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FULL PAPER

Immunology

ELISA using a recombinant chimera of ESAT-6/MPB70/MPB83 for *Mycobacterium bovis* diagnosis in naturally infected cattle

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ABSTRACT. Bovine tuberculosis (bTB) control programs generally rely on intradermal tuberculin tests for the antemortem diagnosis of *Mycobacterium bovis* infection in cattle, but these tests detect only a portion of the infected animals. The aim of the present study was to evaluate the diagnostic coverage of a combination of the bTB antemortem techniques known as the comparative intradermal tuberculin test (CITT) and an ELISA based on a recombinant chimera of ESAT-6/MPB70/MPB83 as the antigen in cattle. The results were compared to postmortem findings based on *M. bovis* culturing and PCR. Paired comparisons of all data (n=92) demonstrated that ELISA and LST results compared to the culturing results did not present significant differences (P=0.27 on McNemar's test and P=0.12 on Fisher's exact test, respectively). Using culturing as the gold standard, the sensitivity and specificity of ELISA were 79.5% (95% CI: 64.5–89.2%) and 75.5% (95% CI: 62.4–85.1%), respectively, whereas LST demonstrated 100% sensitivity (95% CI: 91.03–100%) and 92.5% specificity (95% CI: 82.1–97.0%). The ELISA results did not reveal significant differences in relation to the LST results (P>0.99 on Fisher's exact test). Using the latter as the gold standard, the sensitivity and specificity of ELISA were 79.1% (95% CI: 64.8–88.6%) and 79.6% (95% CI: 66.4–88.5%), respectively. The use of ELISA with the recombinant chimera of ESAT-6/MPB70/MPB83 as the antigen complements the diagnostic coverage provided by CITT and increases the removal of infected animals from herds.

KEY WORDS: bovine tuberculosis, comparative intradermal tuberculin test, ELISA, *Mycobacterium bovis*

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Bovine tuberculosis (bTB) is a chronic infectious disease caused by *Mycobacterium bovis*, which affects domestic species, especially cattle and buffaloes, as well as other wildlife [18]. This disease causes major economic losses, constitutes a sanitary barrier for the international livestock trade [8], and poses a public health risk [17].

In several countries, bTB control programs involve the detection and slaughter of infected animals based on intradermal tests [15]. However, there are concerns regarding the moderate sensitivity of such tests, which can produce false-negatives [10].

Previous studies describing the use of an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-*M. bovis* antibodies revealed low diagnostic specificity [23]. However, new tests have emerged based on defined recombinant proteins that were used as antigens [5]. Since the kinetics of the antibody response in the presence of different *M. bovis* antigens is variable in

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the infection phase [12, 26], the combination of recombinant antigens either as a pool or chimeric antigen (fusion protein) was expected to enable the detection of antibodies from cattle in different stages of *M. bovis* infection [22].

In a previous study, an ELISA based on a recombinant chimera of ESAT-6/MPB70/MPB83 as the antigen demonstrated satisfactory agreement with intradermal testing. This antigen was produced by cloning of the DNA coding sequences for the hydrophilic domains of ESAT-6, MPB70, and MPB83, and then expressed in the *E. coli* Rosetta strain as a 35 kDa fusion protein [22]. However, comparisons between diagnostic tests based on serological and cell-mediated responses are not the most appropriate, as there is evidence suggesting that serological tests are particularly useful for detecting false-negative (anergic) animals on intradermal tests [6].

The aim of the present study was to employ an ELISA based on a recombinant chimera of ESAT-6/MPB70/MPB83 as the antigen for diagnosing *M. bovis* in naturally infected animals and to compare the outcomes with postmortem results, such as lesions suggestive of bTB and microbiological cultures.

MATERIALS AND METHODS

Ethical aspects

Serum samples for ELISA, tissues samples for bacteriological culture and PCR, and comparative intradermal tuberculin test (CITT) results were obtained in compliance with the Brazilian National Program for the Control and Eradication of Brucellosis and Tuberculosis of the Ministry of Agriculture and Food Supply.

Animals

This study was conducted on 92 animals from four herds categorized into three groups based on CITT results. CITT was performed by veterinarians of the official veterinary service following the recommendations of the Brazilian National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis of the Ministry of Agriculture, Livestock and Food Supply [3]. Group A comprised of 39 CITT-negative animals from a TB-free herd. Group B comprised of 21 CITT-positive animals from three herds in the same region. Group C comprised of 32 CITT-negative animals from the same infected herds (herdmates of animals of Group B). Animals with inconclusive CITT results were considered positive in the present study, as recommended elsewhere [30].

Tests

Seven days after the CITT, blood samples were collected from the animals for ELISA. All animals from the three infected herds were sacrificed, while those from the TB-free herd were slaughtered for commercial purposes. Samples of tissues with macroscopic lesions suggestive of tuberculosis (LST) were collected. In the absence of LST, hepatic, mediastinal, mesenteric, retropharyngeal, and tracheobronchial lymph nodes were collected. The samples were processed for culturing and PCR analysis. Animals were considered infected if at least one sample had LST and/or a positive culture.

Culturing and confirmatory PCR

Tissue samples were kept at −30°C until processing. Tissues (between 10 and 25 mg) were macerated with 1.5 m*l* of sterile distilled water in a MagNA Lyser apparatus (Roche, Penzberg, Germany), decontaminated using the Petroff method [19] and cultured in Stonebrink culture medium tubes at 37°C for up to 90 days. Colonies suggestive of *M. bovis* were submitted to conventional PCR with the Mb.400 primers, which were used to amplify a fragment of 400 bp flanking the region of differentiation 4 (RD4) specific to *M. bovis* [21].

Nested-PCR direct detection of M. tuberculosis complex (MTC) in tissues

DNA from macerated tissues was extracted with the DNEasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. A nested-PCR for *rv2807* was used for the direct detection of MTC in tissues [2] from the 39 animals of the TB-free herd.

ELISA

The wells of Costar 3590 polystyrene 96-well plates (Corning, Corning, NY, U.S.A.) were adsorbed with the recombinant chimera of ESAT-6/MPB70/MPB83 [22] in carbonate-bicarbonate buffer, pH 9.6, for 60 min at 37°C. The plates were then blocked with 100 μl/well of phosphate buffer saline with 0.1% Tween 20 (PBST) with 5% skim milk for 60 min at 37°C. After five washes with PBST, 100 μl/well of the control and test sera diluted to 1:600 in PBST with 2% skim milk were incubated for 60 min at 37°C. The plates were washed five times with PBST. Next, 100 μl of anti-bovine IgG (whole molecule) horseradish peroxidase conjugate (A8917, Sigma, St. Louis, MO, U.S.A.) (dilution: 1:80,000 in PBST) were added to each well. The plates were incubated for 30 min at 37°C, washed five times, and 50 μl/well of chromogen/substrate tetramethylbenzidine (T0440, Sigma) was added. The reactions were stopped with 2.5 N of H_2SO_4, and the results were read in an EL-800 ELISA reader (BioTek Instruments, Winooski, VT, U.S.A.) with a 450 nm filter.

Statistics

The cutoff value for the ELISA was determined based on receiver operating characteristic (ROC) analysis (Metz, 1978), using the MEDCALC 10.3.0.0 software program. This analysis was based on 30 serum samples from cattle infected with *M. bovis*

Table 1. Results of comparative intradermal tuberculin test (CITT), ELISA with recombinant chimera of ESAT-6/MPB70/MPB83, lesions suggestive of tuberculosis (LST), and bacteriological culture for *Mycobacterium bovis* in cattle from infected and non-infected herds

Group	Number of animals with positive or inconclusive CTT results/total	Positive for LST	Positive culture	Positive ELISA
A	0/39	0	0	1
B	21/21	18	15	19
C	0/32	25	24	24
Total	21/92	43	39	44

A, bovine tuberculosis-free herd; B, CITT-positive/inconclusive animals from infected herds; C, CITT-negative animals from infected herds.

Table 2. Cattle tissues identified with lesions suggestive of tuberculosis (LST)

Type of tissue	Number with LST	Number with LST and positive microbiological culture (%)
Pulmonary lymph node	21	18 (85.7)
Mesenteric lymph node	7	6 (85.7)
Retropharyngeal lymph node	26	23 (88.5)
Parotid lymph nodes	1	1 (100.0)
Mammary lymph node	8	7 (87.5)
Lung tissue	7	5 (71.4)
Mammary tissue	4	4 (100.0)

(positive CITT, LST, and microbiological culturing results) and 30 serum samples from non-infected cattle (negative CITT, LST, and microbiological culturing results). Comparisons between culturing, ELISA, CITT, and LST results were evaluated using McNemar's or exact Fisher's tests for matched-pair samples, according to the number of discordant pairs, ≥20 or <20, respectively.

RESULTS

Lesions suggestive of tuberculosis

Among the 92 animals studied, 43 (46.7%) exhibited LST: none from Group A (CITT-negative animals from bTB-free herd), 18 (19.6%) from Group B (CITT-positive or inconclusive animals from infected herds), and 25 (27.2%) from Group C (CITT-negative animals from infected herds) (Table 1). Twenty-five animals (58.1%) had a single LST, 14 (32.6%) had two or three lesions, and four (9.3%) had four or more lesions. The type and number of tissues identified with LST are indicated in Table 2.

Culturing and confirmatory PCR

Bacterial culturing was positive for 39 animals (42.4%): 15 (16.3%) from Group B, and 24 (26.1%) from Group C (Table 1). Positivity according to the tissues in which LST were detected is indicated in Table 2. All colonies suspected of *M. bovis* were confirmed by PCR targeting RD4.

Nested-PCR direct detection of MTC in tissues

All tissue samples collected from the 39 animals of the bTB-free herd (Group A) were negative based on the nested PCR.

ELISA

For ELISA, a cutoff value of 0.319 was defined in the ROC analysis. Among the 92 animals included in the present study, 44 (47.8%) were positive: one (1.1%) in Group A (non-infected), 19 (20.6%) in Group B, and 24 (26.1%) in Group C (Table 1).

LST and culturing vs. CITT groups

In Group A, all 39 animals were negative for LST and microbiological culture (100%). In Group B, 15/21 animals (71.4%) had positive LST and culture results, 3/21 (14.3%) were LST+/culture−, and 3/21 (14.3%) were LST−/culture−. In Group C, 24/32 animals (75.0%) were LST+/culture+, 1/32 (3.1%) was LST+/culture−, and 7/32 (21.9%) were LST−/culture− (Table 3).

ELISA and culturing vs. CITT groups

In Group A, 38 of the 39 animals were negative for ELISA and microbiological culture (97.4%), and one animal was ELISA+/culture−. In Group B, 13/21 animals (61.9%) were ELISA+/culture+, 6/21 (28.6%) were ELISA+/culture−, and 2/21 (14.3%) were ELISA−/culture+. In Group C, 18/32 animals (56.2%) were ELISA+/culture+, 6/32 (18.7%) were ELISA+/culture−, 6/32 (18.7%) were ELISA−/culture+, and 2/32 animals (6.2%) were ELISA−/culture− (Table 3).

Table 3. Results of lesions suggestive of tuberculosis (LST), bacteriological culture, and ELISA in cattle from herds infected with *M. bovis* and non-infected herds

Diagnostic evidence	Groups			Total
	A	B	C	
LST+/Culture+	0	15	24	39
LST+/Culture−	0	3	1	4
LST−/Culture+	0	0	0	0
LST−/Culture−	39	3	7	49
Total	39	21	32	92
LST+/ELISA+	0	16	18	34
LST+/ELISA−	0	2	7	9
LST−/ELISA+	1	3	6	10
LST−/ELISA−	38	0	1	39
Total	39	21	32	92
ELISA+/Culture+	0	13	18	31
ELISA+/Culture−	1	6	6	13
ELISA−/Culture+	0	2	6	8
ELISA−/Culture−	38	0	2	40
Total	39	21	32	92

A, bovine tuberculosis-free herd; B, CITT-positive/inconclusive animals from infected herds; C, CITT-negative animals from infected herds.

Paired comparisons of all data (n=92)

Comparing the tests with the culturing results as the gold standard, the ELISA and LST results did not present significant differences (P=0.27 and P=0.12 on McNemar's test and Fisher's exact test, respectively). Accordingly, using culturing as the gold standard, the sensitivity and specificity of ELISA was 79.5% (CI 95%: 64.5–89.2%) and 75.5% (95% CI: 62.4–85.1%), respectively, whereas LST demonstrated 100% sensitivity (95% CI: 91.03–100%) and 92.4% specificity (95% CI: 82.1–97.03%). The ELISA results did not present significant differences in relation to those of the LST results (P>0.99 on Fisher's exact test). Using the latter as the gold standard, the sensitivity and specificity of the ELISA was 79.1% (95% CI: 64.8–88.6%) and 79.6% (95% CI: 66.3–88.5%), respectively. Conversely, the CITT results presented significant differences in relation either to those of the culture (P=0.001, McNemar's test), ELISA (P=0.000009, McNemar's test), and LST (P=0.000032, McNemar's test).

DISCUSSION

In many countries, the control of bTB has primarily relied on the application of intradermal tuberculin tests and the subsequent slaughter of animals found to be positive [7]. Despite this control strategy, reports of persistent outbreaks of the disease remain frequent due to several transmission routes, such as moving infected cattle, re-infection from an environmental reservoir, and the poor sensitivity of the diagnostic tests [4].

The correct identification of animals that are truly infected using antemortem tests is the cornerstone of any disease eradication program. Failure to identify all infected animals hinders progress towards controlling and eradicating the disease. In the case of bTB, the moderate sensitivity of the CITT can result in a "hidden burden" of infection residing within the population [10, 15].

According to Whelan *et al.* (2011), despite the effectiveness of CITT in reducing the incidence of infected animals, animals that are not detected maintain the herd at risk of spreading the infection [28]. The intradermal test is more efficient at detecting the disease in advanced stages, rather than earlier stages [9]. Thus, conventional methods of cellular immune response are flawed with regard to the detection of chronically infected animals by *M. bovis*.

Microbiological culturing is considered the gold standard method by the World Organization for Animal Health for the diagnosis of bTB [16, 20]. In the present study, 24 of the 32 CITT-negative cattle from infected herds exhibited lesions suggestive of tuberculosis, which was confirmed by the microbiological culture. This means that 75% of the CITT-negative cattle from the infected herds were infected and presented false-negative CITT results.

Lesions in lymph nodes of the thoracic region were found, which was in agreement with data described in the literature [14]. In a study on the distribution of lesions in bovines infected with *M. bovis* in 20 dairy herds, 14 animals presented evidence of tuberculosis in the tracheobronchial, mediastinal, or medial retropharyngeal lymph nodes. Gross lesions of tuberculosis were found more frequently in lymph nodes of the thoracic region (60.0%) and head (26.7%) [29].

Lahuerta-Marin *et al.* (2016) found that a large proportion of animals with positive culture results (90.8%; 635/699) also had visible lesions consistent with bTB during meat inspection. Therefore, the inspection of carcasses at slaughter is a method for the direct detection of lesions and the diagnosis of bTB [10]. In the present study, the ELISA results and lesions suggestive of tuberculosis did not demonstrate significant differences. Thus, ELISA can be used for the identification of infected animals in an

antemortem test, which can subsequently be corrected by the postmortem (culture) test.

Using parallel tests with different immune responses increases the diagnostic coverage. Combining traditional techniques based on cellular responses and the detection of antibodies can accelerate the eradication of bTB by assisting in the management of foci and the control of the disease [6].

Bovine tuberculosis predominantly triggers cell-mediated immunity (CMI) in the early and intermediate phases of the infection, elicited by Th1 lymphocytes. Therefore, the main diagnostic methods used in eradication programs worldwide are based on the detection of the CMI response: intradermal tests and interferon gamma (IFN-g) assay. As the disease progresses, the shift from Th1 to Th2 lymphocytes is associated with a decrease in CMI and the development of serological responses [6].

Considering the high prevalence of *M. bovis*-infected dairy herds, ELISA exhibited significantly greater sensitivity than the CITT, which is the official bTB diagnostic test in Brazil. This greater sensitivity may reflect the predominance of a humoral immune response in the advanced stage of the *M. bovis* infection [27], denoted in this study by the presence of gross lesions in 39/53 (73%) of the animals with positive microbiological cultures.

Although more expressive serological responses are expected only in the advanced stage of the *M. bovis* infection, antibodies for MPB83 can be found as early as four to six weeks after experimental *M. bovis* inoculation [12, 26]. A remarkable animal-to-animal variation in the serological response following the experimental *M. bovis* inoculation was observed, along with a variation in the protein profile recognized during the course of infection [24]. These facts suggest that a genetic component may play a role in the early antibody response to *M. bovis.*

The sensitivity of ELISA can be enhanced by the injection of purified protein derivatives (PPDs) from the intradermal tuberculin tests [6, 25], which boost *M. bovis*-specific IgG responses to defined antigens, such as MPB83 and MPB70, and induce an antibody affinity maturation. The booster effect can be further increased by the re-injection of PPDs [25]. It is thought that infection with nontuberculous mycobacteria may lead to false-positive reactions in the ELISA, as heat-inactivated Johne's disease vaccine induces cross-reactions in serological methods based on MPB83 and MPB70 [7]. In the present study, however, nontuberculous mycobacteria were not detected in any microbiological culture.

One of the difficulties in estimating the specificity of diagnostic tests for bTB in a country where disease is endemic is to define truly negative herds, since the culture may present false-negative results and animals in the early stages of infection may not present visible lesions. In this study, samples of cattle with negative culture and/or no visible lesions from infected herds were included, which may have led to an underestimation of the specificity of the ELISA. Considering only herd A, in which the 39 animals were negative for CITT, LST, culture, and nested-PCR, only one had an optical density of 0.450, above the ELISA cutoff of 0.319 (2.6%).

The ELISA was able to detect the majority of infected animals with false-negative CITT results. These animals are generally considered anergic or unresponsive to PPDs in the intradermal tests [11]. Thus, using skin tests as the sole diagnostic tool frequently does not detect all infected animals. This lack of reliability in the diagnosis of infected animals may also be related to a state of anergy in chronically infected herds, in which a number of animals could be in advanced stages of the disease, likely due to a previous diagnostic failure. These false-negative animals could be hosts to bacteria, which compromises the elimination of the main sources of infection [1, 13].

In the particular epidemiological condition of the high prevalence of animals in the advanced stage of *M. bovis* infection, the contribution of CITT in the overall detection of bTB-positive animals was minimal, as only 2/39 (5.1%) animals with positive culture findings were detected exclusively by CITT when comparing all groups. In contrast, 18/39 (46.1%) infected animals were detected exclusively by ELISA. In addition to the advantage of greater sensitivity with ELISA, only a single visit to the farm is necessary, which decreases the economic costs involved in the diagnosis of bTB [5].

The present data demonstrate the potential for the use of ELISA with a recombinant chimera of ESAT-6/MPB70/MPB83 as an antigen for the diagnosis of bTB, especially the detection of animals with false-negative CITT results. The use of serological assays as ancillary tests complements the diagnostic coverage provided by CITT and accelerates the removal of infected animals from herds.

CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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REFERENCES

1. Álvarez, J., Perez, A., Marqués, S., Bezos, J., Grau, A., de la Cruz, M. L., Romero, B., Saez, J. L., del Rosario Esquivel, M., Martínez, M. C., Mínguez, O., de Juan, L. and Domínguez, L. 2014. Risk factors associated with negative in-vivo diagnostic results in bovine tuberculosis-infected cattle in Spain. *BMC Vet. Res.* **10**: 14. [Medline] [CrossRef]
2. Araújo, C. P., Osório, A. L., Jorge, K. S., Ramos, C. A., Souza Filho, A. F., Vidal, C. E., Vargas, A. P., Roxo, E., Rocha, A. S., Suffys, P. N., Fonseca, A. A. J. Jr., Silva, M. R., Barbosa Neto, J. D., Cerqueira, V. D. and Araújo, F. R. 2014. Direct detection of *Mycobacterium tuberculosis* complex in bovine and bubaline tissues through nested-PCR. *Braz. J. Microbiol.* **45**: 633–640. [Medline] [CrossRef]
3. BRASIL 2016. Ministério de Agricultura, Pecuária e Abastecimento. Instrução Normativa N° 19, de 10 de outubro de 2016. Secretaria de Defesa Agropecuária, 4p. Available at http://pesquisa.in.gov.br/imprensa/jsp/visualiza/index.jsp?jornal=1&data=03/11/2016&pagina=7 [accessed on

October 10, 2018].
4. Brooks-Pollock, E., Roberts, G. O. and Keeling, M. J. 2014. A dynamic model of bovine tuberculosis spread and control in Great Britain. *Nature* **511**: 228–231. [Medline] [CrossRef]
5. Casal, C., Díez-Guerrier, A., Álvarez, J., Rodriguez-Campos, S., Mateos, A., Linscott, R., Martel, E., Lawrence, J. C., Whelan, C., Clarke, J., O'Brien, A., Domínguez, L. and Aranaz, A. 2014. Strategic use of serology for the diagnosis of bovine tuberculosis after intradermal skin testing. *Vet. Microbiol.* **170**: 342–351. [Medline] [CrossRef]
6. Casal, C., Infantes, J. A., Risalde, M. A., Díez-Guerrier, A., Domínguez, M., Moreno, I., Romero, B., de Juan, L., Sáez, J. L., Juste, R., Gortázar, C., Domínguez, L. and Bezos, J. 2017. Antibody detection tests improve the sensitivity of tuberculosis diagnosis in cattle. *Res. Vet. Sci.* **112**: 214–221. [Medline] [CrossRef]
7. Coad, M., Clifford, D. J., Vordermeier, H. M. and Whelan, A. O. 2013. The consequences of vaccination with the Johne's disease vaccine, Gudair, on diagnosis of bovine tuberculosis. *Vet. Rec.* **172**: 266. [Medline] [CrossRef]
8. Fieni, F., Grant, C., Gard-Schnuelle, J., Perry, G., Wrenzycki, C. and Blondin, P. 2016. 107 Research priorities for safe sanitary trade of embryo and semen. *Reprod. Fertil. Dev.* **29**: 161–162. [CrossRef]
9. Hirpa, E., Ameni, G., Lawrence, J. C., Tafess, K., Worku, A., Sori, T. and Zewdie, O. 2014. Performance evaluation of *Mycobacterium bovis* antibody tests for the diagnosis of bovine tuberculosis in Ethiopia. *Acad. J. Anim. Diseases* **3**: 33–38.
10. Lahuerta-Marin, A., McNair, J., Skuce, R., McBride, S., Allen, M., Strain, S. A. J., Menzies, F. D., McDowell, S. J. W. and Byrne, A. W. 2016. Risk factors for failure to detect bovine tuberculosis in cattle from infected herds across Northern Ireland (2004–2010). *Res. Vet. Sci.* **107**: 233–239. [Medline] [CrossRef]
11. Lepper, A. W., Pearson, C. W. and Corner, L. A. 1977. Anergy to tuberculin in beef cattle. *Aust. Vet. J.* **53**: 214–216. [Medline] [CrossRef]
12. Lyashchenko, K. P., Pollock, J. M., Colangeli, R. and Gennaro, M. L. 1998. Diversity of antigen recognition by serum antibodies in experimental bovine tuberculosis. *Infect. Immun.* **66**: 5344–5349. [Medline]
13. Medeiros, L. S., Marassi, C. D., Figueiredo, E. E. and Lilenbaum, W. 2010. Potential application of new diagnostic methods for controlling bovine tuberculosis in Brazil. *Braz. J. Microbiol.* **41**: 531–541. [Medline] [CrossRef]
14. Nalapa, D. P., Muwonge, A., Kankya, C. and Olea-Popelka, F. 2017. Prevalence of tuberculous lesion in cattle slaughtered in Mubende district, Uganda. *BMC Vet. Res.* **13**: 73. [Medline] [CrossRef]
15. Nuñez-Garcia, J., Downs, S. H., Parry, J. E., Abernethy, D. A., Broughan, J. M., Cameron, A. R., Cook, A. J., de la Rua-Domenech, R., Goodchild, A. V., Gunn, J., More, S. J., Rhodes, S., Rolfe, S., Sharp, M., Upton, P. A., Vordermeier, H. M., Watson, E., Welsh, M., Whelan, A. O., Woolliams, J. A., Clifton-Hadley, R. S. and Greiner, M. 2018. Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the U.K. and Ireland. *Prev. Vet. Med.* **153**: 94–107. [Medline] [CrossRef]
16. OIE Office International des Epizooties Terrestrial manual. Chapter 2.4.7. Bovine Tuberculosis. World Health Organization for Animal Health. 2009. Available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.07_BOV INE_TB.pdf [accessed on October 10, 2018].
17. Olea-Popelka, F., Muwonge, A., Perera, A., Dean, A. S., Mumford, E., Erlacher-Vindel, E., Forcella, S., Silk, B. J., Ditiu, L., El Idrissi, A., Raviglione, M., Cosivi, O., LoBue, P. and Fujiwara, P. I. 2017. Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*-a call for action. *Lancet Infect. Dis.* **17**: e21–e25. [Medline] [CrossRef]
18. Pesciaroli, M., Alvarez, J., Boniotti, M. B., Cagiola, M., Di Marco, V., Marianelli, C., Pacciarini, M. and Pasquali, P. 2014. Tuberculosis in domestic animal species. *Res. Vet. Sci.* **97** Suppl: S78–S85. [Medline] [CrossRef]
19. Petroff, S. A. 1915. A new and rapid method for the isolation and cultivation of tubercle bacilli directly from the sputum and feces. *J. Exp. Med.* **21**: 38–42. [Medline] [CrossRef]
20. Ramos, D. F., Silva, P. E. A. and Dellagostin, O. A. 2015. Diagnosis of bovine tuberculosis: review of main techniques. *Braz. J. Biol.* **75**: 830–837. [Medline] [CrossRef]
21. Sales, M. L., Fonseca, A. A. J. Jr., Sales, E. B., Cottorello, A. C., Issa, M. A., Hodon, M. A., Soares Filho, P. M., Ramalho, A. K., Silva, M. R., Lage, A. P. and Heinemann, M. B. 2014. Evaluation of molecular markers for the diagnosis of *Mycobacterium bovis*. *Folia Microbiol. (Praha)* **59**: 433–438. [Medline] [CrossRef]
22. Souza, I. I., Melo, E. S., Ramos, C. A., Farias, T. A., Osório, A. L., Jorge, K. S., Vidal, C. E., Silva, A. S., Silva, M. R., Pellegrin, A. O. and Araújo, F. R. 2012. Screening of recombinant proteins as antigens in indirect ELISA for diagnosis of bovine tuberculosis. *Springerplus* **1**: 77. [Medline] [CrossRef]
23. Wadhwa, A., Johonson, R. E., Eda, K., Waters, W. R., Palmer, M. V., Bannantine, J. P. and Eda, S. 2014. Evaluation of ethanol vortex ELISA for detection of bovine tuberculosis in cattle and deer. *BMC Vet. Res.* **10**: 147. [Medline] [CrossRef]
24. Waters, W. R., Vordermeier, H. M., Rhodes, S., Khatri, B., Palmer, M. V., Maggioli, M. F., Thacker, T. C., Nelson, J. T., Thomsen, B. V., Robbe-Austerman, S., Bravo Garcia, D. M., Schoenbaum, M. A., Camacho, M. S., Ray, J. S., Esfandiari, J., Lambotte, P., Greenwald, R., Grandison, A., Sikar-Gang, A. and Lyashchenko, K. P. 2017. Potential for rapid antibody detection to identify tuberculous cattle with non-reactive tuberculin skin test results. *BMC Vet. Res.* **13**: 164. [Medline] [CrossRef]
25. Waters, W. R., Palmer, M. V., Stafne, M. R., Bass, K. E., Maggioli, M. F., Thacker, T. C., Linscott, R., Lawrence, J. C., Nelson, J. T., Esfandiari, J., Greenwald, R. and Lyashchenko, K. P. 2015. Effects of serial skin testing with purified protein derivative on the level and quality of antibodies to complex and defined antigens in *Mycobacterium bovis*-infected cattle. *Clin. Vaccine Immunol.* **22**: 641–649. [Medline] [CrossRef]
26. Waters, W. R., Palmer, M. V., Thacker, T. C., Bannantine, J. P., Vordermeier, H. M., Hewinson, R. G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J. M., Andersen, P. and Lyashchenko, K. P. 2006. Early antibody responses to experimental *Mycobacterium bovis* infection of cattle. *Clin. Vaccine Immunol.* **13**: 648–654. [Medline] [CrossRef]
27. Welsh, M. D., Cunningham, R. T., Corbett, D. M., Girvin, R. M., McNair, J., Skuce, R. A., Bryson, D. G. and Pollock, J. M. 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology* **114**: 101–111. [Medline] [CrossRef]
28. Whelan, C., Shuralev, E., Kwok, H. F., Kenny, K., Duignan, A., Good, M., Davis, W. C. and Clarke, J. 2011. Use of a multiplex enzyme-linked immunosorbent assay to detect a subpopulation of *Mycobacterium bovis*-infected animals deemed negative or inconclusive by the single intradermal comparative tuberculin skin test. *J. Vet. Diagn. Invest.* **23**: 499–503. [Medline] [CrossRef]
29. Whipple, D. L., Bolin, C. A. and Miller, J. M. 1996. Distribution of lesions in cattle infected with *Mycobacterium bovis*. *J. Vet. Diagn. Invest.* **8**: 351–354. [Medline] [CrossRef]
30. Zarden, C. F. O., Marassi, C. D., Figueiredo, E. E. E. S. and Lilenbaum, W. 2013. *Mycobacterium bovis* detection from milk of negative skin test cows. *Vet. Rec.* **172**: 130. [Medline] [CrossRef]