Validation of a new serological assay for the identification of *Mycobacterium tuberculosis* complex-specific antibodies in pigs and wild boar

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ABSTRACT

Background and objectives: Animal tuberculosis (TB) is a multi-host disease involving a wide variety of domestic and wild mammals and causing a significant economic burden and sanitary problems. Wild boar and domestic pigs (*Sus scrofa*) are indicators of the circulation of the *Mycobacterium tuberculosis* complex (MTC) and can play a role in its maintenance. The proper diagnosis of MTC contact in these species is, therefore, a key factor as regards controlling TB. The objective of the current study is to evaluate the diagnostic performance of the protein complex P22 as a candidate for use in an in-house ELISA to identify *M. tuberculosis* complex-specific antibodies for the diagnosis of TB in comparison to the commonly used bPPD-based ELISA (bPPD ELISA) in suids.

Methods: We conducted a retrospective study. Sera were collected from wild boar during hunting season and from domestic pigs during routine handling, and all the animals underwent reference standard tests (detailed necropsy followed by bacteriological culture and isolation). Animal TB was confirmed to be positive in 277 animals and negative in 366 animals based on both reference standard tests. Sera from those animals were tested by P22 ELISA as well as bPPD ELISA.

Results: Both ELISAs yielded a good diagnostic value, however, a higher sensitivity (Se) and specificity (Sp) was achieved with the P22 ELISA (Se: 84.1%; CI_{95%}: 79.3–88.2% / Sp: 98.4%; CI_{95%}: 96.5–99.4%) when compared to the bPPD ELISA (Se: 77.3%; CI_{95%}: 71.9–82.2% / Sp: 97.3%; CI_{95%}: 95–98.3%). An optimum Sp of 100% (CI_{95%}: 98.54–100%) was attained with white pigs for both the bPPD and the P22 ELISA.

Discussion: The results suggest that serological tests for MTC-antibody detection, and particularly the P22 ELISA, are valuable tools in the diagnosis of TB in wild boar and domestic pigs when attempting to detect contact with MTC and thereby facilitate TB control and management.

1. Introduction

Animal tuberculosis (TB) is a globally relevant multi-host disease caused by *Mycobacterium bovis* and other closely related members of the *M. tuberculosis* complex (MTC), which infect a wide variety of mammals including domestic and wild animals (Waters et al., 2016). This disease

causes a significant economic burden and sanitary problems in the farming industry (Chambers, 2013), and is also responsible for zoonotic problems, mainly in developing countries (Michel et al., 2010).

The role of wild and domestic hosts in the epidemiology of animal TB varies between regions (Gortázar et al., 2012). Suids have been recognized to play a significant epidemiological role in animal TB in

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different areas, particularly in the case of the Eurasian wild boar (*Sus scrofa*). Wild boar TB has been reported in many European countries (Gortázar et al., 2015), in addition to Asia (Jang et al., 2017), northern Africa (El Mrini et al., 2016), and South America (Maciel et al., 2018). Moreover, scientific evidence suggesting that this species plays a key role in the maintenance of MTC on the Iberian Peninsula is accumulating (Naranjo et al., 2008; Gortázar et al., 2015, 2017).

Domestic pigs (and feral swine) are often considered as ‘dead end hosts’ for MTC (Corner, 2006; Nugent et al., 2015). However, in certain epidemiological scenarios, several studies have shown that free-ranging domestic pigs can act as maintenance hosts for MTC, becoming a true reservoir (Di Marco et al., 2012) or amplifier host (Coleman and Cooke, 2001). In any case, domestic pigs and feral swine are useful sentinels for MTC (Corner, 2006; Bailey et al., 2013; Nugent et al., 2015). Animal TB has also been reported in other suid species such as bush pigs (*Potamochoerus porcus*) (Hlokwe et al., 2014) and warthogs (*Phacochoerus africanus*) in Africa (Miller et al., 2016) and collared peccaries (*Tayassu tajacu*) in South America (Mayer et al., 2012).

Hence, proper identification of wild and domestic suids infected with TB, followed by implementation of suitable management strategies, is important for an effective TB control (Csivincsik et al., 2016). Standard *in vivo* diagnostic methods included in the current eradication programs of animal TB, generally in cattle and other ruminants, are based on measuring the cell-mediated immune (CMI) response to MTC (Buddle et al., 2009), which can be detected by using the tuberculin skin test or interferon-gamma (IFN γ) release assays (De la Rua-Domenech et al., 2006). Similar CMI-based tests are also available for suids, but have logistic constraints and are **therefore not** the first choice for these species (Jaroso et al., 2010; Pesciaroli et al., 2012; Beltrán-Beck et al., 2014).

Antibody-based diagnosis has been widely used on wild boar and feral swine owing to its ease of use and generally good performance (Boadella et al., 2011; Richomme et al., 2010; Pedersen et al., 2017). Table 1 presents the main serological assays used on wild boar and in pigs/feral swine. The most commonly used antigen is the purified protein derivative obtained from *M. bovis* (bovine PPD or bPPD) (Aurtenetxe et al., 2008; Boadella et al., 2011; Che'Amat et al., 2015); however, recent studies have focused on improving its specificity (Sp) by using more specific antigens, such as MPB83 (García-Bocanegra et al., 2012; Pérez de Val et al., 2017), MPB70 (Cardoso-Toset et al., 2017) or CFP10/ESAT-6 (Roos et al., 2016). New ELISA assays recently performed with an immunopurified subcomplex protein obtained from bPPD (CZ Veterinaria SA, Porriño, Spain), named P22 and composed of several antigens including MPB70, MPB83, ESAT-6 and CFP-10 (Infantes-Lorenzo et al., 2017), have provided a high Sp and sensitivity (Se) in cattle and mice (Casal et al., 2017; Infantes-Lorenzo et al., 2017). Therefore, we hypothesized that P22 ELISA would yield a higher diagnostic performance than bPPD ELISA in terms of Se and Sp for TB diagnosis in suids.

The present study was conducted with the objective of evaluating the diagnostic performance of P22 as a candidate for use in a new serological assay with which to identify *M. tuberculosis* complex-specific antibodies for the diagnosis of TB in wild boar and domestic pig.

2. Materials and methods

2.1. Animals

The study was performed retrospectively with samples from wild boar and domestic pigs collected from several sources. We included samples from wild boar, domestic pig in extensive management system (Iberian pig), as well as domestic pig in intensive management system (white pig) in order to avoid bias in the validation of the test and also to assess the performance of the test in two different systems of management, because of the fact that animals reared under extensive management systems or wildlife have more chances of exposure to MTC and

other environmental mycobacteria, compared to the animals reared under intensive management systems. The animals were not intentionally killed for this study but were rather hunter-harvested (wild boar) during the legal hunting season 2015–16 from September to March or routinely slaughtered (pigs) during the period 2015–16 from the southern and central Spain. No ethical approval was, therefore, deemed necessary for this study. Protocols, amendments and other resources were used according to the guidelines approved by each autonomous government following the R.D.1201/2005 of the Spanish Ministry of Presidency. Blood and tissue samples were collected by veterinarians in compliance with the Ethical Principles in Animal Research. The study was compiled in accordance with STARD guidelines 2015 (Cohen et al., 2016).

All animals were subjected to a detailed post-mortem examination in order to assess the presence or absence of TB compatible lesions (TBL). Samples included in the study were selected using a consecutive sampling technique and sample size for index test validation was determined by incorporating all the samples from wild boar and domestic pigs with confirmed infection status (both positive and negative). The infection status was assessed on the basis of the presence of TBL followed by bacteriological culture and isolation (reference standard test). In this study, a TB-positive animal is defined as the one that has TBL and is also positive to the MTC culture, while a TB-negative animal is negative as regards both TBL and the MTC culture. The reference standard tests were performed in another laboratory and the results of reference standard were available to the assessors of index test and vice versa. Details of animals used for index test validation are summarized in Table 2.

2.2. Bacteriology

Tissue samples were placed in a sterile recipient and stored at -80°C until their bacteriological culture. All tissues with present or suspicious TBLs were cultured for MTC. When TBLs were not observed, a pool of mandibular, tracheobronchial and mediastinal lymph nodes (LNs) from each animal was also cultured. The culture of samples was performed on Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media (Difco, Madrid, Spain) after decontamination with a final concentration of 0.37% hexadecylpyridinium chloride (Corner and Trajstman, 1988). Isolates were identified by means of PCR (Wilton and Cousins, 1992) and spoligotyping (Kamerbeek et al., 1997).

2.3. Serum antibody detection

Blood samples (approximately 10 ml from each animal) were collected in tubes without additives by puncture of the orbital sinus in pigs and the cavernous sinus of the dura mater in wild boar (Arenas-Montes et al., 2013). Samples were centrifuged (1500 g for 10 min) and sera stored at -20°C until testing for the detection of MTC antibodies.

2.3.1. Indirect ELISA with P22

Serum samples were tested by means of an in-house ELISA using P22 as antigen and following the protocol described by Casal et al. (2017) with some modifications. Briefly, after coating the plates with the P22 antigen at a concentration of 10 $\mu\text{g}/\text{ml}$ in phosphate buffered saline (PBS) (ingredients of PBS; Panreac Química S.L.U., Barcelona, Spain), they were stored overnight at 4°C . The wells were subsequently washed with a PBS solution containing 0.05% Tween-20 (PBST) (Tween 20; Sigma Aldrich Inc., Munich, Germany) and blocked for 1 h at room temperature (RT) with 5% skimmed milk powder (commercially available) solution in PBS (SM). Sera were added in duplicate at a dilution of 1:100 in SM, incubated stationary for 1 h at 37°C and subsequently washed three times with PBST. Anti-porcine IgG (HRP)-conjugated (Bethyl Laboratories, Montgomery, USA) at a concentration of 1:20,000 in SM was added and the plates were incubated for 1 h at RT. Following four washes with PBST, color was developed with o-

Table 1
Serodiagnostic tests for the diagnosis of tuberculosis in swine.

Assay test	Sample	N/E	Number of animals (n _{Se} + n _{Sp})	Antigen	Sensitivity (%)	Specificity (%)	References
ELISA- protein G	Eurasian wild boar	N	96 + 104	bPPD	79.2	100	Boadella et al., 2011
ELISA- IgG	Eurasian wild boar	E	17 + 17	bPPD	94.4	100	Beltrán-Beck et al., 2014
	Domestic pig	N	66 + 23	bPPD	86.4	65.2	Mohamed et al., 2012
				Ag85-B	87.9	86.9	
ELISA-IgM	Eurasian wild boar	E	17 + 17	bPPD	77.7	100	Beltrán-Beck et al., 2014
ELISA- protein A&G	Eurasian wild boar	N	16 + 15	bPPD	72.7 ^a	100 ^a	García-Bocanegra et al., 2012
				MPB83	100	100	
	Eurasian wild boar	N	22 + 43	MPB83	86	100	Pérez de Val et al., 2017
	Common warthog	N	16 + 19	bPPD	88	89	Roos et al., 2016
	Common warthog	N	25	bPPD	92		Roos et al., 2018
ELISA TB -VK (Vacunek, Spain)	Eurasian wild boar	N	73 + 112	bPPD	72.6	96.4	Aurtenetxe et al., 2008
	Common warthog	N	16 + 19	bPPD	88	79	Roos et al., 2016
	Domestic pig	N	59 + 88	bPPD	72.9	100	Cardoso-Toset et al., 2017
	Common warthog	N	25	bPPD	86		Roos et al., 2018
ELISA- INgezim TB (INGENASA S.L., Spain)	Domestic pig	N	59 + 88	MPB7, MPB83	74.6	98.9	Cardoso-Toset et al., 2017
Rapid Test DPP WTB (Chembio Diagnostic Systems, Inc., USA)	Eurasian Wild boar	E	16 + 16	MPB83, MPB70	94.11	100	Beltrán-Beck et al., 2014
Rapid Test DPP VetTB (Chembio Diagnostic Systems, Inc., USA)	Eurasian wild boar	N	64 + 113	MPB83, ESAT-6, CFP10	76.6	97.3	Lyashchenko et al., 2008
	Eurasian wild boar	N	96 + 104	MPB83, ESAT-6, CFP10	89.6	90.4	Boadella et al., 2011
	Eurasian wild boar	E	17 + 17	MPB83, ESAT-6, CFP10	77.8	100	Beltrán-Beck et al., 2014
	Common warthog	N	16 + 19	MPB83, ESAT-6, CFP10	75	89	Roos et al., 2016
Rapid Test INgezim TB-CROM (INGENASA S.L., Spain)	Domestic pig	N	59 + 88	MPB83	74.6	98.9	Cardoso-Toset et al., 2017

N/E - Natural or experimental infection, ELISA-Enzyme linked immunosorbent assay, DPP-Dual Path Platform Technology.

n_{Se}- number of TB positive animals used for evaluation of Se, n_{Sp}- number of negative animals used for evaluation of Sp.

^a Se and Sp were evaluated based on mixed species of animals.

Table 2
Details of the animals used for validation of index test.

Sample	Number	TB status ^a
Wild boar	236	TB-positive
	103	TB-negative
Iberian pig	41	TB-positive
	12	TB-negative
White pig	251	TB-negative
Total = 643 (277 TB-positive and 366 TB-negative animals)		

^a TB-positive: presence of TB-compatible lesions and positive MTC culture; TB-negative: absence of TB-compatible lesions and negative MTC culture.

phenylenediamine dihydrochloride substrate (FAST OPD, Sigma Aldrich Inc., Munich, Germany), and the samples were incubated for 20 min in darkness and RT conditions. The reaction was stopped with H₂SO₄ 2 M and the optical density (OD) measured in a spectrophotometer at 450 nm.

With the aim of evaluating the repeatability of the assay, the same positive and negative controls were included in quadruplicate in every plate of the study (which were tested on the same day and on different days, as well as by different operators). The positive controls were obtained from MTC infected wild boar, previously confirmed by the presence of TBL and *M. bovis* culture, as well as by bPPD ELISA (high and low ODs) for MTC-antibodies. The negative controls were obtained from TB-free wild boar from TB-free areas, previously confirmed as MTC negative by culture and without the presence of TBL, and negative to bPPD ELISA for MTC-antibodies.

2.3.2. Indirect ELISA with bPPD

The serum samples were tested by following the same in-house ELISA protocol described above, but using bPPD (CZ Veterinaria SA, Porriño, Spain) as antigen at a concentration of 5 µg/ml. The positive and negative controls used were the same as for P22 antigen. This methodology, with slight modifications, has been previously used for the identification of MTC specific antibodies in wild boar and domestic pigs (Che'Amat et al., 2015; Cardoso-Toset et al., 2017).

2.4. Data analysis

The ELISA results were expressed as an ELISA percentage (E%), calculated using the following formula: [sample E% = (mean sample OD/ (2 x mean of negative control OD)) × 100]. The cut-off value was calculated using a ROC analysis and was defined as the value at which the highest sum of Se plus Sp was obtained (Aurtenetxe et al., 2008). The presence or absence of TBL and mycobacterial culture was employed as a reference standard. The discriminatory indexes for Se and Sp were calculated as the ratio between each one and the complementary indexes of the other (Se/(1-Sp) and Sp/(1-Se), respectively) in order to assess test performance. Confidence intervals for the positive and negative predictive values (PPV and NPV, respectively) of the Se and Sp were calculated using the Clopper-Pearson method (Graphpad prism 5.0, USA). The inter-test agreement between the evaluated assays was calculated by means of Cohen's kappa coefficient (Epitools, Ausvet Pty Ltd., Canberra, Australia). All statistical analyses were performed at a confidence level of 95% (CI_{95%}).

3. Results

The presence of TBL was diagnosed by necropsy of the entire animal with macroscopic inspection of LNs, abdominal and thoracic organs, where lesions were especially prominent in lung, as well as in mandibular and tracheobronchial LNs. The presence of TBL was confirmed or not by isolation of the microorganism through MTC culture. Animals found to be positive to either TBL only or culture only were omitted from the study. Animals positive or negative to both techniques were

Fig. 1. Diagnostic value graphs for the serodiagnosis of tuberculosis in swine (wild boar and domestic pigs) when using the *Mycobacterium bovis*-derived immunopurified P22 or the purified protein derivative (bPPD) as antigens. Sensitivity (Se), specificity (Sp) and their semi-sum are the percentages on the primary Y-axis. Discriminatory indexes for Se [Se/ (1-Sp)] and Sp [(Sp/ (1-Se))] are on the secondary Y-axis.

included in the study for index test validation. The flow of participants used in this study is summarized in a diagram in Supplementary material (Fig. S¹).

The diagnostic value of P22 and bPPD ELISA in swine, obtained by means of a ROC analysis (Fig. 1), shows that the P22 ELISA had a slightly better sum of Se + Sp (184) than the bPPD ELISA (179). The cut-off value was defined as the ratio of the mean sample OD to the double of the mean OD of the negative control. The P22 ELISA with a cut-off value set at 100 E% had both a higher Se (84.1%) and a higher Sp (98.4%) when compared to the values attained with bPPD ELISA (Se: 77.3%, Sp: 97.3%). Modifying the cut-off value (greater than or less than 100 E%) resulted in either a decreased Sp or a constant Se, so that a cut-off value of 100 was chosen for the P22 ELISA. Moreover, both discriminatory indices of Se and Sp reached their optimum value in P22 ELISA at the cut-off value chosen (100 E%), being higher than those achieved in bPPD ELISA and indicating a good level of ability to predict and discriminate the true positive and true negative individuals. The cross tabulation of P22 and bPPD ELISA results by reference standard is summarized in Supplementary material (Table S-2).

Data regarding Se, Sp, PPV, NPV, area under the curve (AUC), and kappa values (K), along with a CI₉₅ for both ELISAs at the chosen cut-off value of 100E% are summarized in Table 3. In this context, if Sp is evaluated within the different management systems, we observe that it was 94.78% (CI₉₅: 88.99–98.06%) and 91.30% (CI₉₅: 84.59–95.75%) for P22 and bPPD ELISA, respectively, in animals reared under extensive management systems (wild boar and Iberian pig). However, an optimum Sp of 100% (CI₉₅: 98.54–100%) was attained with animals

Table 3

Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), area under the curve (AUC) and kappa coefficient (K) for P22 and bPPD ELISA in swine.

Test	Se		Sp		PPV		NPV		AUC		K	
	%	CI ₉₅ ^a	%	CI ₉₅	%	CI ₉₅	%	CI ₉₅		CI ₉₅		CI ₉₅
P22 ELISA	84.1	79.3–88.2	98.4	96.5–99.4	97.5	94.6–99.1	89.1	85.7–92.0	0.94	0.92–0.96	0.84	0.8–0.88
bPPD ELISA	77.3	71.9–82.1	97.3	95–98.3	95.5	92–97.8	85.0	81.2–88.3	0.93	0.91–0.95	0.76	0.71–0.81

Cut-off value chosen for ELISA was 100 E% (ELISA percentage), using the presence of TB compatible lesions and *Mycobacterium tuberculosis* complex culture as reference standards.

^a 95% confidence intervals.

Fig. 2. Histogram of the distribution of the ELISA percentage (E%) values of individual animals tested by ELISA using P22 and bPPD as antigens, respectively, according to their known TB status. (Wild boar - ●, Iberian pig - ⊗, White pig - ○). The horizontal line represents the chosen cut-off value.

reared under intensive systems of management (white pigs), in both bPPD and P22 ELISA. The histogram of the species-wise distribution of the ELISA value (E%) of individual TB positive and negative animals is represented in Fig. 2. Positive and negative predictive values are found to be higher in P22 ELISA than in bPPD (Table 3), which indicates comparatively higher diagnostic accuracy for P22 ELISA and a higher proportion of number of cases identified out of all positive test results.

4. Discussion

This study confirms that serological tests are valuable tools in the diagnosis of TB in wild boar and pigs. Moreover, the new P22 ELISA yielded a high serodiagnostic value in terms of Sp (98.4%) and Se (84.1%), improving the performance of the classic bPPD ELISA (Sp: 97.3%, Se: 77.3%).

Antibody diagnostic tests can be routinely used in the diagnosis and surveillance of TB in swine, as they constitute a rapid and cost-effective screening tool for samples that can be collected both ante and post mortem. Our study reinforces previous findings stating that these tests have a high diagnostic value (Table 1). In addition, antibody based diagnosis has been found to be more reliable in swine when compared with other species. Antibody production is closely related to infection status/lesion severity in swine, as found in several studies (Aurtenetxe et al., 2008; Lyashchenko et al., 2008; Garrido et al., 2011; Roos et al., 2016; Cardoso-Toset et al., 2017). In contrast, this correlation is not consistently reported in ruminants such as red deer (*Cervus elaphus*) (Thomas et al., 2017), fallow deer (*Dama dama*) (Boadella et al., 2012), cattle (Bezoz et al., 2014) or goats (Gutiérrez et al., 1998). This is also evidenced by the higher sum of Se + Sp in the ROC analysis, along with a high kappa agreement with the reference standards when compared to studies carried out on other species (Boadella et al., 2012; Buendía et al., 2013; Kang et al., 2016). The results obtained with the new antigen P22 coincide with recent reports of a high Sp and Se in mice (Infantes-Lorenzo et al., 2017) and a higher Se in cattle (Casal et al., 2017) when compared to bPPD.

The high Sp is attributed to the fact that the P22 antigen shares fewer proteins than bPPD with other mycobacteria not belonging to the MTC (Infantes-Lorenzo et al., 2017). This may minimize the proportion of false positive reactions in non-infected animals (Morés et al., 2006). The Sp of the bPPD ELISA was 100% in some previous studies (see Table 1), but either the sample size used for those studies was very low (experiments), or negative samples were taken from TB free herds. In this context, our study is innovative, since it includes a large sample size and field samples from wild boar and domestic pigs, regardless of their origin. A strict comparison with other previous studies is, therefore, difficult. However, the high Sp found in the aforementioned studies coincides with the finding we obtained in the case of white pigs in the present study, in which the Sp was 100% for both ELISAs. These pigs, which are housed in closed farm buildings, are not having exposure to mycobacteria able to elicit a cross reactive immunity that would influence on test Sp (Estevez et al., 2004). This contrasts with the possibility of exposure in the case of free ranging Iberian pigs and wild boar. Even so, the high global Sp of P22 ELISA (98.4%) observed in this study confirms that it could be used in epidemiological studies focused on farms as the unit of study, where the identification of a seropositive animal indicates with high probability that a herd is truly infected.

A good Se was found in both ELISAs, which is in line with previous findings reporting that 50% of the concentration of the 10 principal proteins of *M. bovis* is found in both bPPD and P22, five of which are predicted to be highly immunogenic (Infantes-Lorenzo et al., 2017).

The higher Se achieved in the P22 ELISA in this study confirms the hypothesis that suggests that replacing bPPD with P22 may increase the Se of the immunological diagnosis of TB because of the high abundance of MPB70 in *M. bovis* and its immunological properties (Harboe et al., 1990; Infantes-Lorenzo et al., 2017). Similarly, in the study of García-Bocanegra et al. (2012), a better Se was obtained with the MPB83 antigen in comparison to bPPD showing that, in addition to the high Sp, it is also possible to have a better Se with purified/specific antigens than with the whole bPPD protein complex.

This study has also limitations for the interpretation of the results. We used presence or absence of TBL as well as MTC culture as reference tests in order to determine the TB status of the animals. This means that the Se might be overestimated, due to the definition of a positive TB status in this study that causes a bias towards advanced cases of TB and its consequence on overall test performance, since the antibody response is more active towards the later stage of the disease (Waters et al., 2016). Moreover, it could be possible to find some infected animals without visible TBLs or false negative results in culture due to the possibility of the presence of microorganisms in the non-cultured tissues or organs which had no visible TBLs or due to decontamination prior to MTC culture that adversely affects the viability of mycobacteria, especially when the number of viable microorganisms is low (Stewart et al., 2013). Mycobacterial culture is often considered as an imperfect gold standard because if the gold standard test itself does not have a 100% Se, errors will arise in defining the Se of new diagnostic assays under validation (Banoo et al., 2008; Maas et al., 2013). Hence, in some cases requiring improved accuracy, the P22 ELISA could be interpreted in parallel to the diagnostic techniques based on CMI response, in order to improve the Se, as reported in other species (Harrington et al., 2008; Casal et al., 2017).

5. Conclusion

In conclusion, the serodiagnosis of TB in suids (pig and wild boar) is an efficient and simple method by which to monitor farmed or wild animal populations for the purpose of TB prevention and control. The immunopurified antigen complex P22 provided a better Se and Sp when compared to bPPD when used in an indirect ELISA for MTC-antibody detection. The P22-based indirect ELISA can be used as a cost effective, rapid and reliable tool for the large-scale screening and assessment of the TB status of wild boar and pigs.

Conflict of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.prevetmed.2018.11.004>.

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