



Bayesian Assessment of the Accuracy of a PCR-Based Rapid Diagnostic Test for Bovine Tuberculosis in Swine

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Infection with the *Mycobacterium bovis* (*M. bovis*) causes a disease referred to as bovine tuberculosis (bTB), which affects a wide range of mammal hosts. Many countries have implemented control and eradication plans that have resulted in variable levels of efficacy and success. Although bTB is a notifiable disease in Argentina, and a control plan that targets cattle herds has been in place for decades, *M. bovis* is still prevalent in cattle, swine, and certain wild species. The aim of the paper here was to assess the sensitivity (Se), specificity (Sp), and positive and negative predictive values (PPV and NPV) of PCR from tissue, which is a test for rapid *M. bovis* detection in swine. Bacteriological culture was also performed for comparison purposes. A Bayesian approach was applied to estimate the accuracy of the diagnostic tests, PCR and bacteriological culture, in 266 swine samples with bTB-like lesions recovered during routine official inspections at slaughterhouses. A one-population model, assuming conditional dependence between test results, and incorporating prior information on the performance of the tests obtained from the literature, was used to estimate the tests Se and Sp. The accuracy of the combined (in parallel) application of both tests was also estimated. The Se of the PCR (82.9%) was higher than the Se of the bacteriological culture (79.9%), whereas the Sp of both tests was similar (88.5 and 89.0%, respectively). Furthermore, when both techniques were assessed in parallel, the Se of the diagnostic system increased substantially (Se = 96.6%) with a moderate Sp loss (Sp = 78.8%; PPV = 92.8%; NPV = 89%). Results suggest that the PCR, or the combined application of bacteriological culture and PCR, may serve as an accurate diagnostic tool to confirm bTB in swine samples. Results here will help the design and implementation of effective surveillance strategies for the disease in swine of Argentina and other settings in which the disease is prevalent.

Keywords: tuberculosis, swine, diagnosis, bacteriological culture, PCR, bayesian modeling

INTRODUCTION

Mycobacterium bovis (*M. bovis*) is a member of the *Mycobacterium tuberculosis* complex (MTC) (1). MTC members cause tuberculosis (TB) in a wide range of host species worldwide, and *M. bovis* is a major causative agent of bovine tuberculosis (bTB), holding a significant zoonotic potential (2). Globally, bTB prevalence is quite heterogeneous and somehow related to the social features of the setting, with the disease being endemic in most developing countries and eradicated from many developed regions (3). Factors suggested to have impaired the efficacy of bTB control programs include limited political willingness, resources scarcity, existence of wildlife reservoirs, and limitations of the available diagnostic tests (4–8).

There are many tests available and widely used to diagnose the disease (9, 10). Many bTB diagnostic tests have been applied for decades, and rely on a variety of biological principles, including the measurement of the cellular response in the host following the application of an intradermal test, the histopathology of postmortem specimens, or the bacteriological culture (BC) of the agent (11). Currently, there are many other testing strategies available, such as the interferon gamma assay, antibody-based assays, or the detection of bTB DNA by PCR, which presents new opportunities to improve or develop control plans, but for which there is still a need to gain understanding of their performance in field conditions (9, 12, 13). However, because those diagnostic techniques have values of sensitivity (Se) and specificity (Sp) that vary on each animal species and the specific epidemiological situation, estimation of the test accuracy is challenging (14–16).

In Argentina, bTB is endemic in both livestock and wildlife populations (17). The protein purified derivative (PPD) skin test and the meat inspection of carcasses at slaughterhouses are the actions approved and used in the bTB's Control and Eradication National plan (SENASA, Res.128/2012). However, PPD testing is compulsory only for dairy cattle, dairy goats, dairy sheep, and genetic nuclei and multipliers. Control activities are voluntary for other species, including swine.

Official records estimated that 0.3% of inspected pigs in Argentina showed TB-like lesions, as observed by the Argentine Animal Health Service (SENASA) inspectors at slaughterhouses. However, evidence suggests that the figure may have been underestimated (18–20).

The BC is considered the reference technique for bTB diagnosis, even though the Se of the test is only ~80% (10, 12), impairing its systematic application on disease control programs. Moreover, it is a laborious technique, which requires high biosecurity facilities and relatively specialized workforce for implementation. Also, because the technique depends on the agent's viability, preservation, and quality of the collected sample drastically affects the results (10, 21). Another key limitation is the relatively long turnaround time of the techniques (on average, between 2 and 3 months), which jeopardizes the ability to inform decision-makers on a timely manner (22, 23). For that reason, the BC has important limitations as a confirmatory test for the macroscopic inspection at slaughterhouses (Table 1).

The direct PCR analysis from tissue samples has been developed as an alternative technique to obtain a relatively fast confirmation of the infection. Direct PCR is believed to allow for the rapid, specific detection of *Mycobacteria*, and it is independent of the agent's viability on the sample. Some studies have reported the performance of the direct PCR in samples from cattle, buffaloes, humans, and some wildlife species (10, 14, 16). However, to the best of the authors' knowledge, the accuracy of the test is yet-to-be-assessed in swine (12).

The evaluation of diagnostic test performance, traditionally, has been based on the comparison of test results against a gold standard, allowing the assessment and validation of new techniques in comparison to a reference test. The limitation of such analytical approach is that the assumption of perfect Se and Sp of the reference test is, typically, questionable for many diseases, including bTB (21). Alternatively, Bayesian methods have been proposed as an analytical option to assess the accuracy of diagnostic tests without the requirement of a reference test (24). Bayesian methods have previously been used to estimate the accuracy of TB diagnostic techniques in bovine populations (8, 10, 25).

The aim of the study here was to estimate the Se and Sp of the BC, and of a rapid diagnostic test (PCR from tissue) on swine TB-like lesions obtained at slaughterhouses, and thereafter to evaluate the combined performance of those tests. Results will inform current discussions regarding the evaluation and potential modifications to the bTB control strategies in the target population and in the context of the Argentine disease control plan. Results may

TABLE 1 | Key features of both diagnostic tests that influence the feasibility of implementation in the context of a control plan.

Test Requirements	Bacteriological culture + identification by PCR	DNA extraction from tissue + identification by PCR
Laboratory biosafety	High: BSL-2. During all process. Special features: Viability and exponential amplification of the agent. Long process. High exposition risks.	High: BSL-2 For initial manipulation. Until extraction and inactivation of the remaining material.
Average turnaround time	2–3 months	3–4 days
Relative cost	1*	1.33 [#]
Trained personnel	Highly dependent on the operator skills (technique sensitivity varies with the level of skills and experience of the operator)	Average training requirements. Allows broader automation. PCR tests are more robust and versatile tests for labs settings.

Relative cost: based on the comparison between average costs provided by 3 local labs. *cost of *Mycobacteriology* culture includes: culture, Ziehl-Neelsen staining, and identification by PCR.

[#]cost of direct PCR from tissue samples, includes: extraction kit and PCR.

also be useful for countries in which bTB is prevalent in swine populations.

MATERIALS AND METHODS

Sample Collection

Swine samples ($n = 266$) showing bTB-like lesions (TBL) were collected in multiple visits to three slaughterhouses located in the Province of Buenos Aires between 2015 and 2017. Those three slaughterhouses processed pigs from the main productive region of Argentina, which includes the provinces of Buenos Aires, Córdoba, Santa Fe, La Pampa, and Entre Ríos. Approximately 4×4 cm cuts of lymph nodes showing bTB-like lesions were collected. Additionally, tissue samples from swine shipped from bTB-free premises were also collected in order to validate the DNA extraction and PCR assay.

All samples were stored at -20°C . BC and PCR were carried out at the Infectious Disease Department's Mycobacterial diagnosis laboratory of the Veterinary School of the University of Buenos Aires. Because samples were collected from animals inspected post-mortem by the national authority and according to national regulations, no ethical or farmer's consent approval was required.

Diagnostic Procedures

Preparation of the Samples for PCR and Culture

Samples (4–7 g) of each individual lymph node were placed into a mortar and crushed with sterile sand and 10 mL of sterile bi-distilled water for homogenization. Two milliliters of this homogenate were transferred into a 15 mL tube and 4 mL of 4% NaOH were added to decontaminate the sample using the Petroff's modified method described elsewhere (26). A portion ($\sim 400 \mu\text{L}$) of the homogenate was separated and frozen at -20°C for further DNA extraction.

DNA Extraction

Invitrogen™ PureLink™ Genomic DNA Mini Kit (Invitrogen, California, USA) was used for DNA extraction directly from tissue, according to the manufacturer's protocol. The obtained DNA was stored at -20°C until use for the PCR assay.

Bacteriological Culture

Bacteriological culture was performed following a protocol established elsewhere (26). Stonebrink and Löwenstein Jensen media were inoculated and incubated up to 60 days at 37°C and examined every 2 weeks. When bacterial growth was observed, Ziehl Neelsen staining was performed to observe acid fast bacilli, and, if positive staining, a loop full of bacteria was suspended in $200 \mu\text{L}$ of bi-distilled water and thermal lysis was performed at 95°C for 45 min. Lysates obtained were stored at -20°C until PCR assay.

Polymerase Chain Reaction (PCR) Assay for *M. bovis* Detection

PCR was conducted in both DNA extracted from tissue and colonies lysates, for the detection of the insertion sequence 6110 (IS6110) characteristic of the MTC (27). Positive (bTB-confirmed sample) and negative controls (bi-distilled water) were also

evaluated by the PCR. The primers used for the amplification, as well as PCR cycling conditions, have been described elsewhere (28). Colonies grown in Stonebrink media and IS6110-PCR positive were subject to spoligotyping to identify and to type the *M. bovis* isolates, following the protocol described by Kamerbeek et al. (29). Spoligotyping was carried out using the spoligotyping kit (Mapmygenome India). *M. tuberculosis* H37Rv (ATCC 27294) and *M. bovis* Bacillus Calmette-Guerin (BCG) (ATCC 27289) were included as reference strains for each assay.

Detection of *Mycobacteria* Other Than Tuberculosis

Ziehl Neelsen staining-positive isolates that were IS6110-PCR negative, were tested for its identification. The IS1245-PCR was used to detect the *Mycobacterium avium* (*M. avium*) complex (30). PCR controls were also conducted using a strain of *M. avium* obtained from a pure culture by thermal lysis as a positive control and bi-distilled water as a negative control.

Statistical Model

Latent Class Analysis

A Bayesian approach was used to estimate the Se and Sp of the BC and the PCR test (24) in samples showing bTB-like lesions ($n = 266$) and in the absence of a gold standard. Samples were considered to have originated from one single population, given that only samples showing bTB-like lesions were evaluated. For the analysis, results from both tests were assumed to be conditionally dependent because, although biological principles of both tests are different (the culture required that the pathogenic agent was viable, whereas the PCR only requires the presence of the genetic material in sufficient quantity), both tests are based on the detection of the mycobacteria. For that reason, we preferred to follow the conservative assumption that results were not independent.

Prior distributions for model parameters (including test Se and Sp and the disease prevalence, p) were initially approximated using information on the expected values and uncertainty around that expectation, from data reported in the peer-reviewed literature (Table 2). Beta distributions of the parameters were fitted using BetaBuster (<https://betabuster.software.informer.com/>), using a most likely value based on the median of estimates published in the literature, and a lower bound of the credibility interval that was approximately three standard deviations below the median, according to the published data (lower 99%CI Se = 0.58; Sp = 0.81). We preferred to use wide prior standard deviations to reflect the uncertainty around the true value of the parameters, considering that uncertainty related to the true value of the parameters is likely larger than simply the 95% CI of the results reported in the literature. Uniform distributions were used for the two co-variances (33). Field data were then used to modify the prior distributions and estimate posterior distributions in a Bayesian framework, using a one-population model and assuming conditional dependence between test results. Posterior distributions were reported as the posterior estimates of the median and posterior probability intervals (95% PPI). The code is provided as **Supplementary Table 2**. All analyses were implemented in the WinBUGS software, version 1.4, and results were computed for 10,000 iterations,

TABLE 2 | Parameters of the beta distribution and source of data used to estimate the accuracy of both bTB tests in swine samples from Argentina.

Parameter	Most likely value	Uncertainty	References	Parameters of the beta distribution		
				A	B	
Bacteriology culture	SeBC	0.79	>0.6	(10, 31)	16.1034	5.0197
	SpBC	0.97	>0.8	(10, 31)	17.2976	1.5041
PCR	SePCR	0.81	>0.6	(10, 12, 14, 15)	13.7759	3.7573
	SpPCR	0.99	>0.8	(10, 12)	14.52192	1.13658
True prevalence	P	0.32	>0.2	(32)	5.025	7.0375

BC and MTC-PCR IS6110 (PCR). Uncertainty was measured as the 95% confidence level that the parameter was higher (>) than a certain value. Se, Sensitivity; Sp, Specificity.

after the first 1,000 were burnt-in. Autocorrelation was eliminated through thinning the chains by collecting one in 10 consecutive samples (<https://www.mrc-bsu.cam.ac.uk/software/bugs/the-bugs-project-winbugs/>). The influence of the selected priors on the posterior distributions was evaluated by comparing the initial models with a model fitted using non-informative uniform (0.1) distributions alternatively for the parameters of each test and the prevalence (**Supplementary Table 1**). The outputs including the MCMC trace-plots, posterior density distribution plots and convergence were visually assessed (**Supplementary Figure 1**).

Agreement between the results obtained from both test was measured using the kappa statistic. The Kappa coefficient, combined Se and Sp of the tests used in series and in parallel, and the positive and negative predictive values of the tests were calculated using the posterior estimates of the model and using the WinEpi software (34) as:

$$Se_{series} = Se_{PCR} \times Se_{BC}$$

$$Se_{parallel} = 1 - (1 - Se_{PCR}) \times (1 - Se_{BC})$$

$$Sp_{(series)} = 1 - (1 - Sp_{PCR}) \times (1 - Sp_{BC})$$

$$Sp_{(parallel)} = Sp_{PCR} \times Sp_{BC}$$

For presenting the results here, we followed the guidelines for reporting of diagnostic accuracy in studies that use Bayesian Latent class models (STARD-BLCM) described elsewhere (35).

RESULTS

Descriptive Results

Most (171/266, 64.8%) samples were culture-positive, and most of those samples (137/171, 80.1%) were also MTC-IS6110+ PCR-positive. Out of the PCR-positive samples (176/266, 66.2%), only some (39/176, 15%) were BC-negative. A few (13/56, 23.2%) of the remaining 21% culture and PCR-negative samples (i.e., 4.9% of all the samples) were *M. avium complex* (IS1245+)-positive (**Table 3**). All IS6110-positive samples showed spoligotypes that were characteristic of *M. bovis* species, due to the absence of the 3, 9, 16, and 39–43 spacers.

Estimation of Tests Se and Sp

The estimated (posterior) Se of the bacteriological culture and of the PCR were 79.9% (95% posterior probability intervals, PPI: 71.69–88.7%) and 82.9% (95% PPI: 74.35–92.3%), respectively. The estimated (posterior) Sp was similar for both tests, with a value of 88.5% (95% PPI: 67.2–99.5%) for culture, and of 89.05% (95% PPI: 69.8–99.1%) for PCR.

Bovine tuberculosis prevalence in TB-like samples was 74.39% (95% PPI: 63.3–83.5%). The agreement of both tests was moderate (Kappa coefficient = 0.395; 95% CI (confidence interval) = 0.304–0.486). The negative and positive posterior correlation estimated between the diagnostic tests was uncertain, –0.02 (95% PPI: –0.2–0.33) and 0.16 (95% PPI: –0.15–0.74), respectively. The low correlations between the two test Se and between the two test Sp for samples showing bTB-like lesions suggests that the results of both tests were independent from each other.

Combination of both tests was evaluated considering the estimated prevalence, obtaining a Se and Sp of 66.2 and 98.7% (positive predictive value, PPV = 92.8%; negative predictive value, NPV = 89%) when the test were combined in series, and a Se and Sp of 96.6 and 78.8% (PPV = 92.8%; NPV = 89%) when combined in parallel. Results for a broad range of hypothetical prevalence values are presented in **Figure 1** to illustrate the expected variation on the PPV and NPV for alternative scenarios of disease prevalence when those two techniques were combined in parallel.

Results were not sensitive to the selection of the prior distributions, as suggested by the relatively consistency (magnitudes of percent differences <9%) in the results when using non-informative priors (**Supplementary Table 1**), to the posterior distribution of Se and Sp for both tests except for the Sp of the tests in which a reduction of 26.6% for the culture and 29.1 for the PCR was estimated.

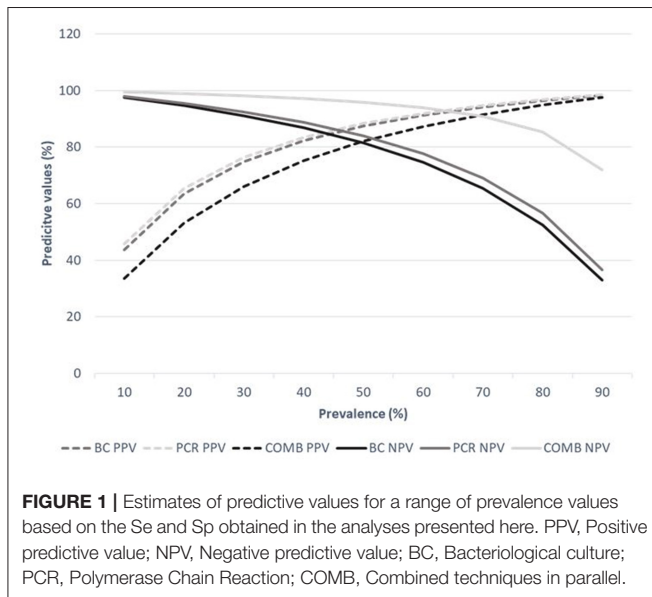
DISCUSSION

Although significant improvements have been made in the last 20 years, control and eradication of bTB continues to be a major challenge for Latin American countries (36, 37). Furthermore, the disease has re-emerged in humans in the region, reinforcing the need to understand the role of domestic and wild animals on the disease epidemiology, and, most importantly, the need for developing new strategies to effectively and efficiently prevent and control disease spread (37–40). Some of the challenges associated with bTB diagnosis in swine include presence of fewer bacilli in bTB lesions compared to other species (41, 42), relatively high susceptibility to avian TB infection, being those lesions undistinguishable from those produced by *M. bovis* (20, 32), and the lack, despite some promising advances (43), of simple, affordable, and sensitive diagnostic tools to identify infected animals at the farm level, like the PPD test use routinely

TABLE 3 | Distribution of the results for both bTB diagnostic tests applied.

	TB positive samples			TB negative samples		Total
	POS _{BC} /POS _{PCR}	POS _{BC} /NEG _{PCR}	NEG _{BC} /POS _{PCR}	NEG _{BC} /NEG _{PCR}		
TBL	137	34	39	56	266	
	51%	13.8%	15%	21%	100	

Samples of swine lymph nodes with TBL obtained from slaughterhouses were processed by BC and MTC-PCR IS6110 +PCR.



in cattle (43–45). Here, we provided evidence suggesting that the PCR may be used as an effective tool for the rapid and effective detection of the infection in swine routinely inspected at slaughterhouses in Argentina. Ultimately, results presented here will help to inform decisions intended to update control strategies in endemic settings.

The high frequency of *M. bovis* infection in bTB-like lesions estimated in this assay (74.39%), suggests that the disease continues to be prevalent in swine populations of Argentina. Furthermore, the figure is substantially higher compared to those reported elsewhere (12, 32), who detected MTC in 32.7% of TBL samples from South West Iberian Peninsula and absence of MTC positive results from samples obtained in Sao Paulo region, respectively. Still, the high frequency (31%) of bTB reported in wild boar samples from the south of Brazil (46) would suggest the need for further studies, increasing the sample size, and targeting specific risk populations. The relative high bTB prevalence in Argentine swine is likely associated with different rearing systems. Such conclusion is also supported by the observation that the risk for bTB is relatively high in pigs reared in extensive, small (<50 sow) farms that are co-located with cattle (unpublished data). In this regard, unfortunately, not much evidence has been reported regarding the interspecies dynamic of transmission of bovine tuberculosis in farms where the cohabitation with other species, like cattle, exists. A report from the Association of Veterinarians of Buenos Aires province

(47) provided preliminary evidence that supports this hypothesis, describing a different frequency of slaughterhouse findings of TBL based on rearing system being more frequent in extensive farms. Biosecurity in extensive farm is usually less strict than in intensive systems, increasing the risk for bTB (48).

The Se of the PCR test estimated here (83%) is similar to that reported for cattle (10), and higher than values previously reported for pig (12). The difference may be explained, at least in part, by differences in the study design, given that previous studies have used BC as the gold standard, whereas we have used a Bayesian approach that does not make use of such a questionable assumption. Another explanation may be that we used a larger sample size (4–7 g range of homogenized tissue for initial extraction) compared to previous studies (10, 12, 49). Increasing the volume of the samples may have increased the analytical Se (and thus the diagnostic test Se) of the test, without affecting the test Sp (13). One study (12) reported a detection rate of 77.3% of culture positive samples by RT-PCR and others (31) showed a Se of 66.1%, compared to 80.1% obtained in this work using direct PCR. Only one other study (49), reported a frequency of PCR-positive results similar to ours, working with bovine samples.

The bTB control program in Argentine swine is voluntary and its implementation is based on the use of PPD test at farm level and identification of bTB-like lesions in swine without the use of a confirmatory test. Here, we estimated that ~25% of the bTB-like lesions observed at the time of slaughtering in swine are non-infected, suggesting a Sp of the inspection of ~75% (95% CI: 63–85%). Furthermore, results suggest that the PCR may be used as a rapid, effective, confirmatory test for bTB-like lesions detected in pigs at the time of slaughtering, given that the test accuracy was similar (and on average, slightly higher) to that reported for the BC. Furthermore, the significant reduction of the turnaround time between the sample submission and the result would facilitate the follow up actions on positive cases by the sanitary authority. As expected, in-series combination of the tests impaired the combined Se value (66.6%), making it not suitable as a screening protocol. Conversely, in scenarios of relatively high bTB prevalence, such as those observed in Argentina, the in-parallel use of the BC and PCR (Figure 1) showed a good performance suggesting that the combined use of those techniques would be appropriate for the confirmation of the disease in bTB-like lesions. Consequently, it is recommended that bTB-like lesions were run by PCR and the positive samples be considered bTB infected, whereas negative samples would be run by BC, and considered as infected if positive, and non-infected

otherwise. Moreover, the NPV (probability that a sample negative to a screening test was non-infected) and PPV (probability that a sample positive to a screening test was infected) of the in parallel use of both tests (Figure 1) showed that this combination would be suitable for a wide range of prevalence. The combination of both tests showed much better NPV than the individual tests (% of increase, 8.5–35.3%) with prevalence values higher than 0.4; however, the combined use of the tests did not substantially impact the PPV, showing <1–8.5% reductions. Similar findings were also reported elsewhere (50–52). Results suggest that the combined use of those techniques would be appropriate for disease confirmation in bTB-like lesions and in the context of a TB control plan. The use of PCR as a routine confirmatory technique is commonly questioned taking into account only the associated direct costs (53). However, direct application of the PCR technique in tissues brings certain benefits such as the reduction of both the laboratory turnaround time and indirect costs associated with maintenance of personnel and facilities. Furthermore, bacteriological culture required significant investments in personnel training and the biosafety protocols and facilities, as it represents a much greater risk of exposure to the agent (Table 1).

In conclusion, these results suggest that bTB is still highly prevalent in swine populations of Argentina, and that the PCR may serve as an effective and rapid test for the confirmation of the agent in bTB-like lesions macroscopically detected at the time of slaughtering in the country. The results here may ultimately help to update current strategies used to prevent and

control of the disease in settings in which the disease is yet-to-be eradicated.

AUTHOR CONTRIBUTIONS

SB, MP, and AP conceived and performed the statistical analysis. SB and MP drafted the manuscript. MJM, MM, NA, MZ, and SB participated in the generation, collection and curation of the data, and collaborated in interpretation of the results. SB and AP designed the study and coordinated the work. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00204/full#supplementary-material>

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