Accuracy of individual serological tests for the diagnosis of bovine brucellosis and covariance of paired-test combinations

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Abstract

Diagnostic sensitivity (DSe) and diagnostic specificity (DSp), and conditional dependence (C^+ and C^-) were estimated using a Bayesian approach for seven serological tests used in the diagnosis of bovine brucellosis. Serum samples divided into six groups: group 1 – 52 serum samples from culture-positive animals; group 2 – non-vaccinated animals (28 serum samples) positive in RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test) selected from herds with an ongoing history of brucellosis; group 3 – 32 sera samples from animals from a brucellosis-free area; group 4 – 114 sera from animals vaccinated with S19 from properties without a history of brucellosis, collected on days 28, 56 and later points (average of 688 ± 406 days) post vaccination; group 5 – 60 serum samples from animals vaccinated with RB51 from properties without a history of brucellosis, 28 and 56 days after vaccination; and group 6 – 42 serum samples from animals inoculated with *Yersinia enterocolitica* group O:9 at 7, 14, 21, 28, 35, 42 and 49 days after inoculation, were tested in parallel by RBT, 2ME, FPA (fluorescence polarization assay), BPAT (Buffered plate antigen test), iELISA_IDEXX (indirect enzyme-linked immunosorbent assay), iELISA_SOD (Superoxide dismutase [Cu-Zn]) and CFT (complement fixation test). The test that exhibited the best DSe was iELISA_SOD [64.55% (95% credibility interval (95% CI): 56.92 – 73.23%)] and the test with the best DSp was FPA [84.02% (95% CI: 80.51 – 87.98%)]. Conditional dependency was exhibited in sensitivity (C^+) in 76% (16/21) and in specificity (C^-) in 81% (17/21) of paired-test assessed combinations, with FPA and iELISA_IDEXX (0.2148) and BPAT and RBT (0.1326) showing the highest covariance C^+ and C^-, respectively. The results demonstrated the conditional dependence between the most adopted serological tests for the diagnosis of bovine brucellosis on a global scale. Furthermore, they provided a solid estimate of these covariances, which are essential for developing effective and accurate diagnostic strategies that involve the use of multiple tests.

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

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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 and 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). Therefore, serological tests offer a more practical means for the diagnostic of bovine brucellosis, with several ones already described and used worldwide (Nielsen and Yu, 2010).

In control and eradication programs, as no test offer a complete certainty 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) (Godfroid et al., 2002; Poester et al., 2010) . Diagnostic tests can also be used in parallel strategy to increase the diagnostic sensitivity, reducing the false negative results (Dohoo et al., 2012). Nonetheless, the implementation of those strategies must take into consideration the possible conditional dependence of the tests, although in most cases they erroneously considered the tests as independent, even when they measure similar biological processes. Indeed, when the conditional dependency of the tests is neglected, this may substantially alter 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 compared with those obtained considering conditional dependence (Thibodeau, 1981; Gardner et al., 2000).

Therefore, determining the covariance, the statistical measure of the conditional dependence of two variables (Gardner et al., 2000), 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 first to estimate the DSe and DSp of the conventional serological tests routinely used in the diagnosis of bovine brucellosis and second their covariance (C^+ and C^-), in order to support their best use (pairwise) in control and eradication programs. The DSe and DSp of paired combination of bovine brucellosis serological tests will be assessed hereafter.

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 six groups of bovines were used, divided into two panels. The first panel consisted of groups 1 and 2, composed of sera from animals classified as positive. The second panel was composed of groups 3, 4, 5, and 6, from sera classified as negative. The groups were composed by: group 1 – naturally infected animals (52 serum samples), from animals with positive culture for *B. abortus*, belonging to the serum bank from Laboratório Federal de Defesa Agropecuária (LFDA - Pedro Leopoldo, Minas Gerais, Brazil) from Ministério da Agricultura e Pecuária (MAPA - Ministry of Agriculture and Livestock, Brazil); group 2 – non-vaccinated animals (28 serum samples) selected from herds with brucellosis history, and that were positive in RBT (Rose Bengal test) and 2ME (2-mercaptoethanol test) tests; group 3 – animals negative for brucellosis (32 serum samples), selected from brucellosis-free herds in the state of Santa Catarina, Brazil (Dorneles et al., 2014), where S19 vaccination is prohibited, kindly provided by Companhia Integrada de Desenvolvimento Agrícola de Santa Catarina (CIDASC); group 4 – calves vaccinated with S19 (114 serum samples), between 3 and 8 months of age, collected at days 28, 56 and later points (average of 688 ± 406 days) post vaccination, from properties without a history of bovine brucellosis; group 5 – calves vaccinated with

RB51 (60 serum samples), between 3 and 8 months, 28 and 56 days post-vaccination; group 6 – serum from heifers older than 24 months of age inoculated with *Yersinia enterocolitica* group O:9 (reference strain YE 383 – NCTC 11174, 3 x 10^{10} colony forming units/animal inactivated with β-propiolactone) (42 serum samples) sampled at 7, 14, 21, 28, 35, 42 and 49 days after inoculation (Corbel and Cullen, 1970). All sera were stored at -20 ºC until the tests. The experimental blood sampling was approved by the ethics committees on animal experimentation of Universidade Federal de Lavras - UFLA or Universidade Federal de Minas Gerais - UFMG under the protocols CETEA 139/2010, CEUA 069/2018 and CEUA 024/2021.

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 showed absence of agglutination were considered negative and presence of partial or total agglutination considered positive.

The CFT (complement fixation test), RBT (Rose Bengal test), 2ME (2-mercaptoethanol test) were also performed based on the procedures described by Alton et al. (1988). The CFT was carried out at the Vet Vida Laboratory (Cuiabá, Mato Grosso, Brazil, PGF:000014.0130228/2020), authorized and accredited by MAPA. 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. In the 2ME tests, samples were considered positive when complete agglutination was observed at dilution ≥ 1:25 (Brasil, 2017).

The FPA (fluorescence polarization assay) was performed as described by Nielsen et al. (1996). The test was performed using the *Brucella* S Antibody Test Kit (B1001BRA, Ellie LLC, USA) and the Ellie Sentry 201 handheld fluorescence polarized (FPA) reader (Ellie Technical Notes, single tube reader, Germantown, USA). The tests were carried out at 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 (Brasil, 2017). Seropositivity was set at > Δ20 mP, according to manufacturer 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 Andrade et al. (2023b). Recombinant SOD [Cu-Zn] protein was commercially synthesized 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 (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 of 0.3945 OD_{450} units.

2.3 Statistical analysis

DSe and DSp were estimated for the seven tests: RBT, 2ME, FPA, BPAT, iELISA_IDEXX, iELISA_SOD and CFT, as well as pairwise sensitivity (C^+) and specificity (C^-) 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) distribution 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). The pairwise covariances for C^+ and C^- were estimated considering the DSe and DSp obtained for all groups taken together. Data analysis was performed using R software version 4.2.2 (Team, 2021) with aid of the packages 'R2jags' (Su and Yajima, 2021) and 'coda' (Plummer et al., 2006).

3. Results

A total of 328 individual serum samples of six populations were tested in parallel for detection of *Brucella* spp. specific antibodies by RBT, 2ME, FPA, BPAT, iELISA_IDEXX, iELISA_SOD and CFT. Bayesian estimation was performed using dichotomized tabulated combination of results from seven different tests. Test result pattern of 328 individual sera samples from the dichotomous result of seven tests for bovine brucellosis is shown in Table 1. The number of possible result combinations for the seven tests are 128 (2^7), however, only 45 were observed in the tested population (Table 1). Among the 328 serum samples analyzed 33 were positive in all tests (10.1%), while 26.5% (87/328) tested negative in all brucellosis tests.

3.1 DSe and DSp of individual tests

Bayesian DSe and DSp estimates and their respective 95% credibility interval (CI) for the whole studied population, not considering the group subdivision, are shown in Table 2. Among the seven tests, those that showed the best DSe were iELISA_SOD [64.55% (95% CI: 56.92 – 73.23%)], BPAT [64.06% (95% CI: 58.36 – 69.99%)] and RBT [61.82% (95% CI: 56.33 – 66.76%)]. For DSp, the tests that exhibited better results were FPA [84.02% (95% CI: 80.51 – 87.98%)], 2ME [83.14% (95% CI: 79.74 – 87.49%)] and iELISA_IDEXX [82.81% (95% CI: 77.12 – 88.25%)].

The DSe and DSp estimates for the seven tests according to the six groups are shown in Table 3. Based on the panel 1 groups (sera from animals classified as positive), specifically group 1 consisting of naturally infected animals, the DSe between the tests varied from 68.46% (CI 95%: 57.12 – 80.65%) to 96.87% (95% CI: 92.17 - 99.99%). The tests that exhibited the best DSe were FPA [96.87% (95% CI: 92.17 – 99.99%)], BPAT [96.85% (95% CI: 91.76 – 99.99%)] and RBT [96.57% (95% CI: 91.11 – 99.97%)]. In group 2 (non-vaccinated positive animals), the tests that showed better DSe were BPAT [95.27% (95% CI: 86.99 – 99.99%)] and 2ME [94.38% (95% CI: 85.86 – 99.95%)]. Whereas in panel 2 (sera from animals classified as negative), specifically in group 3, DSp ranged from 84.63% (95% CI: 72.69 – 98.09%) to 97.21% (95% CI: 92.37 – 99.99%). The tests that showed the best performance were RBT [97.21% (95% CI: 92.37 - 99.99%)] and BPAT [97.17% (95% CI: 92.58 – 99.93%)]. In group 4 (sera from animals vaccinated with S19), the tests that showed the best DSp were FPA [89.40% (95% CI: 80.46 – 97.51%)] and 2ME [88.17% (95% CI: 78.57 – 96.04%)]. It is worth noting that antibodies induced by the S19 vaccination could be detected years after vaccination in some animals, particularly by the iELISA_SOD (Supplementary Figure 1). In group 5 (vaccinated with RB51), iELISA_IDEXX [98.14% (CI 95%: 94.61 – 100.00%)] and FPA [97.63% (95% CI: 92.73 – 100.00%)] showed better DSp. In group 6 (animals inoculated with *Y. enterocolitica* group O:9), the FPA [97.34% (95% CI: 92.32 – 100.00%)] and 2ME [93.05% (95% CI: 80.54 – 99.95%)] exhibited the best DSp among the seven serological tests.

3.2 Covariance

Analyses of the pairwise covariances of sensitivity (C^+) and specificity (C^-) are detailed in Table 4. Mean estimates of C^+ ranged from 0.2148 to -0.1204, with the combination of FPA and iELISA_IDEXX tests (0.2148) with the highest covariance and 2ME and iELISA_SOD (-0.1204) with the lowest covariance. Likewise, mean estimates of C^- ranged from 0.1326 to -0.0182, with the combination of BPAT and RBT tests (0.1326) with the highest covariance and CFT and iELISA_SOD (-0.0182) with the lowest covariance.

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 approach in the diagnosis of bovine brucellosis but carelessness in assuming conditional independence of diagnostic tests even when they are based on the same biological process can lead to biased estimates due to an underestimation of classification errors (Thibodeau, 1981; Gardner et al., 2000). Given that, one of the aims of this study was to estimate the pairwise covariance (conditional dependence) of seven serological tests used for the diagnosis of bovine brucellosis, beside their individual DSe and DSp using a Bayesian approach. Our results confirmed the hypothesis of conditional dependence between the tests and showed FPA and iELISA_IDEXX (0.2148), and RBT and BPAT (0.1326), as the tests with higher conditional dependences in sensitivity (C^+) and specificity (C^-), respectively.

Regarding the characteristics of the tests, four of them (RBT, 2ME, BPAT and CFT) are based on the detection anti-*Brucella* smooth lipopolysaccharide (S-LPS) antibodies (Nielsen and Yu, 2010), whereas the FPA test detects anti-*Brucella* O-chain antibodies (Nielsen et al., 1996), iELISA_SOD detects anti-SOD [Cu-Zn] *Brucella* antibodies (Faria et al., 2020) and the iELISA_IDEXX test detects antibodies against *B. abortus* although it does not inform which antigen(s) is(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), 16 (76%) in C^+ and 17 (81%) in C^- combinations between pairs of the seven assessed tests were within this interval, showing conditional dependence. Conditional dependence was greater in C^+, which may be logical considering that most of the tests used to diagnose bovine brucellosis detect anti-LPS, part of LPS or the O-chain antibodies, and thereby they measure similar biological process, exhibiting a direct relationship among the proportion of false negative results. In opposite, the false positive results may be caused by infection with several other Gram-negative bacteria that can induce cross-reactive antibody responses or by antibody induced by S19 vaccination (Kittelberger et al., 1998; Dorneles et al., 2015). The test combinations that exhibited conditional independence, in both C^+ and C^-, are tests that have more differences in the assessed

biological process (Table 4). Among these combinations, iELISA_SOD is present in four (C^{+}) and two (C^{-}), suggesting that this test could give a gain in the DSe or DSp when used in parallel or series strategies for bovine brucellosis diagnosis together with other conventional tests, even though it did not exhibit 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 S-LPS antigen, which also contribute to minimize cross-reaction with other Gram-negative bacteria (Andrade et al., 2023b). 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 C^{+} were FPA and iELISA_IDEXX (0.2148), RBT and 2ME (0.2124), RBT and BPAT (0.2084), respectively. These results were not unexpected, since, except for iELISA_IDEXX for which the antigen is undisclosed by the manufacturer, all the other pair of tests use very similar antigens. The conditional dependence observed for the RBT and 2ME tests can be justified because both tests use whole-cell antigens from *B. abortus* (strain S1119-3) and focus on the detection of anti-IgG antibodies (Alton et al., 1988). However, this scenario raises concerns regarding the efficiency of the diagnosis strategy adopted by some bovine brucellosis control and eradication programs. This is because some countries adopt the serial testing strategy, in which RBT is used as the screening test, and seroreactive animals are subsequently confirmed by the 2ME test (Saravi et al., 1995; Brasil, 2006). The present results indicates that this procedure would be suboptimal and, in turn, would leads to an increase in costs and time required for controlling and eradicating bovine brucellosis in the country. Likewise, RBT and BPAT tests are classified within the same group of tests, as both use buffered *Brucella* antigen and have similar execution procedures (Alton et al., 1988), although they are introduced as different screening tests in many countries. Indeed, the logic of both tests are very similar, the cell antigen of *B. abortus* S99 or S1119-3, are stained with Rose Bengal (RBT) or Brilliant Green and Crystal Violet (BPAT), and are suspended in a buffer to final pH of 3.65 (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 and Yu, 2010). Furthermore, it is very likely that the antigen used in iELISA_IDEXX is composed by S-LPS or part of S-LPS, which would justify the higher covariance observed between this tests and the other assessed tests, which also uses the O chain of LPS, as observed for FPA (Nielsen et al., 1996).

For the covariances in C^-, the highest covariances in the combinations of tests were in the RBT and BPAT (0.1326), RBT and 2ME (0.1311) and RBT and CFT (0.1262). For the RBT – 2ME and RBT – BPAT, the same reasons already given for explain the covariance in the infected animals (C^+), could be used to justify the high covariance among non-infected animals (C^-). For the high covariance in C^- in the combination of RBT and CFT, the explanation could also be that both tests use as antigens *B. abortus* whole cells and are focused in detecting anti-S-LPS antigens. In fact, several steps used in the production of antigens from both tests are shared and performed from the same strain (S1119-3) (Alton et al., 1988). This dependency is within the same range observed by others when applying similar test combinations to diagnose swine brucellosis (Gardner et al., 2000; Mainar-Jaime et al., 2005). This suggests that the dependence of C^- between these two serological tests (RBT and CFT) remains consistent regardless of the animal species considered. It should be noted that albeit these were the highest covariances observed in non-infected animals, they were approximately half than those observed for C^+.

A Bayesian analysis with the latent class model was used to estimate the DSe and DSp of the seven bovine brucellosis serological tests in six different populations. Considering the whole population on six groups, the DSe values as well as the DSp values were significantly lower than those estimates found in a recent meta-analysis conducted by our research group (Andrade et al., 2023a). The underestimation in the DSe in this study maybe due to the low prevalence of brucellosis in the entire analyzed population, since only two groups, among the six assessed, were from positive animals. The underestimation in the DSp was smaller for the whole population than that observed for

DSe, and the DSe estimates for the group 1 (naturally infected animals) was significantly higher compared to other groups for the majority of the tests (Table 3). Other studies using Bayesian models also achieved similar results, especially in DSe (Muñoz et al., 2012; Getachew et al., 2016; Ahasan et al., 2017; Arif et al., 2018), when using models considering tests as conditionally dependent.

Overall, iELISA_SOD was the test that showed the best performance in DSe (64.55%), followed by BPAT 64.06% and RBT 61.82%. A possible explanation for this performance may be related to the fact that iELISA_SOD is capable of detecting all IgG immunoglobulin isotypes, while BPAT and RBT cannot due to the low final pH of the reaction (3.65), which allow the identification mainly of IgG1 (Alton et al., 1988; Poester et al., 2010). BPAT and RBT tests are traditionally considered to have high DSe and thereby used as a screening test by many countries (WOAH, 2018). On the other hand, the test that showed the best DSp was the FPA 84.02%, used in brucellosis control and certification programs in North America and European Union (Godfroid et al., 2010). FPA was also the test with best DSp in the recent conducted metanalysis (Andrade et al., 2023a).

Considering the DSe estimates in the group 1 of naturally infected animals, the tests that showed better performances were FPA (96.87%), BPAT (96.85%) and RBT (96.57%), while CFT (68.86%) and 2ME (68.46%) were the tests with the worst performances. These finding for DSe were also similar to those observed in the metanalysis performed to assess the accuracy of serological tests for bovine brucellosis diagnosis (Andrade et al., 2023a). Likewise, the DSp estimates for the group 3 (negative animals), indicated that RBT (97.21%), BPAT (97.17%) and FPA (97.15%) were 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 non-infected populations (Table 4 and supplementary Figure S1).

Overall, the present study showed the conditional dependence of serological tests that are the most used for diagnosis of bovine brucellosis worldwide and provided robust estimation of these covariances. These are essential prerequisites in order to be able to design efficient and assertive diagnostic strategies employing multiple tests (in parallel or in series). However, the most suitable combination(s) of tests should be assessed considering different prevalence scenarios, the objectives of the control program, the budget available and the timeline to achieve these objectives. In this perspective, there will be no single combination of tests suited for all situations.

5. Conclusion

The tests that showed better DSe and DSp were FPA and RBT, respectively. The FPA was the test with best accuracy, given its DSp for vaccinated animals and for the animals inoculated with *Y. enterocolitica* O:9. 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 use in multiple tests diagnostic strategies. This warrants further assessment according to the epidemiological situation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ahasan, M.S., Rahman, M.S., Rahman, A.A., Berkvens, D., 2017. Bovine and Caprine Brucellosis in Bangladesh: Bayesian evaluation of four serological tests, true prevalence, and associated risk factors in household animals. Tropical Animal Health and Production 49, 1-11.

Alton, G.G., Jones, L.M., Angus, R., Verger, J., 1988. Techniques for the brucellosis laboratory. Institut National de la recherche Agronomique (INRA).

Andrade, R.S., de Oliveira, M.M., de Sousa Bueno Filho, J.S., Ferreira, F., Godfroid, J., Lage, A.P., Dorneles, E.M.S., 2023a. Accuracy of serological tests for bovine brucellosis: a systematic review and meta-analysis. Preventive Veterinary Medicine, 106079.

310 Andrade, R.S., Faria, A., Andrade, H., Mansur, H.S., Mansur, A.A.P., Lage, A., Dorneles, E.M.S.,
311 2023b. Use of Recombinant Malate Dehydrogenase (MDH) and Superoxide Dismutase
312 (SOD)[Cu-Zn] as Antigens in Indirect ELISA for Diagnosis of Bovine Brucellosis. Journal of
313 Microbiology Methods.
314 Arif, S., Heller, J., Hernandez-Jover, M., McGill, D.M., Thomson, P.C., 2018. Evaluation of three
315 serological tests for diagnosis of bovine brucellosis in smallholder farms in Pakistan by
316 estimating sensitivity and specificity using Bayesian latent class analysis. Preventive
317 Veterinary Medicine 149, 21-28.
318 Brasil, 2006. Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal
319 (PNCEBT). In: técnico., M. (Ed.), Ministério de Agricultura, Pecuária e Abastecimento.,
320 Brasília, 184.
321 Brasil, 2017. Instrução Normativa nº 10, de 3 de Março de 2017. Regulamento Técnico do Programa
322 Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal. . Ministério da
323 Agricultura, Pecuária e Abastecimento - MAPA., Diário Oficial da União, 20 Jun 2017.
324 Chisi, S.L., Marageni, Y., Naidoo, P., Zulu, G., Akol, G.W., Van Heerden, H., 2017. An evaluation
325 of serological tests in the diagnosis of bovine brucellosis in naturally infected cattle in
326 KwaZulu-Natal province in South Africa. Journal of the South African Veterinary Association
327 88, 1-7.
328 Corbel, M., Cullen, G., 1970. Differentiation of the serological response to *Yersinia enterocolitica*
329 and *Brucella abortus* in cattle. Epidemiology & Infection 68, 519-530.
330 Dohoo, I.R., Martin, S.W., Stryhn, H., 2012. Methods in epidemiologic research.
331 Dorneles, E.M., Teixeira-Carvalho, A., Araújo, M.S., Lima, G.K., Martins-Filho, O.A.,
332 Sriranganathan, N., Lage, A.P., 2014. T lymphocytes subsets and cytokine pattern induced by
333 vaccination against bovine brucellosis employing S19 calfhood vaccination and adult RB51
334 revaccination. Vaccine 32, 6034-6038.

335 Dorneles, E.M.S., Teixeira-Carvalho, A., Araújo, M.S.S., Sriranganathan, N., Lage, A.P., 2015.
336 Immune response triggered by *Brucella abortus* following infection or vaccination. Vaccine
337 33, 3659-3666.
338 Faria, A.R., Dorneles, E.M.S., da Fonseca Pires, S., de Andrade, H.M., Lage, A.P., 2020.
339 Immunoproteomics of Brucella abortus reveals potential of recombinant antigens for
340 discriminating vaccinated from naturally infected cattle. Microbial pathogenesis 147, 104345.
341 Gardner, I.A., Stryhn, H., Lind, P., Collins, M.T., 2000. Conditional dependence between tests affects
342 the diagnosis and surveillance of animal diseases. Preventive veterinary medicine 45, 107-
343 122.
344 Getachew, T., Getachew, G., Sintayehu, G., Getenet, M., Fasil, A., 2016. Bayesian estimation of
345 sensitivity and specificity of rose bengal, complement fixation, and indirect ELISA tests for
346 the diagnosis of bovine brucellosis in Ethiopia. Veterinary Medicine International 2016.
347 Godfroid, J., Nielsen, K., Saegerman, C., 2010. Diagnosis of brucellosis in livestock and wildlife.
348 Croatian medical journal 51, 296-305.
349 Godfroid, J., Saegerman, C., Wellemans, V., Walravens, K., Letesson, J.-J., Tibor, A., Mc Millan,
350 A., Spencer, S., Sanna, M., Bakker, D., 2002. How to substantiate eradication of bovine
351 brucellosis when aspecific serological reactions occur in the course of brucellosis testing.
352 Veterinary microbiology 90, 461-477.
353 Kittelberger, R., Bundesen, P.G., Cloeckaert, A., Greiser-Wilke, I., Letesson, J.-J., 1998. Serological
354 cross-reactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9:: IV. Evaluation of
355 the M-and C-epitope antibody response for the specific detection of *B. abortus* infections.
356 Veterinary Microbiology 60, 45-57.
357 Mainar-Jaime, R.C., Muñoz, P.M., de Miguel, M.J., Grilló, M.J., Marín, C.M., Moriyón, I., Blasco,
358 J.M., 2005. Specificity dependence between serological tests for diagnosing bovine
359 brucellosis in Brucella-free farms showing false positive serological reactions due to Yersinia
360 enterocolitica O: 9. The Canadian Veterinary Journal 46, 913.

McDermott, J., Grace, D., Zinsstag, J., 2013. Economics of brucellosis impact and control in low-income countries. Revue scientifique et technique (International Office of Epizootics) 32, 249-261.

Muñoz, P.M., Blasco, J.M., Engel, B., de Miguel, M.J., Marín, C.M., Dieste, L., Mainar-Jaime, R.C., 2012. Assessment of performance of selected serological tests for diagnosing brucellosis in pigs. Veterinary Immunology and Immunopathology 146, 150-158.

Nielsen, K., Gall, D., Jolley, M., Leishman, G., Balsevicius, S., Smith, P., Nicoletti, P., Thomas, F., 1996. A homogeneous fluorescence polarization assay for detection of antibody to *Brucella abortus*. Journal of immunological methods 195, 161-168.

Nielsen, K., Yu, W., 2010. Serological diagnosis of brucellosis. Prilozi 31, 65-89.

Olsen, S.C., Stoffregen, W., 2005. Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert review of vaccines 4, 915-928.

Plummer, M., Best, N., Cowles, K., Vines, K., 2006. CODA: convergence diagnosis and output analysis for MCMC. R news 6, 7-11.

Poester, F.P., Nielsen, K., Ernesto Samartino, L., Ling Yu, W., 2010. Diagnosis of brucellosis. The Open Veterinary Science Journal 4.

Saravi, M., Wright, P., Gregoret, R., Gall, D., 1995. Comparative performance of the enzyme-linked immunosorbent assay (ELISA) and conventional assays in the diagnosis of bovine brucellosis in Argentina. Veterinary Immunology and Immunopathology 47, 93-99.

Su, Y.-S., Yajima, M., 2021. R2jags: Using R to Run 'JAGS'. R package version 0.6-1; 2020. URL https://CRAN. R-project. org/package= R2jags.

Team, R., 2021. A language and environment for statistical computing. In: R Foundation for Statistical Computing, V., Austria. (Ed.), http://www.R-project.org.

Thibodeau, L., 1981. Evaluating diagnostic tests. Biometrics, 801-804.

Wang, C., Lin, X., Nelson, K.P., 2020. Bayesian hierarchical latent class models for estimating diagnostic accuracy. Statistical methods in medical research 29, 1112-1128.

WHO, 2015. World Health Organization. The control of neglected zoonotic diseases: from advocacy to action: report of the fourth international meeting held at WHO Headquarters, Geneva, Switzerland, 19-20 November 2014. World Health Organization.

WOAH, 2018. World Organisation for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 - Measurement Uncertainty., OIE Terrestrial Manual 2018. 206-209.

WOAH, 2022. World Organisation for Animal Health. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*) (Infection with *B. abortus, B. melitensis* and *B. suis*). OIE Terrestrial Manual 2022. 355-398.

Zhang, N., Huang, D., Wu, W., Liu, J., Liang, F., Zhou, B., Guan, P., 2018. Animal brucellosis control or eradication programs worldwide: a systematic review of experiences and lessons learned. Preventive veterinary medicine 160, 105-115.