Testing Eurasian wild boar piglets for serum antibodies against *Mycobacterium bovis*

Che' Amat, A.[a,b], González-Barrio, D.[a], Ortiz, J.A.[d], Díez-Delgado, I.[a,h], Boadella, M.[c], Barasona, J.A.[a], Bezos, J.[e,i], Romero, B.[e], Armenteros, J.A.[a], Lyashchenko, K.P.[f], Venteo, A.[g], Rueda, P.[g], Gortázar, C[a*] christian.gortazar@uclm.es

[a]SaBio IREC, Consejo Superior de Investigaciones Científicas, Universidad de Castilla–La Mancha, Junta de Comunidades de Castilla–La Mancha (CSIC-UCLM-JCCM), Ronda de Toledo, 13071 Ciudad Real, Spain

[b]Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

[c]SABIOtec, Camino Moledores s.n. 13005 Ciudad Real, Spain

[d]Departamento Veterinario, Cinegético e Investigación de Medianilla S.L., Cádiz, Spain

[e]Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Avenida de Puerta de Hierro, 28040 Madrid, Spain

[f]Chembio Diagnostic Systems, Inc., Medford, New York, USA

[g]Inmunología y Genetica Aplicada, S.A. (INGENASA), Hermanos García Noblejas, 39, 28037 Madrid, Spain

[h]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

[i]MAEVA SERVET SL, Alameda del Valle, Madrid, Spain.

* Corresponding author:

Highlights

- We explored several serological rapid immunochromatographic and ELISA tests for detection of antibodies against the *Mycobacterium tuberculosis* complex in wild boar piglets.
- Sensitivity was assessed using tuberculosis compatible lesions and *Mycobacterium tuberculosis* complex culture in naturally infected wild boar piglets.
- Rapid animal-side tests can contribute to TB control strategies by enabling the setup of test and cull schemes or improving pre-movement testing.
- However, sub-optimal test performance in piglets as compared to that in older wild boar should be taken into account.

Abstract

Animal tuberculosis (TB) caused by infection with *Mycobacterium bovis* and closely related members of the *M. tuberculosis* complex (MTC), is often reported in the Eurasian wild boar (*Sus scrofa*). Tests detecting antibodies against MTC antigens are valuable tools for TB monitoring and control in suids. However, only limited knowledge exists on serology test performance in 2 to 6 month-old piglets. In this age-class, recent infections might cause lower antibody levels and lower test sensitivity. We examined 126 wild boar piglets from a TB-endemic site using 6 antibody detection tests in order to assess test performance. Bacterial culture (n=53) yielded a *M. bovis* infection prevalence of 33.9%, while serum antibody prevalence estimated by different tests ranged from 19-38%, reaching sensitivities between 15.4% and 46.2% for plate ELISAs and between 61.5% and 69.2% for rapid immunochromatographic tests based on dual path platform (DPP) technology. The Cohen kappa coefficient of agreement between DPP WTB (Wildlife TB) assay and culture results was moderate (0.45) and all other serological tests used had poor to fair

agreements. This survey revealed the ability of several tests for detecting serum antibodies against the MTC antigens in 2 to 6 month-old naturally infected wild boar piglets. The best performance was demonstrated for DPP tests. The results confirmed our initial hypothesis of a lower sensitivity of serology for detecting *M. bovis*-infected piglets, as compared to older wild boar. Certain tests, notably the rapid animal-side tests, can contribute to TB control strategies by enabling the setup of test and cull schemes or improving pre-movement testing. However, sub-optimal test performance in piglets as compared to that in older wild boar should be taken into account.

Keywords

ELISA, *Mycobacterium tuberculosis* complex, sensitivity, *Sus scrofa*

1. INTRODUCTION

Animal tuberculosis (TB) is caused by infection with *Mycobacterium bovis* (*M. bovis*) and closely related members of the *M. tuberculosis* complex (MTC). The infection is often reported in wildlife, even in developed countries (Gortázar et al., 2012). The native Eurasian wild boar (*Sus scrofa*) is considered the greatest TB hazard of all wildlife species in Europe (Hardstaff et al., 2014) and particularly is the single most important MTC maintenance host species in Mediterranean ecosystems of the Iberian Peninsula (Gortázar et al., 2008; Naranjo et al., 2008). High TB prevalence of above 60% is sometimes reported in wild boar from sites with a high infection pressure in central and southern Spain (Vicente et al., 2013).

The prevalence of *M. bovis* infection increases with age in wild boar. In 7-12 month old yearlings (those harvested by hunters in their first autumn-winter season), prevalences reported ranged from 20 – 52% (Vicente et al., 2006, 2013; Díez-Delgado et al., 2014). Infection of yearling wild boar was also noted in a survey on the MTC member *M. microti* in Italy (11.11%; Chiari et al., 2015). However, published information refers almost exclusively to wild boar over six months of age, since it is generally based on hunter-harvested individuals sampled in autumn and winter. Data on MTC prevalence are almost lacking in piglets (less than 7 months old). Exceptionally, a study based on culled (not hunted) wild boar in the TB endemic Doñana National Park in southern Spain found 3 out of 3 piglets sampled in summer to be *M. bovis* infected (Gortázar et al., 2008). In addition, a recent survey in eastern Spain also based on culled

wild boar showed a summer prevalence of 6.3% in wild boar piglets less than 6 months (Mentaberre et al., 2014). Field observations show that lesion scores are lower in piglets than in yearling or subadult wild boar (Díez-Delgado, pers. comm.). Considering the correlation between lesion scores and serum antibody levels against *M. bovis* reported in wild boar (Garrido et al., 2011) and the predominance of early infections in piglets, serology interpretation may be particularly challenging in this age category.

The gold standard confirmation for MTC infection is microbiological culture and identification (OIE, 2009). However, serological methods are often used in wildlife because of their relatively low cost, simplicity, speed and the advantage of easily conducted retrospective studies (Chambers, 2009). The serology can be performed in live animals and is a suitable test towards the harmonization of wild boar testing for large scale surveys eventually involving several countries (Beerli et al., 2015). A conventional enzyme-linked immunosorbent assay (ELISA) using bovine purified protein derivative (bPPD) has shown a moderate sensitivity (73%) and good specificity (96%) for detection of infected wild boar (Aurtenetxe et al., 2008). This finding led to subsequent improvements of sensitivity (79%) and specificity (100%) with no significant difference between yearling, subadult and adult wild boar (Boadella et al., 2011). In 10-month old domestic pigs experimentally infected with *M. bovis*, the sensitivity of a modified bPPD ELISA using anti-pig immunoglobulin G (IgG) was 94% (Beltrán-Beck et al., 2014).

Rapid immunochromatographic tests, such as the DPP (Dual Path Platform) VetTB and DPP WTB (Wildlife TB) assays (Chembio, New York, USA), were developed for animal-side applications. The DPP assays use MTC antigens, including recombinant MPB70, MPB83, and CFP10/ESAT-6 fusion protein. High diagnostic accuracy was reported for wild boar (77-90% sensitivity and 90-97% specificity; Boadella et al., 2011; Lyashchenko et al., 2008). Recent findings in domestic pigs vaccinated with heat inactivated *M. bovis* and challenged with a field strain showed a range of DPP assay sensitivity from 78 to 94% and 100% specificity (Beltran-Beck et al., 2014). The antibody detection rate in wild boar with macroscopic lesions was five times higher (83%) than that in animals without visible lesions (17%; Lyashchenko et al., 2008).

Since only limited knowledge exists on TB prevalence and serology utility in wild boar piglets, the goal of this study was to assess the performance of several antibody detection tests for *M. bovis* infection in 2 to 6 month-old piglets during summer. We hypothesized that serology test sensitivity might be lower in this age category largely representing relatively recent infections with less frequent and/or early seroconversions.

2. MATERIALS AND METHODS

2.1. Animals and Sampling

This study took place in an intensively managed private hunting estate in Sevilla province, southern Spain. As in other intensively managed estates with hunting purposes, wild boar piglets are captured in early summer and maintained in enclosures for about 3 months until the opening of the hunting season. Piglets are released back into the wild after the first hunting event. This procedure is expected to reduce piglet mortality during the dry summer months and during the first hunting event. A total of 126 wild boar piglets (52 males and 74 females) captured during June and July 2012 and 2013 died during captivity and were submitted to the laboratory. The range of body weights was 3.24 to 15.24 kg (mean = 7.53 ± 2.17 S.D.). Blood was taken and serum samples stored at -20°C until testing. All piglets were necropsied and pooled lymphoid tissues from a subsample of 53 piglets were submitted for microbiology. A TB lesion score ranging from 0 – 26 was calculated based on observation of 13 sites. These included left and right mandibular, left and right bronchial and mediastinal lymph nodes, lungs (7 lobes) and mesenteric lymph nodes (see Díez-Delgado et al., 2014 for further details).

2.2. Bacterial culture and characterization

Bacterial culture was used as gold standard and readers of this test were masked to the serology results. Pooled tissues were cultured for *Mycobacterium bovis* as previously described (Corner and Trajstman, 1988). Briefly, the samples were thoroughly homogenized in sterile distilled water (2 g in 10 ml or equivalent) and decontaminated with hexadecylpyridinium chloride at a final concentration of 0.35% (wt/vol) for 1 h. The samples were centrifuged at 1,500 g for 30 min and the pellets cultured onto Coletsos and 0.20% (wt/vol) pyruvate-enriched Lowenstein-Jensen media (Difco FSM, Madrid, Spain) at 37°C. The culture media were checked weekly over

12 weeks for growth. All isolates were spoligotyped in order to confirm the strain identification (Kamerbeek et al., 1997).

2.3. Antibody detection tests

Table 1 present the tests used for detection of antibodies against MTC antigens. All tests were run and interpreted in presence of the same expert (first author).

2.3.1. ELISA bPPD IgG and IgM

The ELISA tests for IgG and immunoglobulin M (IgM) were carried out with a readjusted procedure used by Hu et al. (2008) and Chen et al. (2005) respectively. Briefly, the testing plates were coated with bovine purified protein derivative (bPPD) tuberculin (CZ Veterinaria S.A., Pontevedra, Spain) at 5 µg/ml and incubated overnight at 4°C. Skim milk powder solution (5% concentration) was added to each well and left 60 minutes at room temperature. Following three washes with phosphate buffered saline (PBS) solution containing 0.05% Tween 20 (PBST), piglet sera were added at 1:100 dilution and incubated for 30 min at 37°C. For the detection of IgG antibodies, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-pig IgG antibodies (Bethyl Laboratories Inc., Texas, USA) at a 1:5000 dilution were added, whereas for the detection of IgM antibodies, 100 µl of HRP-conjugated goat anti-pig IgM antibodies at a 1:10000 dilution were added. After the addition of the respective conjugate, the plates were incubated for 30 min at 37°C and subsequently washed with PBST three times. Color was developed by adding 50 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) (Promega Corp., Madison, USA). Protected for 15 minutes from the light at room temperature, the reaction was stopped with 50 µl of H_2SO_4 (2M). The optical densities (OD) were measured at 450 nm with an ELISA

reader. For positive control ELISA-IgG, pooled anti-PPD–positive serum was obtained from wild boar previously described as *M. bovis* culture positive and negative controls from TB- free wild boar previously described as *M. bovis* culture negative from bTB-free areas. ELISA results for IgG were expressed by using the formula: [sample E% = (mean sample OD / 2 x mean negative control OD) x 100]. Values higher than 100 were considered positive. For ELISA-IgM, since no controls were available, cut-off was determined by plotting a vertical graph of the net-OD. The most evident jump in OD was chosen as positive value (OD > 0.4).

2.3.2. ELISA bPPD Protein-G (IgG detection)

Serum samples were tested for anti-PPD antibodies by means of an ELISA using bPPD as antigen and protein-G horseradish peroxidase (Bethyl Laboratories Inc., Texas, USA) as a conjugate applying the previously described protocol (Boadella et al., 2011). Briefly, after coating the plates (at 5 µg/ml) for 18 hours at 4°C, wells were washed with PBST and blocked for 1 hour at room temperature (RT) with 140 µl to each well of 5% skim milk in PBST. Sera were added directly on plate (100 µl/well) at a dilution of 1:200 in PBS and incubated for 1 hour at 37°C. Samples, blanks, and positive and negative controls were tested in duplicate in each plate. Protein G was added (100 µl/well) at a dilution of 0.5 µg/ml in PBST and incubated at 37°C for 1 hour and 20 minutes. After revealing with FastOPD (SigmaFast™ OPD, Sigma, St. Louis, USA), the reaction was stopped with 50 µl/well of sulfuric acid (H_2SO_4; 3N), and OD was measured in an ELISA reader at 450 nm. Sample results were expressed as an ELISA percentage (E%) and E% values greater than 100 were considered positive.

2.3.3. ELISA-DR

The methodology was developed by INGENASA (Madrid, Spain). The principles of double recognition ELISA (DR-ELISA) was developed with recombinant protein (MPB83) serving both as coating antigen and conjugated form (horseradish peroxidase (HRP) labelled), the signal capture. The results were read by ELISA spectrophotometry and 0.3 was chosen for cut-off OD. This assay is able to recognize not only IgGs but also other immunoglobulins, such as IgMs. Briefly, the recombinant MPB83 expressed in baculovirus system was used as antigen to coat 96-well microtitre plates (25ng/well). After an incubation at 4^{o}C, the wells were blocked and a 1/200 dilution of serum in 0.05% Tween, 0.35M NaCl in Phosphate-Buffered Saline (PBS) was incubated for one hour at RT. Bound antibodies were detected by incubation with MPB83-HRP one hour at RT and subsequent addition of the substrate (TMB-MAX, Neogen Corporation, KY, USA).

2.3.4. DPP Tests

We used two immunochromatographic antibody assays, the DPP VetTB and DPP WTB tests, developed by Chembio Diagnostic Systems, New York, USA. DPP VetTB is USDA-licensed (for cervids and elephants) and commercially available for use in any animal species, while DPP WTB is not licenced but available for investigational assays . Sera were tested for the presence of specific antibodies as previously described (Boadella et al., 2011). Any visible band in the test area 20 min after adding sample buffer, in addition to the control line, was considered an antibody positive result, whereas no test band in presence of control line was considered a negative result.

2.4. Data treatment

Sensitivity and area under curve (AUC; based on ROC curve analysis; Fawcett 2006) of serology were analyzed using MTC culture and tuberculosis-like lesions as reference standards, separately. The accuracy was derived from the AUC value. It represents the probability that the test result for a randomly chosen positive case will exceed the result for a randomly chosen negative case. Agreement between test systems was calculated by using the Cohen kappa (Cohen, 1960) coefficient significance value <0.05. The General Linear Models (one for each serological technique) used serological response (binomial variables, coded as 0 = negative and 1 = positive) as a dependent variable, while culture or lesions where included as categorical factors in separate models. We categorized the tuberculosis-like lesions into 3 categories (Score 0; score less than 5; score more than 5). Parameter estimates (β) were calculated using a reference value of 0 for the '3' level (highest TB score) in the variable 'score group'. We used a binomial error and a logit link. All analyses were computed using SPSS Statistical Package (ver.20.0, IBM Corp., New York, USA).

3. RESULTS

Bacterial culture yielded a *M. bovis* infection prevalence of 33.9%, while serum antibody prevalence estimated by different tests ranged from 19 to 38% (Table 1). Table 2 shows the sensitivity and AUC values of the six serological tests using TB lesion presence and culture as reference, respectively. The Cohen kappa coefficient of agreement between TB lesion presence and MTC culture was 0.82 (Sig. 0.000; $p<0.05$), indicating an almost perfect agreement.

Regarding the serum antibody tests, agreement between DPP WTB and culture was moderate (0.45, $p>0.05$) and all other serological tests used had poor to fair agreements (Table 2). Figure 1 displays the performance of each serological test in detecting antibodies against MTC antigens in wild boar piglets as compared to the culture results. The diagnostic sensitivities varied between 15.4% and 46.2% for plate ELISAs and between 61.5% and 69.2% for DPP tests. Specificity was not calculated since it required true negative animals from zero prevalence areas, which were not available in this study. Figure 2 shows the relationships between TB lesion scores and seropositivity rates. Antibody prevalence increased significantly with score lesion group in the DPP WTB assay ($p=0.004$; β score group 1 = -2.2±0.08; β score group 2 = -1.0±0.007), DPP VetTB assay ($p=0.023$; β score group 1 = -2.1±0.08; β score group 2 = -1.5±0.009) and ELISA-DR test ($p=0.015$; β score group 1 = -1.7±1.0; β score group 2 = -0.6±0.01). The patterns of ELISA-IgG and ELISA Protein-G were different although not significant ($p>0.05$), which groups with highest TB lesion scores showing lower proportions of seropositive results.

4. DISCUSSION

This survey revealed the ability of several tests for detecting serum antibodies against the MTC antigens in 2 to 6 month-old wild boar piglets naturally infected with *M. bovis*. Both traditional plate ELISA tests and rapid animal side tests can be used, although the best performance was demonstrated for the latter. The results supported our hypothesis of a lower sensitivity of serology for detecting MTC-infected piglets, as compared to yearling, juvenile or adult wild boar.

Based on the results of microbiological culture, one third of the annual cohort of wild boar was infected very early in life, at least under the high infection pressure existing in intensely managed hunting estates within TB endemic areas. In general, ELISA test sensitivity in piglets was lower than those reported in previous studies on wild boar of all ages (>70%; Boadella et al., 2011, Lyashchenko et al., 2008) and in pigs (94%; Beltrán-Beck et al., 2014). In human TB, diagnosis in pediatric patients is also difficult by any available method and serology is not an exception. The antibody responses to all mycobacterial antigens in pediatric patients were much lower than in adult patients. This may be due to reduced serological responses to MTC infection in young or early infected individuals (Achkar and Ziegenbalg, 2012).

The generally poor performance of antibody detection tests in piglets means that pre-movement testing during wild boar piglet translocations can easily miss detecting infected individuals. Also, piglets will yield lower prevalences than other age classes in cross-sectional serosurveys. This sub-optimal sensitivity must also be taken into account in longitudinal studies, where tagged piglets are later re-tested at more advanced age: some delayed seroconversions could be due to the insufficient test sensitivity in piglets.

The test based on IgM aimed at detecting early infections. However, this test had the poorest sensitivity. Two explanations can be found. First, the lack of a proper positive control hampered

establishing a proper cut-off. Second, IgG-based tests consistently yielded higher sensitivities, suggesting that the detected contacts of the piglets with MTC were not necessarily recent.

In this study, the antibody levels detected by the bPPD ELISAs for IgG did not correlate with the lesion score. This is in contrast to previous findings where the bPPD ELISA results correlated with the lesion score in wild boar, suggesting that ELISA may be used for classifying infected animals as showing a more or less advanced disease (Garrido et al., 2011). Nevertheless, the findings of this particular study on wild boar piglets should not be compared with studies including older subjects with a more developed humoral immune response. On the other hand, lesion scores did correlate with DPP assay results also detecting serum IgG antibodies. Therefore, the difference observed do not only stem from age or early infection, but may represent an example of inter-assay differences. Our sample also lacked of culture and lesion negative piglets from non-endemic sites, thus precluding a specificity analysis.

All plate tests, ELISA IgG, ELISA protein-G and ELISA-DR had a good agreement among them except the ELISA IgM. Both DPP assays had a better sensitivity than the plate ELISAs. Sera went only through one freeze-thawing cycle, making it unlikely that freeze-thawing negatively affected test performance (Boadella and Gortazar, 2011). A possible explanation for the higher sensitivity of the DPP WTB assay as compared to DPP VetTB assay is that the former has a double amount of the target antigen (MPB83+MPB70) immobilized in the test line, as compared to DPP VetTB assay (MPB83 only). This finding is in agreement with prior studies suggesting

that ESAT-6 and CFP10 (antigen in DPP VetTB assay) are not essential for antibody detection in wild boar (Lyashchenko et al., 2008).

The results described that one third of the wild boar piglets can become infected at early age in a high TB prevalence sites. While we show that serology is less sensitive in piglets than generally observed in adult wild boar, performance varies among tests. Certain tests, notably the rapid animal-side tests, can contribute to TB control strategies by enabling the setup of test and cull schemes or improving pre-movement testing. However, sub-optimal test performance in piglets as compared to older wild boar should be taken into account.

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Figure captions

Figure 1: Performance of six serological assays in two to six month-old Eurasian wild boar (*Sus scrofa*) piglets as compared to culture: two immunochromatographic tests, DPP WTB and DPP VetTB assays (Chembio, NY, USA); three in-house enzyme-linked immunosorbent assays (ELISA) for immunoglobulin G (IgG), Protein G and immunoglobulin M (IgM); and the ELISA-DR prototype (Ingenasa, Madrid, Spain).

Figure 2: Seropositivity in relation to three tuberculosis (TB) lesion score categories (0, <5, ≥5) in two to six month-old Eurasian wild boar (*Sus scrofa*) piglets using two immunochromatographic tests, DPP WTB and VetTB assays (Chembio, NY, USA); three in-house enzyme-linked immunosorbent assays (ELISA) for immunoglobulin G (IgG), Protein G and immunoglobulin M (IgG); and the ELISA-DR prototype (INGENASA, Madrid, Spain). Bars represent the standard error (S.E.).

Tables

Table 1: Prevalence of serum antibodies against the *Mycobacterium tuberculosis* complex (MTC) according to six different tests; prevalence of *M. bovis* infection assessed by culture; and prevalence of tuberculosis-compatible lesions in two to six month-old wild boar piglets.

Test system	**Details**	**No. of positive samples**	**MTC antibody prevalence (95% CI)**
DPP WTB	Immunochromatographic single band test, MPB70 and MPB83 antigens, Chembio Diagnostic Systems, Inc.	48/126	38.0% (29.5-46.4)
DPP VetTB	Immunochromatographic two-band test, MPB83 and CFP10/ESAT-6 antigens, Chembio Diagnostic Systems, Inc.	48/126	38.0% (29.5-46.4)
ELISA-IgG	Plate test, PPDb antigen, HRP conjugated goat anti-pigs IgG, in-house ELISA	37/126	29.3% (21.3-37.2)
ELISA-Protein-G	Plate test, PPDb antigen, Protein-G HRP conjugate, in-house ELISA	26/126	20.6% (13.5-27.6)
ELISA-DR	Plate test, MPB83 antigen, INGENASA® Madrid, Prototype	26/126	20.6% (13.5-27.6)
ELISA-IgM	Plate test, PPDb antigen, HRP conjugated goat anti-pigs IgM, in-house ELISA	24/126	19.0% (12.1-25.8)
M. bovis culture	VISAVET, Madrid	18/53	33.9% (21.1-46.6)
TB-compatible lesion	IREC-UCLM-JCCM	27/126	21.4% (14.2-28.5)

Table 2: Sensitivity and area under curve (AUC) of six tests for the detection of serum antibodies against the *Mycobacterium tuberculosis* complex (MTC), using tuberculosis (TB)-compatible lesions and MTC antigens culture as references, respectively.

Test system	**TB lesion**			**MTC culture**		
	Sensitivity	**AUC**	**K (sig <0.05)**	**Sensitivity**	**AUC**	**K (sig <0.05)**
DPP WTB	66.7%	0.682	0.283 (0.001)	69.2%	0.735	0.452 (0.004)
DPP VetTB	59.3%	0.635	0.210 (0.011)	61.5%	0.678	0.342 (0.029)
ELISA-IgG	48.1%	0.620	0.211 (0.016)	46.2%	0.638	0.288 (0.067)
ELISA-DR	40.7%	0.628	0.259 (0.004)	23.1%	0.578	0.186 (0.160)
ELISA-ProteinG	37.0%	0.604	0.212 (0.018)	23.1%	0.523	0.051 (0.736)
ELISA-IgM	25.9%	0.544	0.091 (0.305)	15.4%	0.466	-0.076 (0.613)

K: Kappa agreement expressed as value and significant <0.05

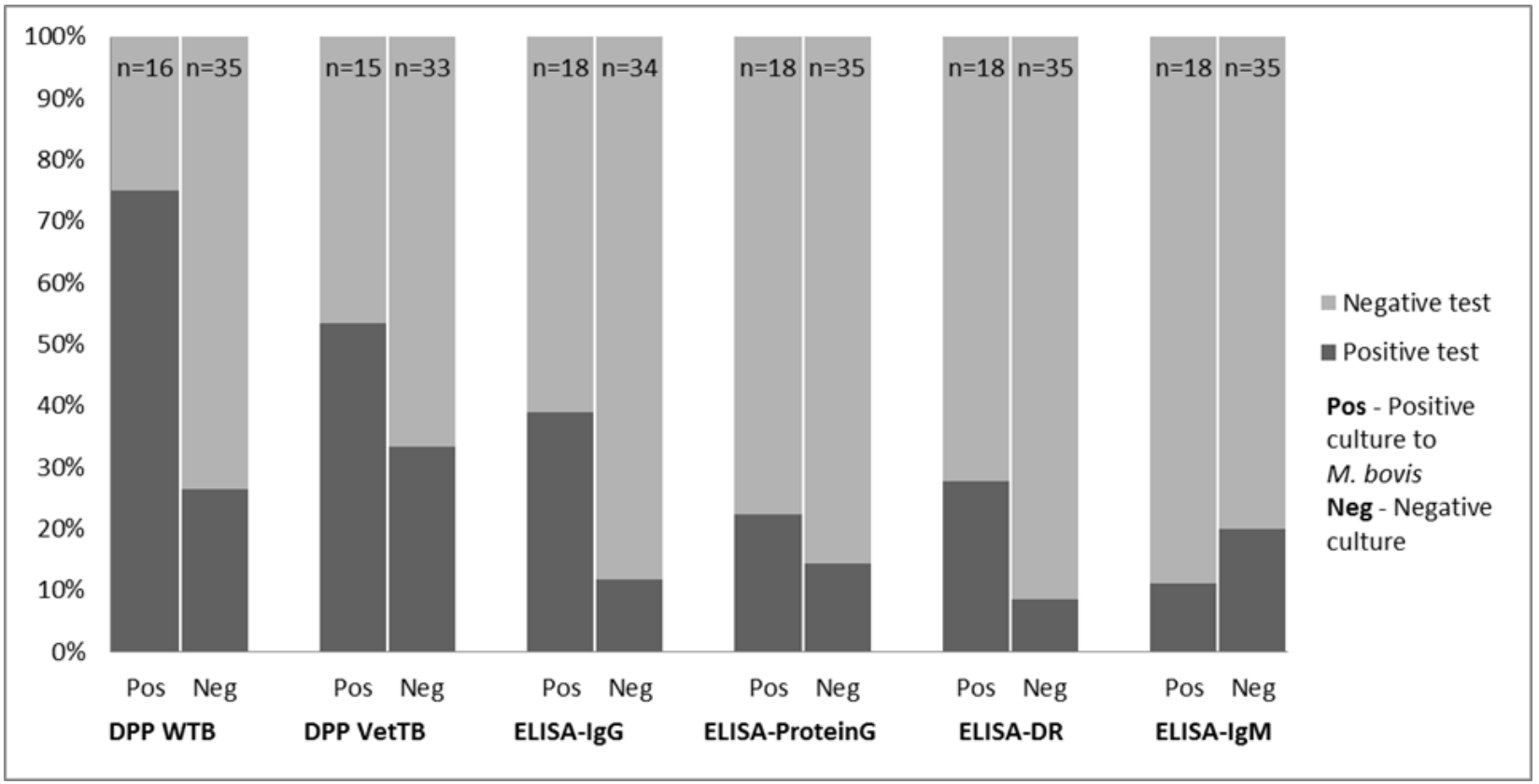
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
n=16
n=35
n=15
n=33
n=18
n=34
n=18
n=35
n=18
n=35
n=18
n=35
Pos
Neg
DPP WTB
DPP VetTB
ELISA-IgG
ELISA-ProteinG
ELISA-DR
ELISA-IgM
Negative test
Positive test
Pos - Positive culture to M. bovis
Neg - Negative culture

Figure 1

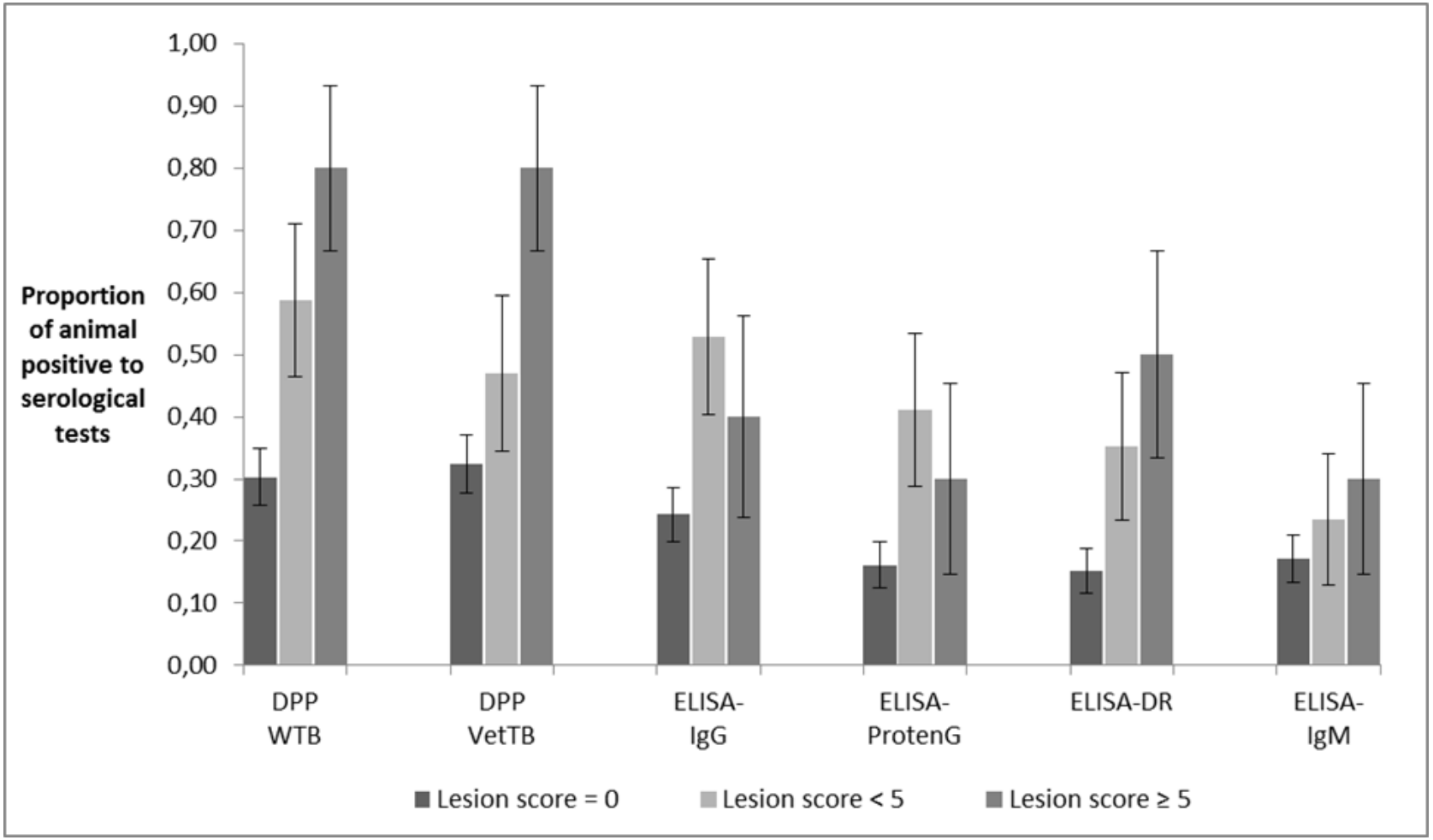
Proportion of animal positive to serological tests
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
DPP WTB
DPP VetTB
ELISA-IgG
ELISA-ProtenG
ELISA-DR
ELISA-IgM
Lesion score = 0
Lesion score < 5
Lesion score ≥ 5

Figure 2