2009	Reagent	100	100	Positive
A20	Guzerá	NI	Reagent	100	100	Positive
A21	Guzerá	NI	Reagent	100	100	Positive
A22	Guzerá	01/10/2011	Reagent	100	100	Positive
A23	Guzerá	NI	Reagent	200	200	Positive
A24	Guzerá	NI	Reagent	200	200	Positive
A25	Guzerá	NI	Reagent	200	200	Positive
A26	Guzerá	NI	Reagent	200	200	Positive
A27	Gir	NI	Reagent	200	200	Positive
A28	Gir	NI	Reagent	200	200	Positive
A29	Gir	NI	Reagent	200	200	Positive
A30	Gir	NI	Reagent	200	200	Positive
A31	Gir	NI	Reagent	200	200	Positive
A32	Gir	30/12/2015	Reagent	200	200	Positive
A33	Gir	02/11/2011	Reagent	200	200	Positive
A34	Gir	27/08/2016	Reagent	200	200	Positive
A35	Gir	30/01/2016	Reagent	200	200	Positive
A36	Gir	18/03/2017	Reagent	200	200	Positive
A37	Gir	24/04/2008	Reagent	200	200	Positive
A38	Gir	08/04/2017	Reagent	200	200	Positive
A39	Gir	09/04/2016	Reagent	200	200	Positive
A40	Guzerá	NI	Reagent	200	200	Positive
A41	Guzerá	NI	Reagent	200	200	Positive
A42	Guzerá	NI	Reagent	200	200	Positive
A43	Guzerá	NI	Reagent	200	200	Positive
A44	Guzerá	NI	Reagent	200	200	Positive

A45	Guzerá	NI	Reagent	200	200	Positive
A46	Girolando	NI	Reagent	200	200	Positive
A47	Girolando	NI	Reagent	200	200	Positive

NI: Not informed; RBT: Rose Bengal Test; SAL: Slow agglutination test in tubes; 2ME: 2-mercaptoethanol

Article 5

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Phenotypic and genotypic characterization of *Brucella abortus* biovar 4 isolates from cattle in Brazil

Caracterização fenotípica e genotípica de isolados de *Brucella abortus* biovar 4 em bovinos no Brasil

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ABSTRACT

The aim of the present study was to characterize (phenotypically and genotypically) two strains of *Brucella abortus* identified as belonging to biovar 4 isolated from cattle in Brazil. The strains were isolated from tissues and stomach contents from cattle in the states of Pará and Rio Grande do Sul, respectively. In the phenotypic identification, the isolates were positive in CO_2 requirement, produced H_2S, were resistant to basic fuchsin (20 µg / mL) and sensitive to thionin (20 µg / mL and 40 µg / mL) and presented M surface antigen, but A surface antigen is absent. The isolates were positive in the PCR for the *bcsp31* gene (genus-specific) and in the AMOS-enhanced PCR, both isolates showed a band profile consistent with *B. abortus* biovar 1, 2 or 4. Moreover, both isolates also showed restriction patterns identical to the reference strain when tested by the *omp2b* PCR-RFLP. In genotyping using *Multiple Locus Variable Number of*

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Tandem Repeat (VNTR) Analysis - MLVA (MLVA16), the isolates showed differences in several *loci* (Bruce42, Bruce19, Bruce04, Bruce16 and Bruce30); by *Multiple Locus Sequence Typing* (MLST), they also exhibited differences in sequence type (ST), strain 16/02 ST1 (2-1-1-2-1-3-1-1-1) and strain 128/11 ST (22-1-1 -8-9-3-1-1-1). The extensive typing of *B. abortus* strains isolated from cattle in Brazil using different approaches confirmed the occurrence of rare *B. abortus* biovar 4 in the country.
Keywords: Brucellosis; *Brucella abortus* biovar 4; genotyping; MLVA; MLST.

RESUMO

O objetivo do presente estudo foi caracterizar (fenotipicamente e genotipicamente) duas cepas de *Brucella abortus* identificadas como pertencentes à biovar 4 isolada de bovinos no Brasil. As cepas foram isoladas de tecidos e conteúdo estomacal de bovinos dos estados do Pará e Rio Grande do Sul, respectivamente. Na identificação fenotípica, os isolados foram positivos na exigência de CO_2, produziram H_2S, foram resistentes à fucsina básica (20 µg / mL) e sensíveis à tionina (20 µg / mL e 40 µg / mL) e apresentaram antígeno de superfície M, mas o antígeno de superfície A ausente. Os isolados foram positivos na PCR para o gene *bcsp31* (gênero específico) e na PCR amplificada por AMOS, ambos os isolados apresentaram perfil de banda consistente com *B. abortus* biovar 1, 2 ou 4. Além disso, ambos os isolados também apresentaram padrões de restrição idêntica à cepa de referência quando testada pelo *omp2b* PCR-RFLP. Na genotipagem usando *Multiple Locus Variable Number of Tandem Repeat* (VNTR) - MLVA (MLVA16), os isolados apresentaram diferenças em vários *loci* (Bruce42, Bruce19, Bruce04, Bruce16 e Bruce30); no *Multiple Locus Sequence Typing* (MLST), os isolados também exibiram diferenças na sequência tipo (ST), amostra 16/02 ST1 (2-1-1-2-1-3-1-1-1) e amostra 128/11 ST (22-1-1-8-9-3-1-1-1). A extensa tipagem de cepas de *B. abortus* isoladas de bovinos no Brasil por diferentes abordagens confirmou a ocorrência da rara *B. abortus* biovar 4 no país.
Palavras-chave: Brucelose; *Brucella abortus* biovar 4; genotipagem; MLVA; MLST.

INTRODUCTION

Brucellosis is a worldwide zoonotic disease caused by bacteria of the genus *Brucella*, which infect a wide variety of wild and domestic animals, as well as humans (ALTON et al., 1988). In cattle, infection is mainly caused by *Brucella abortus* (CORBEL, 2006). Due to the impact of *B. abortus* infection on livestock and public health, the control and eradication of bovine brucellosis is an important goal of several countries where the disease is endemic,

including Brazil, that since 2001 has implemented the Programa Nacional de Controle e Erradicação de Brucelose e Tuberculose - PNCEBT (National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis) (FERREIRA NETO et al., 2016).

The diagnosis of *Brucella* spp. in brucellosis control and eradication programs is generally based on bacteriological and serological tests (ALTON et al., 1988). Although important for the diagnosis of the disease, phenotypic typing methods generally have less discriminatory power compared with genotypic methods and, therefore, make it difficult to track outbreaks and control the spread of the disease (MINHARRO et al., 2013). In this context, the intraspecific characterization of *B. abortus* at biovar and molecular levels are fundamental for a better understanding of the disease epidemiology, for formulation effective strategies of infection control and eradication and solving outbreaks (DORNELES et al., 2014; OLIVEIRA et al., 2017).

By means of phenotypic techniques, it is possible to classify bacteria of the genus *Brucella* into biovars. The *Brucella* International Taxonomy Subcommittee recognizes seven *B. abortus* biovars,1 to 6 and 9 (HOLT, 1984; ALTON et al., 1988). In Brazil, the biovars of *B. abortus* most frequently found, in descending order, were 1, 3, 6 and 2, and, so far, only one single strain of *B. abortus* biovar 4 was identified in the country (MINHARRO et al., 2013). Molecular techniques have been developed for differentiation of *Brucella* strains and biovars (BAILY et al., 1992; BRICKER & HALLING, 1995; CLOECKAERT et al., 1995) that complement the conventional methods used to define the phenotypic profile. Among the molecular typing methodologies commonly used for *Brucella* spp., *Multiple Locus Variable Number Tandem Repeat* (VNTR) *Analysis* (MLVA) and *Multiple Locus Sequence Typing* (MLST) are well-adapted techniques that have proved to be valuable tools in source tracking and in the intraspecific classification of *Brucella* spp. isolates (OLIVEIRA et al., 2017).

In this study, we performed a wide phenotypic and genotypic characterization of two strains of *B. abortus* biovar 4 first described in cattle from Brazil, in order to support PNCEBT by providing high resolution epidemiologic data on rare *B. abortus* isolates among cattle in the country.

MATERIAL AND METHODS

Brucella strains

Two *B. abortus* strains, 16/02 and 128/11, are described in this study, being strain 16/02

previously reported by MINHARRO et al. (2013). The 16/02 strain was isolated from the stomach of an aborted fetus of European breed cow, in Rio Grande do Sul in 2002, and the strain 128/11 was isolated and characterized by the Laboratório Federal de Defesa Agropecuária (LFDA / MG) in 2011, from cervical ligament bursitis of a Nellore cattle slaughtered in Pará. The reference strains *B. abortus* biovar 4 292 = ATCC 23451, *B. abortus* biovar 1 544 = ATCC 23448^{T}, *B. abortus* biovar 1 2308, *B. abortus* biovar 1 S19, *B. abortus* biovar 1 RB51, *B. melitensis* biovar 1 16M = ATCC 23456^{T}, *B. ovis* Reo 198 and *B. suis* biovar 1 1330 = ATCC 23444 were used as controls in different tests.

Identification and biotyping

Phenotypic identification of the two isolates was performed according to international standards (ALTON et al., 1988), using the following procedures: (i) examination of colony morphology, Gram stain; (ii) metabolic tests based on catalase, oxidase, urease, nitrate reduction and citrate activity; (iii) requirement for supplementary carbon dioxide (CO_2) and the production of hydrogen sulfide (H_2S); (iv) sensitivity to thionin (20 and 40 mg / mL) and basic fuchsin (20 mg / mL) dyes in serum dextrose medium; and (v) agglutination with *Brucella* A and M monospecific antisera (Table 1).

Identification by PCR assays

In addition to identification by phenotypic routine tests (Alton et al., 1988), the isolates were also tested by *bcsp31* PCR (BAILY et al., 1992), AMOS-enhanced PCR (BRICKER & HALLING, 1995) and *omp2b* PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms), with restriction by *Taq*I, to confirm them as *B. abortus* biovar 4 strains (CLOECKAERT et al., 1995; GARCIA-YOLDI et al., 2005).

DNA of the strains were obtained from colonies suspended in 100 µL TE buffer (Sigma-Aldrich, USA) (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), inactivated at 65 °C for 1 hour in a water bath, and subjected to genomic DNA extraction by guanidine method according to PITCHER et al. (1989). DNA of reference strains were used as positive controls in each PCR assay. PCR reagents without DNA were also included as negative controls.

Visualization of the amplified products of all PCR reactions was performed in 1.0 % agarose gel in tris-borate-EDTA buffer (TBE) (Sigma-Aldrich, USA) (89 mM Tris Base, 89 mM boric acid, 2 mM EDTA, pH 8.0) stained with ethidium bromide (Sigma-Aldrich, USA) (0.5 mg / mL). Following electrophoresis, the gels were visualized under ultraviolet light and

photographed (L-PIX EX, Loccus Biotechnology, Brazil). The molecular marker 100 bp DNA ladder (100 bp DNA Ladder, New England Biolabs, USA) was used in all electrophoresis.

MLST and MLVA genotyping

MLST was performed as previously described by WHATMORE et al. (2007). Nine distinct genomic fragments were PCR amplified (*loci: gap, aroA, glk, dnaK, gyrB, trpE, cobQ, omp25* and int-*hyp*). Products were separated by agarose gel electrophoresis to check for efficiency of amplification and to ensure that only a single product of the expected size was present. Then, they were purified using a PCR purification kit (Invitek, USA) and sequenced using Big Dye™ 3.1 (Applied Biosystems, USA) on an ABI-3500 automatic sequencer (Applied Biosystems, USA). Sequences were edited using Seqman Pro (Laser Gene, USA) and aligned and edited using BioEdit (HALL, 1999).

To evaluate the genetic relationships among the isolates from this study, *B. abortus* reference strains for each biovar and other *B. abortus* biovar 4 strains, we used the MLST profiles of twenty-three *B. abortus* strains deposited in PubMLST database (https://www.pubmlst.org/) (13 biovar 4 strains) and MLST genotypes obtained from the genome of four strains (Ba col-B012, 68-3396P, 90-0775 and 01-4165) available on PATRIC (https://patricbrc.org/job/) and NCBI plataform (https://www.ncbi.nlm.nih.gov/) (Table 2).

The MLVA was carried out as described by AL DAHOUK et al. (2007) (MLVA16). The MLVA16 *loci* were divided into three panels: panel 1 (P1) or MLVA8 composed of eight minisatellites (Bruce06, Bruce08, Bruce11, Bruce12, Bruce42, Bruce43, Bruce45 and Bruce55); panel 2A (P2A) composed of three microsatellites (Bruce18, Bruce19 and Bruce21); and panel 2B (P2B) with five microsatellites (Bruce04, Bruce07, Bruce09, Bruce16 and Bruce30). The PCR conditions for MLVA16 were as previously described by AL DAHOUK et al. (2007).

The amplified products were submitted to electrophoresis in 2 % or 3 % agarose gel, for the mini and microsatellites, respectively, in Tris-borate-EDTA 1X (TBE) buffer, stained with 0.5 mg / mL ethidium bromide, visualized under UV light, and photographed (L-Pix EX, Loccus Biotecnologia, Brazil). DNA ladders 100 bp (100 bp DNA Ladder, New England Biolabs, USA) and 25 bp (25 bp DNA Step Ladder, Promega, USA) were used to estimate the tandem repeat unit length (MINHARRO et al., 2013).

Band size estimates were converted into number of repeat units for each *locus* (AL DAHOUK et al., 2007; DORNELES et al., 2014) and compared with the internal standard

strains (*B. abortus* RB51 and *B. melitensis* 16M), using the software BioNumerics 7.6 (Applied Maths, Belgium). Clustering analysis was performed using the same software based on the category coefficient and the unweighted pair group method with arithmetic mean (UPGMA) algorithm (AL DAHOUK et al., 2007; DORNELES et al., 2014). The minimum spanning tree (MST) built was the one with the highest overall reliability score and was calculated using UPGMA associated with the priority rule and the bootstrap resampling (BioNumerics 7.6).

Besides the *B. abortus* biovar 4 strains assessed in the present study, all three MLVA16 (BCCN#95-31, BCCN R7#* and 292 ATCC 23451) genotypes of *B. abortus* biovar 4 available in the MLVAbank 2020 (http://mlva.i2bc.paris-saclay.fr/brucella/), including the *B. abortus* biovar 4 strain 292, were used in clustering and MST analyses.

RESULTS

The two field isolates studied showed a phenotype consistent with *Brucella* spp. and a biochemical and metabolic pattern identical to the reference strain of *B. abortus* biovar 4 292 (Table 1). Both isolates were Gram-negative, coccobacilli, non-mobile, non-fermentative, oxidase and catalase positive. The colonies also exhibited whitish color, smooth and shiny surface, and were small and non-hemolytic. The two isolates also showed specific characteristics of *B. abortus* biovar 4: CO_2 requirement, H_2S production and growth in the presence of basic fuchsin (20 µg / mL), but not in the presence of thionin (20 µg / mL and 40 µg / mL) (ALTON et al., 1988). Moreover, in agglutination tests, the isolates agglutinated with monospecific antiserum M, but not with monospecific antiserum A (Table 1).

Also, amplification of the *bcsp31* gene confirmed the isolates as *Brucella* spp. (Baily et al., 1992) (Fig. 1-A) and AMOS-enhanced PCR (Fig. 1-B) results were compatible with *B. abortus* biovar 1, 2 or 4 for both strains (BRICKER & HALLING, 1995). In the PCR-RFLP for the *omp2b* gene, the field strains showed an identical restriction pattern to that of the reference strain *B. abortus* biovar 4 292 (CLOECKAERT et al., 1995; GARCIA-YOLDI et al., 2005) (Fig. 1-C).

The MLST analysis showed different genotypes for both isolates (16/02 and 128/11), strain 16/02 depicted a ST1 (2-1-1-2-1-3-1-1-1) and strain 128/11 did not show a ST (22-1-1-8-9-3-1-1-1) with complete correspondence with any other ST previously described in the PubMLST database for *Brucella* spp. (accessed December 17, 2021) (Fig. 2A-2). The STs of both strains were deposited in the PubMLST.

Likewise, the analysis of the MLVA *loci* revealed different genotypes among the field isolates (16/02 and 128/11). Patterns obtained in the sixteen VNTR *loci* are summarized in Fig.

2B-1. Genotyping based on MLVA8 and MLVA11 identified previously described genotypes in MLVAbank 2020 (access on May 22th 2020) for both strains, 16/02 (MLVA8 = 28, MLVA11 = 75) and 128/11 (MLVA8 = 32, MLVA11 = 182). The MLVA16 genotypes for the isolates 16/02 and 128/11 did not match any of those deposited in the MLVAbank 2020 (access on May 22th 2020). The differences found in MLVA16 between field isolates compared with the *B. abortus* biovar 4 reference strain 292 occurred in all panels (P1, P2A and P2B), the strain 16/02 showed addition of one repeat unit in Bruce19 and Bruce30, addition of two repeat units in Bruce04 and deletion of one repeat unit in Bruce42 (Fig. 2B-1). For the strain 128/11, the comparison with the reference strain 292 revealed addition of one repeat unit in Bruce16 and deletion of one repeat unit in Bruce19 and Bruce30 (Fig. 2B-1). The MST created based on MLVA16 genotypes is shown in Fig. 2B-2.

DISCUSSION

Intraspecific characterization of *Brucella* spp. circulating strains is critical for elimination of outbreaks, tracking infection spread and periodic assessment of anti-brucellosis strategies (BRICKER & HALLING, 1994; DORNELES et al., 2014). Therefore, the objective of this study was to characterize phenotypically and genotypically two isolates of *B. abortus* biovar 4, rare in cattle from Brazil, as part of the actions to support PNCEBT, the program for the control and eradication of bovine brucellosis in place in the country.

The two strains of *B. abortus* isolated from cervical bursitis exhibited different biochemical and molecular tests than *B. abortus* biovar 1, the most common strain causing bovine brucellosis in Brazilian territory (MINHARRO et al., 2013; OLIVEIRA et al., 2017). All the tests used allow us to state without doubt that the two isolates are in fact *B. abortus* biovar 4, being the first isolated strains of this biovar in Brazil (MINHARRO et al., 2013). Considering that both strains were isolated after the implementation of the PNCEBT, that brucellosis is endemic with medium/high prevalence in a large part of the Brazilian territory and that many states have not been able to significantly reduce the prevalence of the disease in recent years (FERREIRA-NETO et al., 2016), it is possible to suggest that *B. abortus* biovar 4 is still currently circulating in the Brazilian cattle herd, although it is not possible to state this, since the strains were isolated in 2002 and 2011.

Despite the low frequency of this biovar worldwide compared with other more prevalent *B. abortus* biovars (1, 2, 3 and 6) (BRICKER & HALLING, 1994), biovar 4 strains were previously identified in some countries, such as Argentina, Chad, Chile, Colombia, Cuba, El Salvador, Ecuador, France, India, Iraq, United States, Mexico, Nicaragua, and Venezuela

(LUCERO et al., 2008; HIGGINS et al., 2012; TORRES HIGUERA et al., 2019). The host mainly associated with the isolation of this biovar is cattle (LUCERO et al., 2008; MINHARRO et al., 2013; DARSHANA et al., 2016), however *B. abortus* biovar 4 have also been isolated from elk (ETTER & DREW, 2006), bison (HIGGINS et al., 2012), Rocky Mountain bighorn sheep (KREEGER et al., 2004), dogs (FORBES, 1990) and, sheep and goats (DARSHANA et al., 2016).

Considering that classical epidemiological tools alone usually do not have sufficient resolution to allow a complete understanding of the dynamics of zoonotic infectious diseases with multiple hosts, such as brucellosis, genotyping data contribute indicating the direction of transmission between hosts and assist in the decision-making process for the management of wildlife populations (HIGGINS et al., 2012). Therefore, to increase reliability and complement the results of phenotypic and molecular tests, we genotyped the strains using MLST and MLVA techniques, which allowed the differentiation of *B. abortus* biovar 4 strains into genotypes and the drawing of some inferences on their epidemiological relationships.

The MLST analysis showed different genotypes for both isolates (16/02 and 128/11), the genotype of the strain 16/02 was ST1, which has a global distribution, being widely distributed in many continents (WHATMORE et al., 2016; WHATMORE & FOSTER, 2021). Strain 128/11, on the other hand, exhibited a different and unique genotype, not described in the PubMLST database and far from any other profile already described for *B. abortus* biovar 4 (Fig. 2A), suggesting the absence of an epidemiological link between the isolate (128/11) of the present study and others already described in other countries. In fact, despite the low number of strains analyzed, the allelic profile observed for the strain 128/11 indicate the existence of several polymorphisms at the individual *locus* and at the level of the combined alleles, compared with other STs already described for *B. abortus* biovar 4 (Fig. 2A). Likewise, the comparison of ST between both Brazilian biovar 4 isolates also suggests a lack of epidemiological relationship between these two strains, due to the large genetic distance observed (Fig. 2A).

Similarly, MLVA results showed a great genetic diversity among the studied strains using MLVA8, MLVA11 and MLVA16. The differences observed between the genotypes of the field strains were not limited to the differences at the most variable *loci* (Bruce19, Bruce04, Bruce16 and Bruce30), but they were also observed in more conserved *locus*, such as Bruce42 (Fig. 2B-1). In fact, the MST (Fig. 2B-2) analysis shows that the Brazilian isolates (16/02 and 128/11) are very distant genetically considering the MLVA16 markers, as each one is at one end of the tree. Taking into account all the *B. abortus* biovar 4 genotypes available at

MLVABank, the strain 16/02 was closest to the isolate from Italy (BCCN#95-31), whereas the strain 128/11 to the strains isolated in the United Kingdom (BCCN R7#* and 292-ATCC 23451).

This large genetic distance in MLST and MLVA genotypic profile in the comparison between the two *B. abortus* biovar 4 strains isolated from Brazil, together with the absence of an epidemiological link between them, strongly suggest that both strains originated from a different ancestor. Indeed, considering the great geographic distance between the place of origin of the isolates, since Pará and Rio Grande do Sul are far opposite states in the Brazilian territory (north and south, respectively, more than 3000 km apart, approximately 1864 miles), and also the difference in the productive profiles and historical origin of the cattle herds between the these two states, it is very likely that the strains have different origins. Cattle herd from Rio Grande do Sul has a historical influence from neighboring countries, Uruguay and Argentina, which make up the herd mainly from European breeds (*Bos taurus*), on the other hand Pará has a large part of the territory occupied by the Nellore breed (*Bos indicus*), influenced by the proximity to states of high representativeness in the national livestock, such as Mato Grosso, Goiás and Mato Grosso do Sul (LÁU, 2006; CANOZZI et al., 2019).

In this context, it is tempting to speculate that the studied strain 16/02 isolated in the state of Rio Grande do Sul may be associated with animal import and transport, since to date there have been 17 isolates characterized as *B. abortus* biovar 4 in the world, from which 52.94% (9/17) belong to ST1, even the reference strain *B. abortus* biovar 4 292 from United Kingdom. Nevertheless, albeit the 16/02 strain depicted the ST1, considered to be widespread in many continents, other *B. abortus* biovar 1 and 2 also exhibited ST1 and have shown to be historically closely related genetically (GARGANI & LOPEZ-MERINO, 2006). Indeed, from 54 *B. abortus* isolates from Brazil deposited in the PubMLST 75.92% (41/54) showed ST1, although not classified biovar 4. Based on these findings, it is not yet possible to identify distinct genetic lineages corresponding to these biovars (WHATMORE et al., 2016; WHATMORE & FOSTER, 2021).

Another possible origin of the strain 16/02, although there is no epidemiological or molecular evidence, since very few strains of *B. abortus* biovar 4 were genotyped by MLST or MLVA16, could be *B. abortus* biovar 4 in neighboring countries, such as Argentina, where this biovar has already been found (LUCERO et al., 2008). However, it is important to emphasize that these strains identified and classified as *B. abortus* biovar 4 were not genotyped, making it impossible to trace their origin.

Regarding the transmission chain associated with the 128/11 strain isolated in Pará, it is

difficult to state a hypothesis for the origin of the strain based on MLST and MLVA results, due to the scarcity of available data. MLST data of the *B. abortus* biovar 4 strain Ba col-B012 isolated in Colombia (TORRES HIGUERA et al., 2019), which is at the northern Brazilian border, on the contrary, indicate the isolates are unrelated.

Considering the different results obtained between MLST and MLVA, it is important to highlight that these techniques characterize the isolates at different levels of resolution, being the MLST based on polymorphism observed in conserved portions of the genome, while MLVA is built by a set of VNTRs (non-coding regions). Thereby, they are complementary from an epidemiological point of view. Moreover, it is important to mention that the identification *B. abortus* biovar 4 isolates is uncommon, despite the easy availability of serological data on bovine brucellosis in Brazil and worldwide. This is probably due to the largely limited data on its etiological agent, considering the complexity of handling, as it is a level 3 agent, leaving information on the prevalent species and biovars of *Brucella* obscure. Additionally, the complementation of these findings with genotypic analysis by MLST or MLVA is even rarer. In fact, only seventeen sequence types (thirteen available in PubMLST and four obtained from whole genome sequencing by NCBI) and three MLVA16 genotypes are available for *B. abortus* biovar 4 strains (Table 2). Although HIGGINS et al. (2012) also genotyped *B. abortus* biovar 4 isolates, they used different VNTR *loci* (HOOF-Print1; HOOF-Print3; HOOF-Print4; HOOF-Print8; VNTR2; VNTR5A; VNTR5B; VNTR16; VNTR17; VNTR21) precluding any comparison among their and other studies.

The identification and characterization of *Brucella* species and biovars that affect animals and humans is of fundamental importance to understand the epidemiological situation of the brucellosis, allowing the improvement of control and eradication strategies.

CONCLUSION

The typing of *B. abortus* strains isolated from cattle in Brazil confirmed the occurrence of *B. abortus* biovar 4 in the country, providing support for surveillance of the pathogen within the program for the control and eradication of bovine brucellosis in the country.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHOR'S CONTRIBUTION

RSA wrote the paper; EMSD and APL conceived and critically reviewed the manuscript for important intellectual content; SM, PGS and MC performed research; PMSF, CRP and AAFJ did the analysis and interpretation of data.

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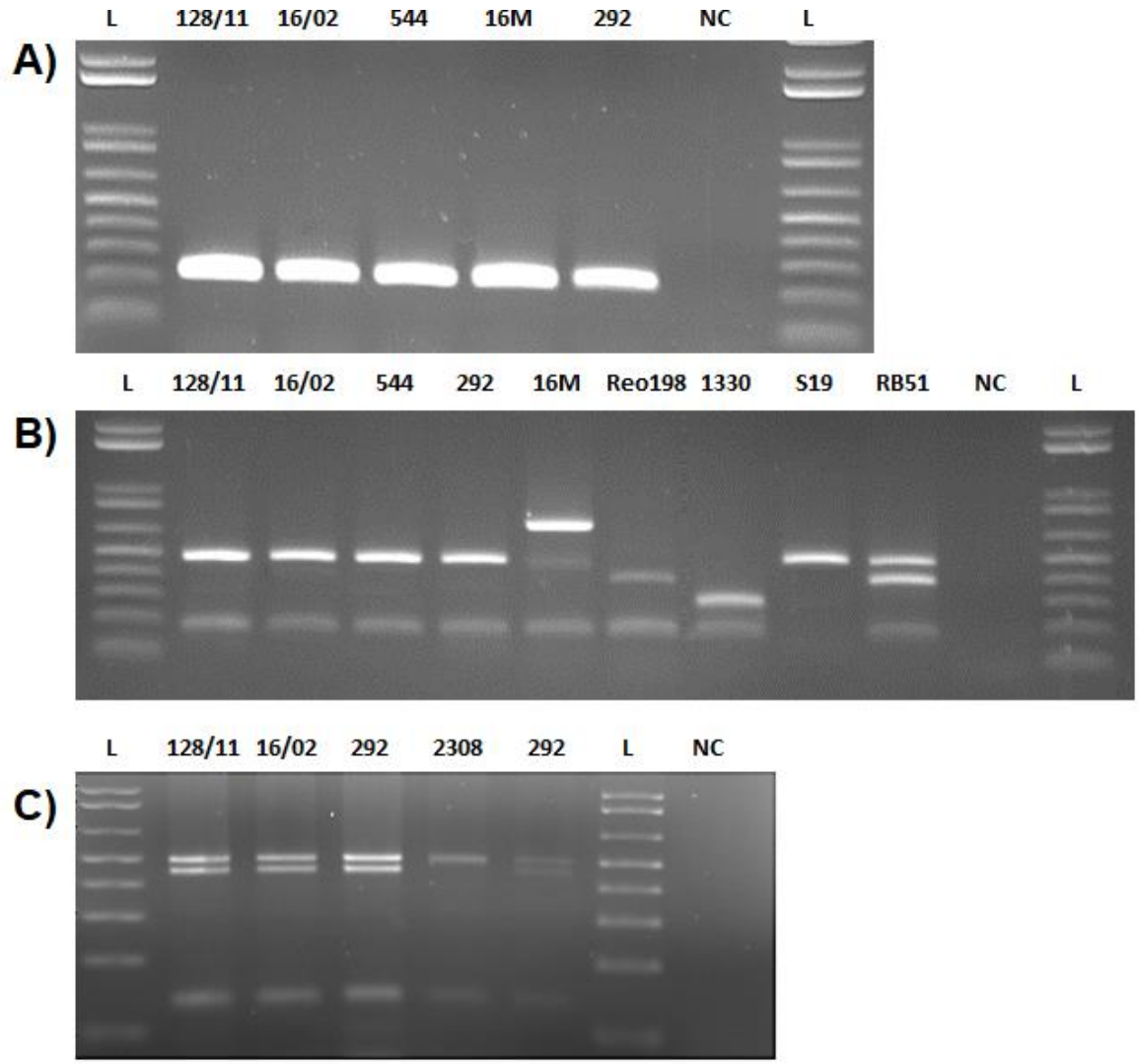

Figure 1 (A) Agarose 1% gel showing PCR amplification for *bcsp31* gene (*Brucella* genus specific) stained with ethidium bromide (0.5 mg / mL). Lanes L - 1Kb plus DNA Ladder molecular weight marker (Invitrogen, USA); lanes 2 e 3 - field isolates 128/11 e 16/02, respectively; lanes 4, 5 and 6 - positive controls *B. abortus* biovar 1 544, *B. melitensis* biovar 1 16M, *B. abortus* biovar 4 292, respectively; NC - negative control. (B). Agarose 1% gel showing amplification of ethidium bromide stained AMOS-enhanced-PCR (0.5 mg / mL). Lanes L - molecular weight marker 1Kb plus DNA Ladder (Invitrogen, USA); lanes 2 and 3 - field isolates 128/11, 16/02, respectively; lane 4 - *B. abortus* biovar 1,544; lane 5 - B. *abortus* biovar 4 292; lane 6 - *B. melitensis* biovar 1 16M; lane 7 - *B. ovis* Reo 198; lane 8 - *B. suis* biovar 1 1330; lane 9 - *B. abortus* biovar 1 S19; lane 10 - *B. abortus* biovar 1 RB51; NC - negative control. (C) Restriction patterns of the PCR-amplified *omp2b* gene digested with *TaqI* enzyme. Lanes L - 1Kb plus molecular weight marker (Invitrogen, USA), lane 2 - *B. abortus* strain 128/11, lane 3 - *B. abortus* strain 16/02, lane 4 = B. *abortus* biovar 4 292; 5 = *B. abortus* biovar 1 2308; laen 6 - *B. abortus* biovar 4,292 (ATCC 23451); NC - Negative control

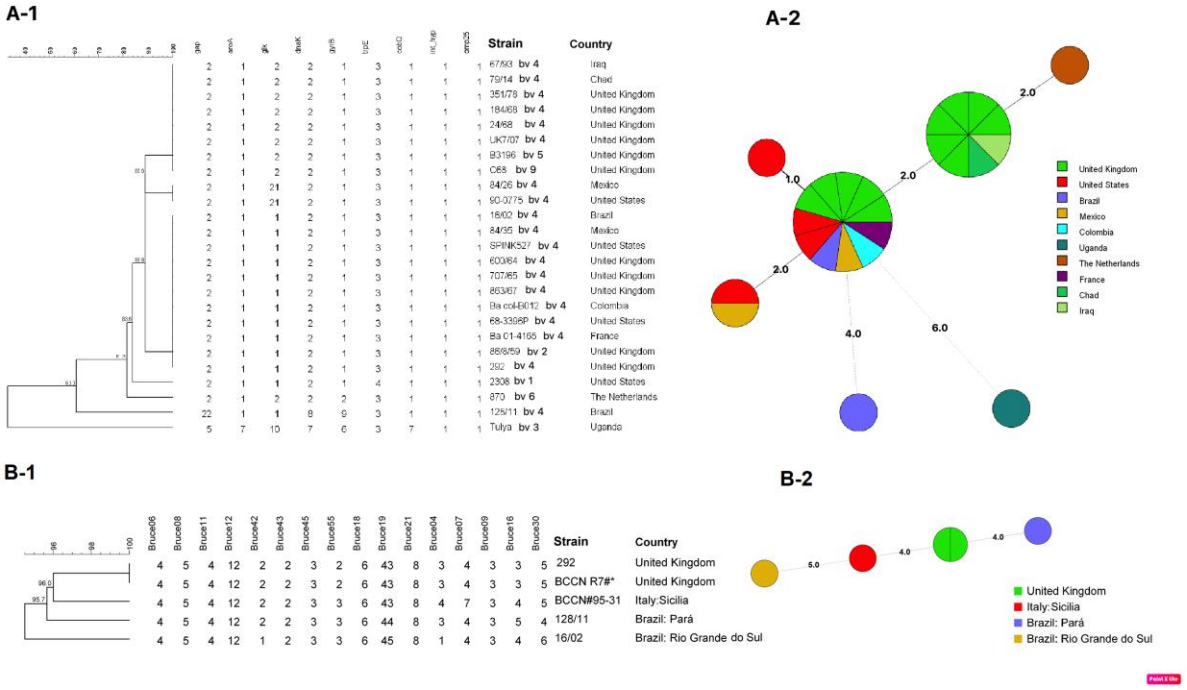

Figure 2 Cluster analyzes of *Brucella abortus* biovar 4 by molecular typing methods MLST and MLVA with the aid of the Bionumerics software (version 7.6, Applied-Maths, Belgium). A-1) Dendrogram based on the MLST genotyping test showing relationships of *B. abortus* reference strains for each biovar and other *B. abortus* biovar 4 strains (thirteen available in PubMLST and four obtained from whole genome sequence in the NCBI) and the two isolates from this study (128/11 and 16/02). A-2) Minimal Spanning Tree (MST) analysis of *B. abortus* strains using MLST data. B-1) Dendrogram based on MLVA16 for all three *B. abortus* biovar 4 MLVA16 genotypes (BCCN # 95-31, BCCN R7 # * and 292 ATCC 23451) available at MLVAbank 2020 and the two isolates from this study (128/11 and 16/02). B-2) MST analysis of *B. abortus* biovar 4 isolates using MLVA16 data.

Table 1 Growth characteristics of *Brucella abortus* isolated from cervical ligament lesions and lymph nodes from cattle slaughtered in Rio Grande do Sul and Pará, Brazil, in 2002 and 2011.

Strain	CO_2 requirement	H_2S production	Growth on dyes			Agglutination in sera	
			Thionin (20 µg/mL)	Thionin (40 µg/mL)	Basic fuchsin (20 µg/mL)	Anti-A	Anti-M
16/02	+	+	-	-	+	-	+
128/11	+	+	-	-	+	-	+
B. abortus biovar 4 292 = ATCC[1] 23451	+	+	-	-	+	-	+
B. abortus biovar 1 544 = ATCC	+	+	-	-	+	+	-

[1] ATCC = American type culture collection

Table 2 Information on *B. abortus* biovar 4 isolates available in databases (PubMLST, NCBI, PATRIC and MLVABank)*

Method	Strain	Host	Species	Biovar	Country	Continent	Year[1]	Plataform
MLST	84/35	Human	*B. abortus*	4	Mexico	North America	1984	PubMLST
MLST	SPINK527	Unknown	*B. abortus*	4	United Kingdom	North America	1951	PubMLST
MLST	600/64	Bovine	*B. abortus*	4	United Kingdom	Europe	1964	PubMLST
MLST	707/65	Bovine	*B. abortus*	4	United Kingdom	Europe	1965	PubMLST
MLST	863/67	Bovine	*B. abortus*	4	United Kingdom	Europe	1967	PubMLST
MLST	84/26	Human	*B. abortus*	4	Mexico	North America	1984	PubMLST
MLST	67/93	Buffalo	*B. abortus*	4	Iraq	Asia	1967	PubMLST
MLST	79/14	Bovine	*B. abortus*	4	Chad	Africa	1979	PubMLST
MLST	351/78	Unknown	*B. abortus*	4	United Kingdom	Europe	1978	PubMLST
MLST	184/68	Unknown	*B. abortus*	4	United Kingdom	Europe	1968	PubMLST
MLST	24/68	Bovine	*B. abortus*	4	United Kingdom	Europe	1968	PubMLST
MLST	UK7/07	Human	*B. abortus*	4	United Kingdom	Europe	2007	PubMLST
MLST	Ba 01-4165	Bovine	*B. abortus*	4	France	Europe	Unknown	PubMLST
MLST	2308	Unknown	*B. abortus*	1	United States	North America	2008	PubMLST
MLST	86/8/59	Bovine	*B. abortus*	2	United Kingdom	Europe	1959	PubMLST
MLST	Tulya	Human	*B. abortus*	3	Uganda	Africa	1958	PubMLST
MLST	B3196	Bovine	*B. abortus*	5	United Kingdom	Europe	1959	PubMLST
MLST	870	Bovine	*B. abortus*	6	The Netherlands	Europe	1959	PubMLST
MLST	C68	Bovine	*B. abortus*	9	United Kingdom	Europe	1958	PubMLST
MLST	Ba col-B012	Bovine	*B. abortus*	4	Colombia	South America	1997	NCBI and PATRIC

MLST	68-3396P	Unknown	*B. abortus*	4	USA	North America	1968	NCBI and PATRIC
MLST	90-0775	Bovine	*B. abortus*	4	USA	North America	1990	NCBI and PATRIC
MLST	01/65	Bovine	*B. abortus*	4	France	Europe	Unknown	NCBI and PATRIC
MLVA	BCCN R7#*	Bovine	*B. abortus*	4	United Kingdom	Europe	Unknown	MLVABank
MLVA	BCCN#95-13	Bovine	*B. abortus*	4	Italy: Silicia	Europe	1995	MLVABank
MLST/MLVA	292 ATCC[2] 23451	Bovine	*B. abortus*	4	United Kingdom	Europe	1961	PubMLST/ MLVABank
MLST/MLVA	128/11	Bovine	*B. abortus*	4	Brazil: Pará	South America	2011	This study
MLST/MLVA	16/fev	Bovine	*B. abortus*	4	Brazil: Rio Grande do Sul	South America	2002	This study

[1] Year of isolation;
[2] ATCC = American type culture collection
* PubMLST database (https://www.pubmlst.org /), NCBI plataform (National Center for Biotechnology Information) - (https://www.ncbi.nlm.nih.gov/), PATRIC (Pathosystems Resource Integration Center) - (https://patricbrc.org/job/) and MLVABank (http://mlva.i2bc.paris-saclay.fr/brucella/). -

GENERAL CONSIDERATIONS

The works that composed this thesis address interconnected themes in which we can conclude among them that:

1. Among the most used serological tests for the diagnosis of bovine brucellosis worldwide, those that showed the best DSe and DSp were the iELISA (BS) and the FPA, respectively. DSp and particularly DSe were overestimated due to the case-control study design used in most studies involving the validation of diagnostic tests for bovine brucellosis, which must be take into account in their application in disease control and eradication programs.

2. The conventional serological tests used in the diagnosis of bovine brucellosis evaluated showed conditional dependence, emphasizing the importance of considering the covariance of the tests in their validation and proposed use in effective diagnostic strategies. The tests that showed better DSe and DSp were BPAT and FPA, respectively.

3. iELISA_SOD exhibited could be used for the diagnosis of infected animals, increasing the range of serological tests available for the diagnosis of bovine brucellosis, with the advantage of being free of S-LPS. In contrast, the iELISA_MDH showed low usefulness as diagnostic test for bovine brucellosis, as well as for differentiate infection from vaccination.

4. *Brucella* spp. it is not present in ovaries of seropositive bovines for brucellosis, suggesting the safety of the use of reproductive biotechnologies from the follicular aspirate of these animals and for the laboratorians who will handle these oocytes. In addition, the results also indicate that genetically superior animals diagnosed with brucellosis can be oocyte donors for IVP, potentially not transmitting the disease to the recipient and to the fetus.

5. The typing of *B. abortus* strains isolated from cattle in Brazil confirmed the occurrence of *B. abortus* biovar 4 in the country, providing subsidies for the surveillance of the pathogen within the program for the control and eradication of bovine brucellosis in the country.