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Enzyme-linked immunosorbent assay as complement of intradermal skin test for the detection of mycobacterium bovis infection in cattle

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A B S T R A C T

Diagnostic tests based on cell-mediated immunity are used in programs for the control and eradication of bovine tuberculosis (bTB), which is mainly caused by Mycobacterium bovis. Additional serological assays could be performed as an ancillary method to detect an infected animal that fails to produce an immune response against the intradermal reaction (IDR), the official bTB test. In this study, we evaluated the effectiveness of an enzyme-linked immunosorbent assay (ELISA) that uses bovine PPD as a capture antigen as a complement to the IDR in herds with confirmed cases of bTB. The study was conducted in two stages. First, a panel of 200 serum samples was analyzed by ELISA. The sensitivity and specificity obtained were 60% and 99%, respectively. The subsequent stage consisted of evaluating 7,494 bovines from 14 selected dairy farms. The number of animals yielding a IDR negative/ELISA positive result were 200. A necropsy analysis of 33 of these IDR negative/ELISA positive animals revealed that 30 (91%) presented granulomatous lesions and positive M. bovis isolation. This finding confirmed bTB in most cases. Altogether, the results obtained in the present study suggest that the combined use of IDR and ELISA is an effective strategy to improve the control of bTB in endemic herds.

1. Introduction

Bovine tuberculosis (bTB) is one of the most important infectious diseases in the world and Mycobacterium bovis (M. bovis) is its main causative agent in cattle. This disease is a zoonosis with important implications in public health as well as in production. Indeed, bTB causes direct and indirect economic losses in the livestock because of decreases in milk and meat production as well as a consequence of commercial restrictions on cattle products [1,2].

The control and eradication of bTB, which in Argentina is considered an endemic disease, is based on detection and subsequent elimination of infected animals. Different strategies can be used to detect bTB.The main diagnostic tool used worldwide is the intradermal reaction (IDR) or tuberculin skin test. The IDR is usually applied on the base of the tail and this evaluation is known as the caudal fold tuberculin (CFT) single intradermal test. De la Rua Domnech [3] reported that the sensitivity (Se) and specifity (Sp) of the CFT ranges from 63.2% to 100%, (median of 83.9%) and 75.5%–99.0% (median of 96.8%), respectively. An ancillary diagnostic tool [4,5] to further assess the cell-mediated immune response against bTB is the gamma interferon (IFN-γ) release assay. This assay evaluates the levels of IFN-γ released through purified protein derivative (PPD) stimulation of blood supernatants of infected animals.

Any attempt to improve the identification of cattle infected with M. bovis is important. During the last decades, researchers have designed several serological tests to detect antibodies to M. bovis antigens [6,7]. The researchers focused their studies on identifying animals with false negative to IDR (i.e. anergic animals) [8]. Anergy is associated with severe pathological conditions as well as with an increase in mycobacterial load and excretion [3,9]. The identification of these animals constitutes one of the main challenges in bTB diagnosis. Harboe et al. [10] demonstrated that the application of IDR stimulated the production of antibodies in infected cattle and this in turn significantly increased the sensitivity of humoral diagnostic tests. This booster or anamnestic response caused by the application of the IDR could improve the sensitivity of bTB serodiagnosis [7,11]. Casal et al. [7] have addressed this concept after demonstrating that the IDR booster effect on antibody levels in periferial blood of cattle with bTB increased ELISA sensitivity from 24% to 70% 15 days after IDR application. Waters et al. [16] also reported the importance of the booster effect for an effective serological diagnosis.

The aim of the present study was to evaluate the effectiveness of applying an indirect ELISA as an ancillary test to the CFT in order to detect infected animals with negative reactions to CFT alone. To achieve this aim, we first evaluated the sensitivity and specificity values associated to the assay and subsequently evaluated the proposed

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diagnostic algorithm (negative CFT followed by ELISA) in herds with endemic bTB.

2. Materials and methods

2.1. Study design

This study was carried out in two stages. The first stage included the evaluation of a serum panel previously characterized to determine sensitivity (Se) and specifity (Sp) values of the assessed ELISA. The second stage involved the evaluation of an indirect ELISA after CFT implementation in 7494 animals from 14 dairy herds with confirmed cases of bTB. The CFT and blood sampling were performed by accredited veterinary services in accordance with the institutional guidelines established in Argentine legislation [12].

2.2. Enzyme-linked immunosorbent assay protocol

A previously described indirect ELISA that uses PPD as a capture antigen [13] was performed after implementing CFT. The following factors were evaluated: antigenic concentration, serum dilution and conjugate concentration. The binding step consisted of incubating the antigen (12 μL per well of PPD: 1μg/mL) over night at 4 °C in a wet chamber. Then, the wells were washed 5 times with saline solution (PBSTween 0.5%, 1% of skimmed milk) and the different sera (100 μL; diluted 1:100) were added to the wells and incubated for 1h at 37 °C. More washing procedures (5) were carried out before adding and incubating 100 μL of the conjugate (dilution 1:7000) for 1h at 37 °C. A final round of 5 washes was performed and 100 μL of substrate (Citrate Buffer + ABTS + H_2O_2) was incubated for 10min in the dark. The reaction was evaluated at 405 nanometers (nm).

2.3. Estimation of the sensitivity and specificity

Sensitivity and specifity were assessed using 200 serum samples. Half of the evaluated samples were from CFT positive animals with gross lesions compatible with tuberculosis according to necropsy results. The M. bovis infection was subsequently confirmed by culture and PCR for IS6110. The remaining 100 samples (negative sera) corresponded to CFT negative animals from herds officially declared free of bTB. These herds were from the province of Tierra del Fuego, an island declared free of bTB since 2011 [14].

The results obtained were statistically analyzed by means of the Receiver Operating Characteristic Curve (ROC) (GraphPad Prism software version 7.02). The cut-off point and the sensitivity and specificity were established by determining the value of the area under the curve (AUC) [15].

2.4. Combined diagnostic strategy: CFT-ELISA

First, the sensitivity and specificity of the proposed diagnostic algorithm were estimated by using the results from the CFT (test 1 or T1) and assuming values previously described (Se: 83.9% and Sp: 96.8%) [3]. The assessment of the ELISA (test 2 or T2) was performed using sera from CFT negative bovines coming from herds with confirmed bTB. The strategy consisted of applying the following formulae provided for "combined series tests" [16]:

Sensitivity: 1- (1-Se of T1) x (1- Se of T2)
Specificity: Sp of T1 x Sp of T2

2.5. Field evaluation of the combined diagnostic strategy

The diagnostic strategy was tested under field conditions following the scheme detailed in Table 2. The analyzed animals came from 14 dairy farms with history of confirmed bTB cases in adult cattle (cows and heifers post-service). All farms applied the CFT with an average frequency of 4 months during the year prior to the beginning of this study. Eleven farms were located in Buenos Aires, two in Córdoba and one in Santa Fe. The farms contained an average of 533 animals. First, officially accredited veterinarians performed the CFT to the 7,494 animals according to current regulations. Animals with reactions equal or higher to 3 millimeters were considered positive [12]. Fifteen to 20 days after CFT, blood samples were collected from animals with negative CFT results (booster effect). The assessment of the ELISA was performed with 6,907 sera according to a previously described protocol [13].

The financial consequences of applying the bTB control programme, with the concomintant slaughterhousing of bovine reactors, are entirely borne by the producer. However, CFT negative animals are not condemned to the slaughterhouse. With this in mind, in the present study we randomly selected a group of the CFT negative/ELISA positive animals from each farm to perform an exhaustive necropsy analysis.

Therefore, 33 animals were euthanized by administering a sedative (Xylacin 20 mg/mL, Richmond VetPharma®; 0.5–1mL/100 kg) previous to the barbiturate solution (Euthanyle, Brouwer S.A). The necropsy was developed with special emphasis on two systems: the respiratory (retropharyngeal lymph nodes, lung and tracheobronchial and mediastinic lymph nodes) and digestive (liver, hepatic and mesenteric lymph nodes). Any focus or nodule, either single, multifocal or confluent, circumscribed, and solid or necrotic, with or without apparent caseation and mineralization and with yellow–white appearance was considered a macroscopic tuberculous lesion, according to a previously described report [17]. Tissue samples with or without injuries were collected for histopathology, bacteriology and PCR for IS6110 sequence (performing DNA extraction directly from tissues). Other organs or tissues with lesions compatible with bTB were additionally sampled. A pattern of generalized lesions (or multi-organic) was considered when granuloms involved at least two systems such as respiratory and digestive.

For the bacteriology analysis, tissue samples were aseptically collected and grouped into the following four sets: head (retropharyngeal and submandibular lymph nodes (LN)), respiratory (tracheobronchial and mediastinal LN, and lung sample), digestive (mesenteric and hepatic LN, and liver sample), and mammary (udder and supra mammary LN). The samples were homogenized mechanically for 3 min (Basic Masticator, IUL Instruments type 470, Spain), decontaminated by Petroff's method [18] and cultured in triplicates on the egg-based Stonebrink solid media at 37 °C for at least 8 weeks. The cultures were observed twice weekly and the positive cultures were stained with Ziehl-Neelsen to identify acid-fast bacteria [19]. Histopathology samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. The paraffin plugs were cut in 5 μm-thick sections, deparaffinized, hydrated and stained with hematoxylin-eosin and Ziehl-Neelsen.

PCR performed from different tissues was carried out according to the method described by Zumárraga et al. [20]. DNA extraction previous to PCR was performed by using a commercial kit (DNA PuriPrep T-KIT, Inbio Highway, Argentina). An animal was considered positive when M. bovis isolation or macroscopic lesions compatible with bTB were supported by a positive histopathology and/or PCR result. Moreover, a molecular characterization of the obtained isolates by spoligotyping to confirm the presence of M. bovis and to identify the spoligotypes (www.mbovis.org) [21].

The correlation between the proportion of positive animals to CFT and ELISA of each analyzed farm was estimated by the Pearson correlation coefficient (r).

2.5.1. Cell-mediated immune response by IFN-γ assay

Heparinised blood samples were collected from the 33 studied animals prior to euthanasia. The detection of IFN-γ was performed with a commercial kit (Bovigam™TB kit, ThermoFisher Scientific) and

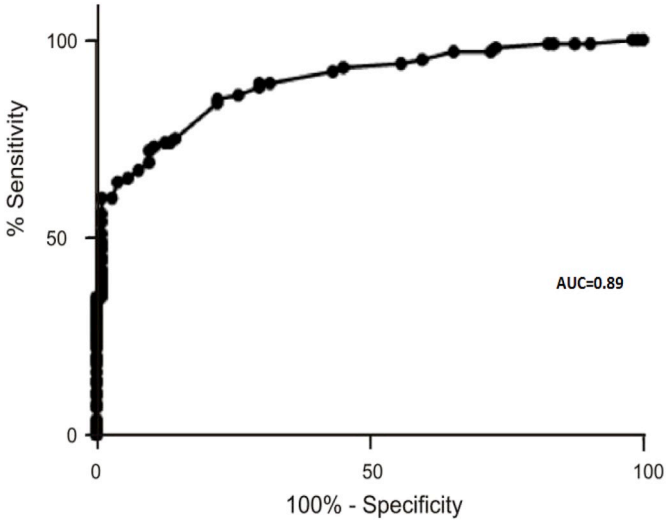

Fig. 1. ROC curve made from the analysis of the serum panel.

compared with the CFT results.

3. Results

3.1. ELISA test

The cut-off point from the serum panel was set at an optical density of 0.42. This cut-off point allowed the optimization of the sensitivity and specificity and yielded the lower number of false negative and false positive results. The obtained sensitivity and specificity were 60% and 99%, respectively. The analysis through the ROC curve yielded an AUC value of 0.89 (Fig. 1). This value indicates a high probability of distinguishing between animals with or without bTB when using this ELISA.

3.2. Combined diagnostic strategy: CFT-ELISA

According to the obtained sensitivity and specificity values of the assessed ELISA (60% Se and 99% Sp), the predicted sensitivity and specificity for the combined tests (negative CFT followed by ELISA) were 93.5% and 95.8%, respectively (Table 1).

3.3. Field evaluation of the combined diagnostic strategy

The 14 analyzed farms exhibited an average of 7.8% (0.5–22%) CFT positive reactors, including 587 CFT positive bovines with in the whole analyzed population. The remaining CFT negative animals (6,907) were analyzed by ELISA; this analysis yielded 200 positive cases (Table 2). The average CFT negative/ELISA positive bovines was 2.9% (0.6–8%). According to the results presented in Table 3, the proportion of CFT positive animals in each farm directly correlated with that of ELISA-positive animals ($r = 0.92\%$; p value < 0.0001).

A subset of the 200 CFT negative/ELISA positive animals was necropsied for further analysis. From the 33 necropsied animals were infected with M. bovis and the remaining three lacked the typical macro

Table 1
Theoretical calculation of the combined series used for CFT followed by ELISA.

TEST	SENSITIVITY	SPECIFICITY
CFT - T1[a]	83.9%	96.8%
ELISA - T2	60%	99%
CFT followed by ELISA	93.5%	95.8%

[a] Median value as described by De la Rua Domenench [3].

Table 2
Strategy used in combined series tests (CFT followed by ELISA) including the 7,494 animals analyzed in the present study.

TEST	POSSIBLE RESULTS		
CFT - T1	587 positive	6,907 negative	
ELISA - T2	T2 was not performed	6,707 negative	200 positive
Final interpretation	POSITIVE	NEGATIVE	POSITIVE

and microscopic lesions of the disease (Table 3). Regarding the IFN-γ assay, 25 (76%) of the necropsied animals were also negative to this ancillary test.

Of the 30 confirmed M. bovis infected animals, 22 (73%) exhibited generalized lesions during the inspection. In addition, 10 (30%) animals displayed pathologic changes in the mammary glands and 3 (9%) in uterus (Fig. 2). Regarding histopathology 28 (84%) animals presented microscopic lesions.

Regarding the bacteriology analysis, M. bovis was isolated in 25 (76%) of the necropsied animals, including those exhibiting generalized lesions. Five animals presumably negative showed microscopic changes compatible with bTB, acid fast bacilli within the necrotic tissue or within the cytoplasm of Langhans giant cells and a positive result to PCR for IS6110 sequence. The molecular typing confirmed the presence of SB0140, SB0120, SB0145 and SB0484 spoligotypes.

4. Discussion

In this study, the sensitivity and specificity for the analyzed ELISA were 60% and 99%, respectively. The sensitivity value was lower than those previously reported in other studies [22,23]. This may be explained by the different ELISA protocols performed or by the different capture antigen used in each case. Whereas in our study the antigenic mix comprised hundreds of proteins that form part of the tuberculin-PPD, Waters and coworkers [22] obtained sensitivity of 63% by using recombinant mycobacterial proteins MPB70 and MPB83 in the antigenic matrix. A similar antigenic proposal (ESAT-6/MPB70/MPB83 chimera) yielded values of 83.2% [23]. De la Rua Domenech et al. [3] emphasize that the incorporation of the MPB70 and MPB83 antigens into the assays would allow a greater specificity, although this procedure would not significantly increase the sensitivity of the serological diagnosis.

Harboe et al. [10] reported that the application of IDR stimulated the production of antibodies in infected cattle, thus significantly increasing the sensitivity diagnosis based on humoral response to MPB70. Recent studies reported an anamnesic response or booster effect with enhanced M. bovis antibody titers after applying the skin test. Indeed, Casal et al. [7] demonstrated that IDR induced the booster effect at antibody levels in periferial blood of cattle with bTB. Furthermore, in that study, the ELISA sensitivity increased from 24% to 70% in sera collected 15 days after performing the IDR. Waters et al. [11] also reported the booster effect as one of the main aspects for an effective serological diagnosis. In both studies this effect was verified through the specific antibody response to MPB70 and MPB83. On the other hand, Ritacco et al. [6] and Hanna et al. [24] described a booster effect when using bovine PPD as an antigen in the ELISA test.

In the first stage of the present study, in which a 60% of sensitivity was obtained, the sera was collected at the time of necropsy or sampling. Thus the time elapsed from CFT application and therefore the booster effects have not been considered. In the second stage the booster effect was considered, increasing the sensitivity to 91%.

Waters et al. [22] evaluated a commercial ELISA composed of different samples donated in collaboration by several reference laboratories without taking into account the booster effect. The sensivity and specifity obtained in that study were of 63% and 98%, respectively.

The composition of the serum panel used to evaluate serological

Table 3
Additional information of the 33 animals (CFT negative-/ELISA positive) analyzed during the second stage of the study.

Herds	Bovine	Size of herd	Prevalence IDR	Prevalence ELISA	γ IFN	Lesions	Bacteriology	Histophatology	PCR
1	1	607	8.6% (52+/607)	3.5% (19+/555)	–	Generalized	+	+	+
	2				+	Respiratory	+	+	+
2	3	498	22% (109+/498)	7.2% (28+/389)	+	Generalized	+	+	+
3	4	426	1% (5+/426)	0.9% (4+/421)	–	Generalized	+	+	+
	5				–	Generalized	+	+	+
4	6	689	3.5% (24+/689)	3.3% (22+/665)	–	Generalized	+	+	+
	7				–	Generalized	+	+	+
	8				–	Generalized	+	+	+
	9				–	Generalized	+	+	+
	10				–	Generalized	+	+	+
5	11	1031	20% (206+/1031)	5.6% (46+/825)	+	Generalized	+	+	+
	12				–	Generalized	+	+	+
6	13	296	11% (32+/296)	3% (8+/264)	+	Generalized	+	+	+
	14				–	LN retrop.[b]	NG[e]	+	+
7	15	360	1% (4+/360)	1% (4+/356)	–	Digestive	NG[e]	+	+
	16				–	Digestive	+	–	+
8	17[a]	638	1.4% (9+/638)	0.6% (4+/631)	–	Digestive[c]	NG[e]	–	–
	18				–	Generalized	+	+	+
9	19	550	3% (17+/550)	1.6% (8+/533)	–	Generalized	+	+	+
	20				–	Generalized	+	+	+
10	21	590	0.5% (3+/590)	0.6% (4+/587)	–	LN retrop.[b]	NG[e]	–	+
	22[a]				–	NAL[d]	NG[e]	–	–
11	23	377	16.5% (62+/377)	8% (26+/315)	–	Generalized	+	+	–
	24				–	Digestive	NG[e]	+	–
12	25	472	6% (28+/472)	2.7% (12+/444)	+	Generalized	+	+	+
	26				+	Generalized	+	+	+
	27				+	Generalized	+	+	+
13	28	612	2.8 (17+/612)	1% (6+/595)	–	Generalized	+	+	+
	29				–	Respiratory	NG[e]	+	+
	30				–	Generalized	+	+	+
	31[a]				–	Digestive[c]	NG[d]	–	–
14	32	348	5.5% (19+/348)	2.7% (9+/329)	–	Generalized	+	+	+
	33				+	Respiratory	+	+	+

[a] Animals considered negative (3/33).
[b] Retropharyngeal lymph nodes.
[c] Digestive: suspicious lesion in the liver, not specific to bTB.
[d] NAL: No Apparent Lesions.
[e] NG: No Growth after the incubation period.

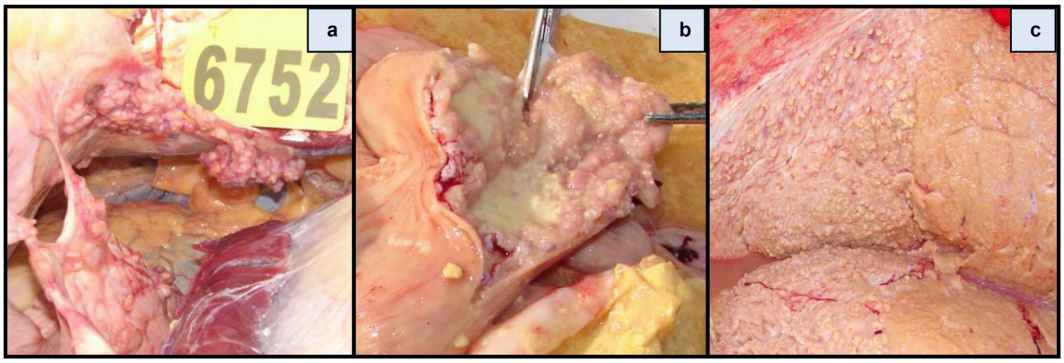

Fig. 2. Lesions compatible with bovine tuberculosis found during necropsies (a)Respiratory (visceral and parietal pleural), (b)Uterus, (c)Mammary gland.

tests is essential because it will determine test validity. Therefore, serum collections should be representative enough of the animals to be analyzed. In several studies, the results obtained in the ELISA were estimated based on the results obtained from positive IDR cattle [7,25,26]. However, we believe that it would be more appropriate to evaluate the use of the ELISA, and its true impact, on negative IDR cattle, in order to optimize the detection of infected cattle. This is the reason why the second stage of this study was carried out with negative CFT cattle of endemic bTB herds. In this way, we would identify anergic animals (false negatives to the CFT).

Previously, Waters et al. [27] have suggested that the strategic application of IDR and ELISA combined could improve of the sensitivity of the diagnostic system and this in turn could enhance the surveillance and control of bTB. Therefore, this proposed scheme would decrease the persistence of animals false negative reactor but infected. Considering the obtained values of sensitivity and specificity (60% and 99% respectively), together with the average values of the IDR (83.9% Se - 96% Sp), we expected to obtain a synergy between both techniques. The expected theoretical calculation yields a sensitivity of 93.5% and specificity of 95.8%. The sensivity obtained in the second stage of the present study were similar to the theoretical calculation performed by combining both tests (CFT negative followed by ELISA: 91% and 93.5%, respectively).

At the herd level, those farms with a higher proportion of positive CFT reactors also showed a higher number of ELISA positive animals. Herds with high prevalence of CFT reactors are expected to have higher

proportion of anergic animals as evidenced by Lilenbaum et al. [25]. Thus, the application of ELISA as a complement to the cell-mediated response-based test CFT seems to be an useful diagnostic strategy to detect additional infected animals missed by CFT.

Out of 200 animals exhibiting a CFT negative/ELISA positive result, a subset of 33 were necropsied for further analysis. Most of these examined animals (76%; 25/33) were also negative to the IFN-γ analysis. Thus, these animals would be exhibiting a deficiency in the cellular immune response. Different factors can give rise to false-negative responses in the tuberculin skin test. For example, these false-negative individuals may be in the early stages of M. bovis infection. Other factors giving rise to false positives are concurrent viral infection, the use of immunosuppressive drugs, incorrectly administered test, etc. [3,28,29]. A generalized infection, nutritional and transport stress could also contribute to anergy. Previous studies of cellular-mediated immunity have shown that the intradermal test and the IFN-γ assay detect overlapping, but also distinct, populations of M. bovis in infected animals [9,30,31]. This could explain the divergence in the results obtained in both tests. Indeed, 24% of the CFT negative animals were positive by the IFN-γ analysis.

The presence of anergic cattle in endemic herds has been previously described [8,25,27]. The macroscopic bTB lesions in 91% (30/33) of the analyzed bovines support the use of ELISA in the identification of these animals, as proposed by Plackett et al. [8] and Whipple et al. [32]. It is interesting to note that the CFT was repeatedly negative in all the animals that presented generalized lesions (73%). The false negative results in this screening test could represent a focus of reinfection at the herd level. This situation, in addition to the economic damages inflicted to farmers, causes a negative effect on the sanitation process and progress in the control of bTB. The lesions found in the mammary glands of 30% of the necropsied animals would indicate a possible shedding of mycobacteria through milk. The 14 farms of the study supplied raw milk to their calves and therefore some of these CFT negative bovines may represent a risk of infection to these young animals [33,34]. This finding highlights the risk of the oral transmission which could disseminate the Btb and could affect the economic sustainability of these farms. This also represents a potential risk to humans taking into account that consumption of raw milk is still common in rural areas.

The analysis of the humoral response revealed similar optical density values (0.9–1.2) among the 33 animals analyzed. However, in this preliminary study, the data of this necropsy analysis comes from a small sample size and therefore should be reinforced with a larger analysis in future studies. Furthermore, it should be investigated the possible relationship between the values of optical densities and the extent of the lesions found in positive animals.

The results obtained in the present study confirm that the ELISA can detect humoral responses against M. bovis in CFT false negative cattle. Thus, this ELISA can successfully complement the classic tools for bTB diagnostic in endemic bTB herds. This would allow diagnostic synergism and would be a more effective identification of M. bovis infected cattle with positive implicancies in the control of the disease. Future studies should be carried out to validate this diagnostic test based on the detection of specific antibodies against the bovine PPD.

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Declarations of interest

The authors declare no conflict of interest.

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Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.05.006.

References

[1] Magnano G, Severina W, Macias A, Sanchez J, Sticotti E, Macio M, Schneider M, Bérgamo E, Giraudo J. Impacto de la tuberculosis bovina sobre la producción láctea en un establecimiento de la provincia de Córdoba. Vet Argent 2016;XXXIII:336.
[2] Boland F, Kelly GE, Good M, More SJ. Bovine tuberculosis and milk production in infected dairy herds in Ireland. Prev Vet Med 2010;93:153–61.
[3] De la Rua Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS. Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci 2006;81(2):190–210. Oct.
[4] Rothel JS, Jones SL, Corner LA, Cox JC, Wood PR. A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. Aust Vet J 1990;67(4):134–7. Apr.
[5] Wood PR, Corner LA, Rothel JS, Baldock C, Jones SL, Cousins DB, McCormick BS, Francis BR, Creeper J, Tweddle NE. Field comparison of the interferon gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis. Aust Vet J 1991;68:286–90.
[6] Ritacco V, López B, Barrera L, Nader A, Fliess E, de Kantor. IN. Further evaluation of an indirect enzyme-linked immunosorbent assay for the diagnosis of bovine tuberculosis. J Vet Med 1990;37:19–27.
[7] Casal C, Díez-Guerrier A, Alvarez J, Rodriguez-Campos S, Mateos A, Linscott R, Martel E, Lawrence JC, Whelan C, Clarke J, O'Brien A, Domínguez L, Aranaz A. Strategic use of serology for the diagnosis of bovine tuberculosis after intradermal skin testing. Vet Microbiol 2014;170:342–51.
[8] Plackett P, Ripper J, Corner LA, Small K, de Whitte K, Melville L, Hides S, Wood PR. An ELISA for the detection of anergic tuberculous cattle. Aust Vet J 1989;66:15–9.
[9] Vordermeier M, Goodchild A, Clifton-Hadley R, de la Rua Domenech R. The interferon-gamma field trial: background, principles and progress. Vet Rec 2004;155:37–8.
[10] Harboe M, Wiker HG, Duncan JR, Garcia MM, Dukes TW, Brooks BW, Turcotte C, Nagai S. Protein G-based enzyme-linked immunosorbent assay for anti-MPB70 antibodies in bovine tuberculosis. J Clin Microbiol 1990;28(5):913–21. May.
[11] Waters WR, Palmer MV, Stafne MR, Bass KE, Maggioli MF, Thacker TC, Linscott R, Lawrence JC, Nelson JT, Esfandiari J, Greenwald R, Lyashchenko KP. 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. ClinVaccine Immunol 2015;22(6):641–9. Jun.
[12] de Agricultura Secretaría. Dirección de Sanidad animal, Argentina. “Plan nacional de Control y erradicación de la Tuberculosis bovina,” resolución Nº 128/2012. SENASA/SAGPyA; 2012.
[13] Ritacco V, de Kantor IN, Barrera L, Nader A, Bernardelli A, Torrea G, Errico F, Fliess E. Assessment of the sensitivity and specificity of Enzyme-linked Immunosorbent Assay (ELISA) for the detection of Mycobacterial antibodies in bovine tuberculosis. J Vet Med A 1987;34:119–25.
[14] Secretaría de Agricultura. Dirección de Sanidad Animal, Argentina. “Declaración de la provincial de Tierra del Fuego, Antártida e Islas del Atlántico Sur como libre de brucelosis y tuberculosis bovina”. 2011. Resolución Nº 100/2011, SENASA/SAGPyA.
[15] Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993;39(4):561–77. Apr.
[16] León EA, DuffySJ. Pruebas diagnósticas: principios y métodos para su evaluación e interpretación. Temas de Zoonósis III. 2006;48:416–21. Cap.
[17] Laisse CJM, Gavier-Widén D, Ramis G, Bila CG, Machado A, Quereda JJ, Ågren EO, van Helden PD. Characterization of tuberculous lesions in naturally infected African buffalo (Syncerus caffer). J Vet Diagn Investig 2011;23:1022–7.
[18] de Kantor IN. Bacteriología de la Tuberculosis humana y animal. CEPANZO, OPS/OMS. Serie de Monografías. 1989;11:63.
[19] Manual de diagnóstico de micobacterias de importancia en medicina veterinaria. Comisión Científica de Micobacterias, Asociación Argentina de Veterinarios de Laboratorio de Diagnóstico; 2005. p. 20–8.
[20] Zumárraga MJ, Meikle V, Bernardelli A, Abdala A, Tarabla H, Romano MI, Cataldi A. Use of touch-down polymerase chain reaction to enhance the sensitivity of Mycobacterium bovis detection. J Vet Diagn Investig 2005;17:232–8.
[21] Kamerbeek L, Schouls A, Kolk M, van Agterveld D, van Soolingen S, Kuijper A, Bunschoten H, Molhuizen R, Shaw M, vanEmbden GJ. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and

epidemiology. J Clin Microbiol 1997;35:907–14.
[22] Waters WR, Buddle BM, Vordermeier HM, Gormley E, Palmer MV, Thacker TC, Bannantine JP, Stabel JR, Linscott R, Martel E, Milian F, Foshaug W, Lawrence JC. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. ClinVaccineImmunol 2011;18:1882–8.
[23] Souza II, Melo ES, Ramos CA, Farias TA, Osório AL, Jorge KS, Vidal CE, Silva AS, Silva MR, Pellegrin AO, Araujo FR. Screening of recombinant proteins as antigens in indirect ELISA for diagnosis of bovine tuberculosis. SpringerPlus 2012;1(1):77.
[24] Hanna J, Neill SD, O'Brien JJ. ELISA tests for antibodies in experimental bovine tuberculosis. Vet Microbiol 1992;31:243–9.
[25] Lilenbaum W, Fonseca L. The use of Elisa as a complementary tool for bovine tuberculosis control in Brazil. Braz J Vet Res Anim Sci 2006;43:256–61.
[26] Thakur MK, Sinha DK, Singh BR. Evaluation of complementary diagnostic tools for bovine tuberculosis detection in dairy herds from India. Vet World 2016;9(8):862–8. Aug.
[27] Waters WR, Vordermeier HM, Rhodes S, Khatri B, Palmer MV, Maggioli MF, Thacker TC, Nelson JT, Thomsen BV, Robbe-Austerman S, Bravo Garcia DM, Schoenbaum MA, Camacho MS, Ray JS, Esfandiari J, Lambotte P, Greenwald R, Grandison A, Sikar-Gang A, Lyashchenko KP. Potential for rapid antibody detection to identify tuberculous cattle with non-reactive tuberculin skin test results. BMC Vet Res 2017;13(1):164. Jun 7.
[28] Snider JR. The tuberculin skin test. Am Rev Respir Dis 1982;125(3 Pt 2):108–18. Mar.
[29] Buddle BM, Livingstone PG, de Lisle GW. Advances in ante-mortem diagnosis of tuberculosis in cattle. NZ Vet J 2009;57(4):173–80. Aug.
[30] Coad M, Downs SH, Durr PA, Clifton-Hadley RG, Hewinson RG, Vordermeier HM, Whelan AO. Blood-based assays to detect Mycobacterium bovis-infected cattle missed by tuberculin skin testing. Vet Rec 2008;162:382–4.
[31] Neill SD, Cassidy J, Hanna J, Mackie DP, Pollock M, Clements A, Walton E, Bryson DG. Detection of Mycobacterium bovis infection in skin test-negative cattle with an assay for bovine interferon-gamma. Vet Rec 1994;135:134–5.
[32] Whipple DL, Bolin CA, Davis AJ, Jarnagin JL, Johnson DC, Nabors RS, Payeur JB, Saari DA, Wilson AJ, Wolf MM. Comparison of sensitivity of the caudal fold skin test and commercial gamma-interferon assay for diagnosis of bovine tuberculosis. Am J Vet Res 1995;56:415–9.
[33] Evangelista TBR, De Anda JH. Tuberculosis in dairy calves: risk of Mycobacterium spp. exposure associated with management of colostrum and milk. Prev Vet Med 1996;27:23–7.
[34] Garro C, Garbaccio S, Cobos Roldán M, Oriani S. Tuberculosis in calves: results of a prospective study. Rev Electron Vet 2011;12.