The tonsils of the soft palate do not mediate the response of pigs to oral vaccination with heat inactivated *Mycobacterium bovis*
Abstract

*Mycobacterium bovis* causes animal tuberculosis (TB) in cattle, humans, and other mammalian species, including pigs. The goal of this study was to experimentally assess the response of pigs with and without tonsillectomy to oral vaccination with heat-inactivated *M. bovis* and challenge with a virulent *M. bovis* field strain; to compare pig and wild boar responses using the same vaccination model as previously used in Eurasian wild boar (*Sus scrofa*); and to evaluate the use of several ELISA and lateral flow tests for in vivo TB diagnosis in pigs, and verify if these tests are influenced by oral vaccination with inactivated *M. bovis*. At necropsy, lesion and culture scores were 20-43% higher in the controls compared to vaccinated pigs. Massive *M. bovis* growth from thoracic tissue samples was observed in 4 out of 9 controls, but in none of 10 vaccinated pigs. No effect of the tonsils was observed on these scores, suggesting that tonsils are not involved in the protective response to this vaccine in pigs. Serum antibody levels increased significantly only after challenge. At necropsy, the estimated sensitivity of the ELISAs and DPP assays ranged from 89% to 94%. In the oral mucosa, no differences in gene expression were observed in the control group between pigs with and without tonsils. In the vaccinated group, the mRNA levels for CCR7, IFN-β and MUT were higher in pigs with tonsils. Complement factor 3 mRNA levels in PBMC increased with vaccination and decreased after *M. bovis* challenge. This information is relevant for pig production in *M. bovis* endemic regions and for TB vaccine research.

Key words: Heat-inactivated vaccine; Swine; Tonsils of the soft palate; Vaccination and challenge experiment.
Introduction

Animal tuberculosis (TB) is caused by infection with *Mycobacterium bovis* and closely related members of the *M. tuberculosis* complex (MTC). Although cattle are the main concern regarding animal TB in developed countries, several other species of mammals, including humans, can be infected [1,2].

Globally, *M. bovis* is one of the ten most important causes of pig losses [3]. Pigs can come in contact with other MTC hosts if raised in free range, open air or backyard systems with limited biosafety. Recent evidence from free-ranging domestic pigs on the Italian island of Sicily showed that naturally infected pigs develop lung lesions and can contribute to *M. bovis* maintenance in mixed farming systems [4]. Evidence of *M. bovis* infection in domestic pigs is also available from other countries in Europe [5], Africa [6], and South America [7]. Moreover, feral pigs are *M. bovis* hosts in several regions worldwide [8,9,10]. This, along with the well-established role of its ancestor the native Eurasian wild boar (*Sus scrofa*) in MTC maintenance [11], makes pigs an important subject to study in relation with their response to MTC infection and the possibility of protecting them by vaccination.

Tonsils are known to play an important role in the detection and initiation of the immune response to pathogens entering through the mouth and nares, and also in maintaining a systemic immune response by producing lymphocytes, cytokines and chemokines [12,13,14]. The main pharyngeal mucosal-associated lymphoid tissues in swine are the tonsils of the soft palate, which constitute a portal of entry for microorganisms [15].
Previous studies in wild boar analyzed the tonsils as a target organ for mycobacteria, demonstrating that: (1) gene expression differs among tuberculous and non-tuberculous animals and between tonsils and lymph nodes after natural exposure to *M. bovis*, but also between vaccinated and non-vaccinated animals under experimental conditions, and (2) differentially regulated molecules of the mandibular lymph nodes and the tonsils are involved in stress/inflammatory responses to mycobacterial infection and may be used as markers for TB diagnosis in wild boar [16,17,18,19,20].

In vivo detection of MTC infection in wild boar is possible through the detection of cell-mediated response [17,21] and through the detection of specific antibodies by different ELISA and animal side DPP tests (e.g. [17,22]). In pigs, tests based on cell-mediated immunity are common use in some countries (e.g. Italy, [23]). However, antibody detection by ELISA and/or immunochromatographic tests is not frequent in pigs.

Our aim was to experimentally assess the response of pigs with tonsillectomy to oral vaccination with heat-inactivated *M. bovis* and challenge with a virulent *M. bovis* field strain; to compare pig and wild boar responses using the same vaccination model as previously used in wild boar [17]; and to evaluate the use of several ELISA and DPP tests for in vivo TB diagnosis in pigs, and verify if these serodiagnostic tests are influenced by oral vaccination with inactivated *M. bovis*.

**Methods**
Ethics Statement

Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish legislation (R.D. 223/1988, R.D. 1021/2005). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Regional Agriculture Authority (Comunidad de Madrid, Permit number CM180112-01).

Animals and experimental design

Twenty 3-4-month-old piglets were purchased from an animal experiment facility (Centro de Cría y Suministro de Animales de Experimentación “La Chimenea”, IMIDRA. Madrid) known to be free of mycobacterial infections. All animals were tested by ELISA to detect \textit{M. bovis} infection [22] on arrival yielding a fully negative result.

Tonsillectomy took place one month prior to the start of the vaccination and challenge experiment. The soft palate tonsils were removed at “Hospital General Universitario Gregorio Marañón” (Madrid, Spain). Premedication was performed using ketamine (15 mg/kg IM; Ketolar®, Pfizer Ireland Pharmaceuticals, Dublin, Ireland) and atropine (0.033 mg/Kg IM; Braun Medical SA, Rubi, Barcelona, Spain). Animals were monitored using capnography, pulse-oximetry and electrocardiogram. Anesthetic induction was performed with propofol (1.66 mg/kg; Propofol Lipomed ®, Fresenius Kabi, Barcelona, Spain), fentanyl (3 mg/kg; Fentanest ®, Kern Pharma, Terrassa, Barcelona, Spain) and atracurium besilat (0.60 mg/kg; Inibsa, Llisà de Vall, Barcelona, Spain) intravenously through the auricular dorsal vein. Then, the animals were
connected to a ventilator (SA 1; Dräger, Dräger Hispania S.A., Madrid, Spain).

Anesthesia was maintained with continuous infusion of propofol (12 mg/kg/h), fentanyl (0.30 mg/kg/h; Fentanest®, Kern Pharma, Terrassa, Barcelona, Spain) and atracurium besilat (0.05 mg/kg/h). Animals received Ringer lactate solution® at (5-6 ml/kg/h; Braun, Rubí, Barcelona, Spain) as required. The oral cavity was opened and fixed with Tuffier rib spreaders (Aesculap®, Braun, Rubí, Barcelona, Spain). For the surgical approach, a cold light source connected to a column of endoscopy (Fiegert Endotech®, Xenon, Tuttingen, Germany) and laparoscopic equipment (Endoshears®, Autosuture, Covidien, Dublín, Ireland) providing sterile cautery (ME 200; Martin Medizin Technik, Tuttingen, Germany) was introduced into the oral cavity to perform a perimeter incision in the area of the soft palate. The postoperative consisted in the administration of ceftriaxone (25 mg/kg; GES, Laboratorios Torlan S.A, Cerdanyola del Vallès, Barcelona, Spain) and Dexketoprofen (2 mg/kg; Enantyum®, Laboratorios Menarini, Badalona, Barcelona) intramuscularly for 3 days. One piglet died during anesthesia.

The animals were housed in level III bio-containment facilities (BSL3) where they had ad libitum food and water. Piglets were randomly assigned to one of four treatment groups: Group 1, vaccinated with tonsils (V; N=5); Group 2, vaccinated with tonsillectomy (TE-V; N=5); Group 3, unvaccinated with tonsils (C; N=4); Group 4, unvaccinated with tonsillectomy (TE-C; N=5).

After an acclimatization period in the BSL3 facility, animals were handled and blood was collected five times during the experiment, including vaccination (T0,
day 1), re-vaccination (T1, day 28), challenge (T2, day 57), one bleeding (T3, day 111; 54 days after challenge), and necropsy (T4, day 195; 138 days after challenge). For the challenge, 2 ml of a suspension containing $10^5$ colony forming units (cfu) of an *M. bovis* field strain (spoligotype SB0295) were administered by the oropharyngeal route as described in previous experiments [17,24].

### Preparation of inactivated vaccine

The *M. bovis* strain used (Neiker 1403; spoligotype SB0339) was a first passage level culture isolated from a naturally infected wild boar in Coletsos medium. The isolate was propagated in Middlebrook 7H9 broth enriched with OADC for 2–3 weeks. Cells were harvested by centrifugation at 2500 x $g$ for 20 minutes and washed twice in PBS. The bacterial pellet was re-suspended in PBS. The turbidity of this suspension was adjusted to an optical density of 1 McFarland unit. Before inactivation, ten-fold serial dilutions were prepared and plated in agar-solidified 7H9 with OADC in quadruplicate to assess the number of cfu in the inoculum. The inoculum was then inactivated in a water bath at 80ºC for 30 minutes. Animals in Groups 1 and 2 were orally administered with approximately $10^7$ bacteria according to cfu counts, diluted in 2 ml of PBS. This inactivated vaccine was again cultured in duplicate to assure absence of viable *M. bovis*.

### Necropsy, sample collection and histopathology

Pigs were anesthetized by intramuscular injection of Zoletil®, and euthanized by captive bolt. A thorough post-mortem examination was done to detect the
presence of macroscopic lesions. Samples for culture were immediately processed and duplicates were frozen at -80°C for mRNA isolation. After collection of samples for mRNA analysis, all main lymph nodes (LNs) and the tonsils were serially sliced into 1–2 mm thick slices and carefully inspected for visible TB-compatible lesions. Organs were also carefully inspected, and each lung lobe was considered separately. TB-compatible lesions were classified based on lesion distribution and lesion intensity, and scored as previously described [24]. Samples of individual tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin–eosin by use of standard procedures. An additional section of those tissues with lesions indicative of tuberculosis was stained by Ziehl–Neelsen (ZN) procedure to detect the presence of acid-fast organisms (AFO).

Microbiology

The tissues collected were as follows: head lymphoid tissues including the soft palate tonsil (only Groups 1 and 3), oral mucosa lateral to the soft palate tonsil, and both mandibular, parotid, and retropharyngeal lymph nodes (LNs); lung (each lobe), tracheobronchial LNs and mediastinal LN; spleen, ileocaecal valve, and mesenteric and hepatic LNs. When suspicious lesions were observed in liver, kidney and LNs from other locations were also cultured. Samples were thoroughly homogenized in sterile distilled water (2 g in 10 ml or equivalently) and decontaminated with hexadecylpyridinium chloride at a final concentration of 0.75% weight/volume (w/v) for 18h. Samples were centrifuged at 1500 x g for 30 min and pellets cultured onto Coletsos and 0.20% (w/v) pyruvate-enriched Löwenstein–Jensen media (Difco FSM, Madrid, Spain) at 37°C. Culture media
were checked weekly during 12 weeks for growth. All isolates were spoligotyped in order to confirm the strain [25]. We used a culture score for *M. bovis* infection, as defined in Garrido et al. [17] for wild boar. This score is the number of LN or organ samples yielding a *M. bovis* isolate out of the total number of culture attempts (N≥7 samples cultured per individual; score range 0–7). Each culture sample with more than 50 cfu was considered as massive growth.

### Serum antibody detection

Serum samples were tested for immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies by means of an in-house ELISA. Purified protein derivative (PPD) derived from *M. bovis* (bPPD; CZ Veterinaria, Porriño, Spain) was used as antigen and IgG and IgM antibodies (Bethyl Inc., Montgomery, Texas) as conjugates, and the protocol described by Boadella et al. [22] was applied. Sample results were expressed as an ELISA percentage (E%) that was calculated using the formula: Sample E% = [mean sample OD / (2 x mean negative control OD) x 100]. Samples with E%> 100 were considered positive.

The DPP® technology was developed by Chembio Diagnostic Systems, Inc. using selected *M. bovis* antigens. Serum samples were tested with DPP®BrockTB assay (formerly DPP BovidTB) and DPP®VetTB as previously described [26] and results were read 20 min after adding sample buffer for both tests. The presence and intensity of either the sole T band (a mixture of MPB70 and MPB83 protein) of the DPP®BrockTB, or the 2 separate test bands (1, MPB83 antigen; 2, CFP10/ESAT-6 fusion protein) of the DPP®VetTB, were
evaluated by a DPP optical reader (in relative light units, RLU). Reactivity above
the cut-off value of 15.0 RLU in any of the test bands was considered a positive
result for the presence of antibody [27].

Interferon gamma test (IFNγ)

At T2 and T3, blood samples were collected into tubes with lithium heparin.
Within 8h of collection, stimulation of whole blood with PBS (nil control), and the
avian and bovine purified protein derivative (PDD; CZ Veterinaria, Porriño,
Spain) was performed as described for other species [28,29]. In addition, we
used ESAT-6/CFP-10 (EC) and Rv3615c antigens in a concentration of
55μg/ml. Detection of IFNγ in the supernatant was performed using a
quantitative ELISA (Pierce Endogen, Rockford, IL, USA) following
manufacturer's recommendations.

RNA isolation and real time RT-PCR

Total RNA was extracted from peripheral blood mononuclear cells (PBMC;
Buffy coat) and oral mucosa samples using TRI reagent (Sigma, Madrid, Spain)
and the RNeasy kit (Qiagen, Izasa, Madrid, Spain), respectively, following
manufacturer's recommendations. RNA was used for real-time RT-PCR
analysis of mRNA levels of selected genes in individual samples. Real-time RT-
PCR was performed with gene-specific primers (Table 1) using the One-Step
RT-PCR Kit with SYBR Green and the CFX thermal cycler (Bio-Rad, Hercules,
CA, USA) following manufacturer's recommendations. A dissociation curve was
run at the end of RT-PCR reaction to ensure that only one amplicon was formed
and that the amplicon denatured consistently at the same temperature range for
every sample [30]. The mRNA levels were normalized against *S. scrofa* cyclophilin, β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPHD) using the genNorm ddCT method [31]. Normalized Ct values were compared between groups by Student’s t-test with unequal variance (p=0.05).

We selected for mRNA analysis in oral mucosa at T4 genes involved in innate immunity (C3; NLR family, pyrin domain containing 3, NLRP3; Toll-like receptor adaptor molecule 1, TRIF; interleukin 1-beta, IL-1β; interferon gamma, IFNγ; and interferon beta, IFN β), mucosal immunity (Perforin, PERF and chemokine (C-C motif) receptor 7, CCR7), and methylmalonyl CoA mutase, MUT (Table 1) based on our previous results on differential gene expression in wild boar in response to *M. bovis* infection and vaccination and other studies [17,18,19,24,32,33,34].

The C3 mRNA levels of pigs were characterized from peripheral blood mononuclear cells (PBMC; buffy coat) before vaccination (T0), after vaccination and before challenge (T2) and at the end of the experiment (T4) in vaccinated and control animals, grouping together animals with and without tonsillectomy after observing that tonsils had no effect on C3 mRNA levels in PBMC and oral mucosa.

Characterization of serum cytokine levels

The cytokine concentration in pooled pig sera was determined at T0, T2 and T4 using the Quantibody® porcine cytokine array (RayBiotech Inc, Norcross, GA, USA), an array-based multiplex ELISA system for the simultaneous quantitative
measurement of multiple cytokines. Using this system, standard cytokines
(Table 2) and samples were assayed in each array simultaneously through a
sandwich ELISA procedure, following the recommendations of the manufacturer
(http://www.raybiotech.com/porcine-cytokine-array-q1-1.html). The signals were
visualized using a Gene Pix 4100A laser scanner (Molecular Devices,
Sunnyvale, CA, USA) and data were extracted by GenePix Pro 6 software
(Molecular Devices). Finally, the quantitative data analysis was performed using
the Quantibody® Q-Analyzer software (RayBiotech Inc). Two replicates were
tested for each sample.

Statistical analyses
Lesion scores among groups were compared with the non-parametric Mann-
Whitney U and Median tests. Fisher’s tests were used to compare culture
scores between controls and vaccinated pigs. Differences in antibody levels
over time and between groups were analyzed by ANOVA and Median Tests.
Correlations between antigens, lesion and culture scores were performed using
Pearson (parametric data) and Spearman (non-parametric data) tests. The
mRNA levels between groups were compared by Student’s t-test as described
above.

Results
Clinical signs and pathology
No pig showed apparent clinical signs such as emaciation or coughing during
the experiment. At necropsy, visible TB compatible lesions were recorded in all
pigs from all groups but for one vaccinated pig belonging to Group 2. Total
lesion and thorax lesion scores were highest in the two control groups (Figure 1). Three controls and one vaccinated pig had severe visible lesions (score>10) in the lung parenchyma. Histopathology (H/E staining) confirmed the presence of tuberculous granulomas in all samples with visible lesions. Z/N staining revealed that only three vaccinated and two control pigs had no AFO in lung tissues. All controls and 8/10 vaccinated pigs had moderate visible abdominal lesions (score<10). The mean lesion score was 33.67 (95%CI 22.59-44.74) for control pigs and 20.50 (11.48-29.52) for vaccinated ones. The observed reduction of the mean total and thorax lesion scores in vaccinated pigs compared with the controls was of 39.11% (12.33-65.89) and 43.28% (4.43-82.13), respectively. Differences between vaccinated and control pigs in total and thorax lesion scores were not significant (U test, p>0.05). No differences in total and lung lesion scores were evidenced among the four treatment groups (vaccine/control and tonsillec tomy/not; Median test, Chi²=3.75, p>0.05). Also, no differences were found between vaccinated pigs with or without tonsillectomy (U test; total score p=0.69, thorax score p=0.55) and between control pigs with and without tonsillectomy (U test; total score p=0.29, thorax score p=0.73).

M. bovis isolation

All isolates belonged to the same spoligotype as the M. bovis strain used for challenge, indicating it was the same strain. Total culture scores and thorax culture scores were highest in the control groups [5.78 (4.77-6.79) and 1.67 (1.36-1.97)] compared with the vaccinated groups [4.60 (3.15-6.04) and 1.30 (0.90-1.70)]. We observed reductions of both total mean culture score and
Thorax culture score as compared to the controls [20.41% (0-45.42) and 22.15% (0-45.92), respectively]. However, massive *M. bovis* growth on solid media (>50 cfu) was observed among 6/9 controls and 2/10 vaccinated pigs (Fisher’s test, p=0.054). Additionally, when considering only thoracic region tissues (tracheobronchial, mediastinal LNs and lung tissue), more than 50 cfu were observed in 4 out of 9 controls, but in none of the 10 vaccinated pigs (Fisher’s test, p=0.032). Again, no differences were found between vaccinated pigs with and without tonsillectomy (U test; total culture score p=0.55, thorax culture score 0.84) and between control pigs with and without tonsillectomy (U test; total culture score p=0.11, thorax culture score p=0.73).

Serum antibody response

No detectable antibody response was evidenced at T1 and T2. With the exception of one pig belonging to Group 2, all four groups consistently responded to challenge producing detectable antibodies at bleeding (T3) and reaching the highest antibody levels at the time of necropsy (T4). No significant differences between groups were found at T3 and T4. At T3 and T4 we found positive correlations between some of the antibodies and lesion and culture scores (Table 3, Figure 2).

From a diagnostic perspective, at T3 (54 days after challenge), sensitivity of the IgG and IgM bPPD ELISA were 94.44% (72.35-99.99) and 77.78% (54.25-91.53), respectively. Regarding DPP, sensitivity was 94.11% (71.08-99.99) for the DPP®BrockTB and 77.8% (54.25-91.53) for DPP®VetTB. Specificity was 100% (85.23-100) for all tests at T0. At the time of necropsy (T4), 17 out of 18
confirmed infected pigs were detected both by bPPD ELISA (IgM and IgG) and DPP®BrockTB, while 16 by DPP®VetTB. In the DPP®VetTB, 16 pigs reacted to test 1 (MPB83) and only 3 also reacted to test 2 (ESAT-6/CFP10 fusion protein). This means a sensitivity of 94.44% (72.35-99.99) for both bPPD ELISA and DPP®BrockTB, and 88.89% (65.95-98.14) for DPP®VetTB.

IFNγ response

Similar responses regarding IFNγ production were obtained whether blood was stimulated with bPPD, EC or Rv3615c antigens. All pigs tested negative to IFNγ at T2. All PBS controls also yielded consistently low results (mean OD 0.10; 0.07-0.11). At T3, 54 days after challenge, all but one pig of Group 2 showed a clear positive IFNγ response. No significant correlations between IFNγ responses and ZN positive tissues, culture or lesion scores were found. No differences in IFNγ response were found between vaccinated pigs with and without tonsillectomy (U test, bPPD, EC and Rv3615c p=0.55, p=0.69, and p=0.15, respectively) and between control pigs with and without tonsillectomy (U test, bPPD, EC and Rv3615c p=0.90, p=0.69, p=0.20, respectively).

RNA isolation and real time RT-PCR

In the oral mucosa, no differences were observed in the control group between animals with and without tonsillectomy but the mRNA levels for CCR7, IFN-β and MUT were higher in vaccinated pigs with tonsils when animals with and without tonsillectomy were compared (Figure 3A and data not shown). However, the comparison between vaccinated and control animals when individuals with and without tonsillectomy were grouped together gave similar
gene expression levels between the two groups, and only IL-1β expression in the oral mucosa was higher in vaccinated animals when compared to controls (Figure 3B). C3 mRNA levels in PBMC increased with vaccination and decreased after challenge with M. bovis (Figure 3C).

Characterization of serum cytokine levels

Serum IL-1β protein levels increased after vaccination and infection in vaccinated animals while IL-6 levels increased after vaccination but decreased after infection (Figure 3D). No significant differences were found for IL-4, IL-8, IL-10, IL-12, GM-CSF, IFNγ, TGFβ1 and TNFα.

Discussion

This experiment produced three main results. First, the response of pigs to oral vaccination with inactivated M. bovis and challenge with an M. bovis field strain was similar to the one recently described for its ancestor, the wild boar [17]. Second, the consistent lack of differences between experimental groups with and without tonsillectomy implies that the role of the tonsils of the soft palate in the pig response to oral vaccination and to infection is smaller than expected. Third, serological tests were found highly sensitive for MTC infection diagnosis in pigs, and vaccination did not interfere.

In general terms, pigs showed more severe lesions after challenge with field M. bovis than wild boar challenged with similar doses in previous trials [17,35,36]. Also, abdominal lesions were more evident in pigs. As in previous experiments in wild boar, lung lesions and culture scores were higher in control pigs and no
massive growth of *M. bovis* in tissues of the thoracic region was recorded among vaccinated pigs. The high challenge dose used might contribute to the severity of the observed lesions [17]. However, these comparisons should be interpreted with caution since data are derived from different experiments.

The finding of no effect of tonsillectomy on pig response to vaccination and challenge was unexpected, since it is known that gene expression differs between tonsils and lymph nodes after natural exposure to *M. bovis* and after vaccination and challenge [16, 17, 19, 20]. The tonsils of the soft palate have been recognized by many authors as the main pharyngeal mucosa-associated lymphoid tissues in swine [12, 14, 15, 37]. However, this finding could indicate that other mucosa-associated lymphoid tissues located in the oropharyngeal area including other tonsils could also play a significant role in initiating immune responses against antigens contacting with mucosal surfaces [38].

As far as we know, this is the first time that bPPD ELISA and DPP TB tests have been used for TB-diagnostics in pigs. The findings of 94% sensitivity with a 100% specificity indicate that both the plate ELISA and the lateral flow DPP assay are as suitable for TB diagnosis in pigs as they are in wild boar [22]. However, studies on uninfected free-ranging pigs and pigs known to be infected with non-tuberculous mycobacteria would be necessary to precisely define the specificity. The fact that the bPPD ELISA and the DPP®BrockTB already detected >90% only 54 days after challenge shows that serology allows early detection, at least under our experimental conditions. Although both vaccination and tonsillectomy could potentially affect the natural antibody response of
normal pigs to M. bovis infection, in this study the vaccination effect and the presence of tonsils did not influence the accuracy of the serological tests. The positive correlations between pathology and serum antibody response also agree with previous results in wild boar [17]. The lack of antibody response prior to challenge suggests that oral vaccination with heat-inactivated M. bovis does not elicit serum antibody production in pigs. This is rather counter-intuitive, since it is known that wild boar do develop detectable antibodies against orally administered antigens [39].

The levels of IFNγ in response to the three different antigens increased in all confirmed-infected pigs at 54 days post-challenge (T3). Thus, the IFNγ assay yielded a sensitivity of 100%. Despite that this sensitivity was higher than the one reported for free ranging domestic pigs in Italy, which was 79-85% [23], the conditions of the two studies are different, i.e. controlled laboratory vs. field conditions. It is also important to note that all pigs tested negative to IFNγ at T2, prior to challenge. This implies that IFNγ tests suffer no interference due to oral vaccination with heat inactivated M. bovis.

The differences in the mRNA levels for CCR7, IFNβ and MUT observed in vaccinated pigs but not in the controls when animals with and without tonsillectomy were compared, supports that tonsils have a role in the expression of some genes in response to vaccination [40]. However, in agreement with the fact that tonsils did not affect the protective response in pigs, the expression of these genes was similar between vaccinated and control animals when individuals with and without tonsils were grouped together.
The complement component C3 has been associated with resistance to TB in wild boar [18,19,33] and its gene expression increases after oral vaccination with BCG or heat inactivated *M. bovis* [17,24]. In this study, C3 mRNA levels in PBMC increased with vaccination and decreased after challenge with *M. bovis*. These results agree with previous findings in wild boar [17,24], in which C3 mRNA levels in PBMC also increased with vaccination and decreased after challenge with *M. bovis*.

When pigs with and without tonsils were grouped together, only IL-1β expression in the oral mucosa was higher in vaccinated animals when compared to controls. This result suggests a role for this molecule in protection against MTC in pigs. It has been shown that IL-1β is an important mediator of innate immunity against mycobacterial infection but can also promote inflammatory tissue damage [41]. The IL-1β produced by dendritic cells preserves and expands IL-22(+) immature natural killer cells, potentially influencing mucosal innate immunity during infection [42]. Furthermore, the expression of IL-22 was recently correlated with protection against bovine TB in cattle vaccinated with BCG [43]. These results suggest that higher IL-1β expression in the oral mucosa and increase in serum cytokine levels in vaccinated pigs may constitute a protective response to infection enhanced by the vaccine, likely through its effect on stimulating C3 production, which in turn has a role against mycobacterial infection [17,18,19,24,33,44,45,46,47].

In conclusion, the reduction of TB total lesion, thorax lesion and thorax culture scores in heat-inactivated *M. bovis* vaccinated pigs (39%, 43%, and 22%,...
respectively) was lower than in wild boar immunized in the same way (43%, 76%, and 33%, respectively), suggesting differences between the pig and the wild boar model [17]. However, antibody and IFNγ responses behaved in the same way in pigs and in wild boar. Also, the high sensitivities of the serological tests for the diagnosis were similar to those previously found in wild boar [22].

Regarding gene expression studies, the results suggest that tonsils have a role in the expression of some genes in response to vaccination in pigs, but are not associated with protection which correlated with C3 expression as previously shown in wild boar [17,24]. In summary, the results show that oral vaccination with inactivated \textit{M. bovis} does not interfere with serodiagnosis and that the response of pigs to oral vaccination with heat-inactivated \textit{M. bovis} is similar than in wild boar.

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Figure 1. Lesion scores and massive culture growth produced by *M. bovis* in experimentally infected pigs. Total and thorax lesion scores in the 4 different groups: vaccinated and tonsil-ectomized (TE-V), vaccinated not tonsil-ectomized (V), unvaccinated and tonsil-ectomized (TE-C) and unvaccinated (C). Black squares indicate animals with massive *M. bovis* growth (≥ 50 cfu).

Figure 2. Serum antibody response and thorax culture score in experimentally infected pigs. The thorax culture score ranged from 0 to 2. Panels (A) and (B) display the serum antibody response in terms of optical density (OD) in the bovine PPD (bPPD) ELISA using anti-IgG and anti-IgM as conjugate, respectively; and panels (C) and (D) display the serum antibody response in terms of relative light units (LRU) in the DPP®VetTB and DPPBrock®TB tests, respectively. Diamonds represent control animals and squares represent vaccinated animals. White figures are for tonsil-ectomized animals and black figures for non-ectomized pigs. High serum antibody levels were consistently detected in the ten pigs with a high culture score.

Figure 3. Gene expression in vaccinated and control pigs. The mRNA levels of selected genes were analyzed by real-time RT-PCR in the oral mucosa of vaccinated and control pigs (A, B). Two independent comparisons were conducted between (A) pigs with and without tonsillectomy (N=5 each) and (B) vaccinated and control pigs when individuals with and without tonsillectomy were grouped together (N=10 and 9, respectively). The mRNA levels were normalized against *S. scrofa* cyclophilin, β-actin and GAPHD and normalized Ct values were represented as mean ± S.D and compared between groups by
Student’s t-test with unequal variance (*p≤0.05). (C) C3 mRNA levels were analyzed by real-time RT-PCR in PBMC of vaccinated and control pigs. (D) Cytokine protein levels were determined in pooled sera from vaccinated and control pigs at T0, T2 and T4 using the Quantibody® porcine cytokine array. For data presented in (A) and (B), samples were taken at the end of the experiment (T4).
Table 1. Oligonucleotide primers and RT-PCR conditions for the analysis of mRNA levels for selected genes.

<table>
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<th>GenBank accession number</th>
<th>Gene</th>
<th>Primers</th>
<th>PCR annealing conditions</th>
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<td>NM_214009</td>
<td>Complement component 3, C3</td>
<td>SsC3-L acaaattgaccgccacgctagg, SsC3-R gcacgtctcggctgtgctga</td>
<td>55ºC, 30s</td>
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<tr>
<td>NM_214405</td>
<td>Methylmalonyl CoA mutase, MUT</td>
<td>SsMUT-L gttgcaacagggaagagt, SsMUT-R aatgacctcaaggacgcat</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>AY373815</td>
<td>Perforin 1, PRF1</td>
<td>Ss-PerF gctccacgtagtcagca, Ss-PerR agttctccacgctctttg</td>
<td>57ºC, 30s</td>
</tr>
<tr>
<td>NM_0011001532</td>
<td>Chemokine (C-C motif) receptor 7, CCR7</td>
<td>Ss-CCR7F ttgtcctcagagctgtc, Ss-CCR7R aagggtcaggaggaag</td>
<td>57ºC, 30s</td>
</tr>
<tr>
<td>NM_182919</td>
<td>Toll-like receptor adaptor molecule 1, TRIF</td>
<td>Hs-TRIFF caggagctggagagatgag, Hs-TRIFR cgggagttgctgctga</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>NM_001243133</td>
<td>NLR family, pyrin domain containing 3 (NLRP3)</td>
<td>Hs-NLRP3F ctctctctgagctccgcag, Hs-NLRP3R gcatgaactgaaagga</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>JN391525</td>
<td>Interferon beta, IFN-β</td>
<td>Ss-IFNBF tcagaagctcctggacagt, Ss-IFNBR atcgtcccatcaagttc</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>DQ913893</td>
<td>Interferon gamma, IFN-γ</td>
<td>SsIFNg-L ctctcattgctgtgaaat, SsIFNg-R accttgctgctgtta</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>NM_214055</td>
<td>Interleukin 1-beta, IL-1β</td>
<td>SsIL1beta-L ccaaagggacatggagaa, SsIL1beta-R ttcattgctcggccttg</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>NM_001206359</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase, GAPDH</td>
<td>Ss-GAPDHF gtcggttgaagctgctg, Ss-GAPHDR agtctgcaagaggtctgt</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>DQ452569</td>
<td>β-actin</td>
<td>Ss-BactinF gagnctcggcactactca, Ss-BactinR ggcagctgctactcctc</td>
<td>55ºC, 30s</td>
</tr>
<tr>
<td>AY008846</td>
<td>Cyclophilin</td>
<td>SsCyclophilin-L agcactgggagaaagatt, SsCyclophilin-R cttggagctgcaatagaa</td>
<td>55ºC, 30s</td>
</tr>
</tbody>
</table>
Table 2. Porcine cytokines (10) assayed by the Quantibody® porcine cytokine array (RayBiotech Inc, Norcross, GA, USA)

<table>
<thead>
<tr>
<th>Cytokine Name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 1-beta (IL-1β)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-4 (IL-4)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-8 (IL-8)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-10 (IL-10)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-12 (IL-12)</td>
<td></td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor (GM-CSF)</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>Interferon gamma (IFNγ)</td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor beta 1 (TGFβ1)</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor alpha (TNFα)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Correlations between serum antibody responses, and lesion and culture scores. Correlations between serum antibody responses and lesion and culture scores in pigs at bleeding (T3) and necropsy time (T4) using bPPD ELISA (anti-pig IgG and anti-pig IgM), DPP Brock®TB and DPP®VetTB tests. Bold text indicates non-parametric spearman correlations.

<table>
<thead>
<tr>
<th>Test</th>
<th>Total lesion score</th>
<th>Thorax lesion score</th>
<th>Total culture score</th>
<th>Thorax culture score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OD anti-IgM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T3)</td>
<td>0.689**</td>
<td>0.545*</td>
<td>0.575*</td>
<td>0.652**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.001</td>
<td>0.016</td>
<td>0.015</td>
<td>0.002*</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>(T4)</td>
<td>0.484*</td>
<td>0.356</td>
<td>0.523*</td>
<td>0.848**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.036</td>
<td>0.135</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td><strong>OD anti-IgG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T3)</td>
<td>0.657**</td>
<td>0.537*</td>
<td>0.585**</td>
<td>0.670**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.002</td>
<td>0.018</td>
<td>0.009</td>
<td>0.002*</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>(T4)</td>
<td>0.384</td>
<td>0.29</td>
<td>0.449</td>
<td>0.777**</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.095</td>
<td>0.229</td>
<td>0.054</td>
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</tr>
<tr>
<td>N</td>
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<td>19</td>
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<tr>
<td><strong>DPP®BrockTB</strong></td>
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</tr>
<tr>
<td>Mixture of MPB70 and MPB83 protein</td>
<td>0.436</td>
<td>0.272</td>
<td>0.630**</td>
<td>0.479**</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.071</td>
<td>0.276</td>
<td>0.005</td>
<td>0.044</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.18</td>
<td>0.18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
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<td>19</td>
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<tr>
<td><strong>DPP®VetTB</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPB83, CFP10/ESAT-6</td>
<td>0.362</td>
<td>0.211</td>
<td>0.537*</td>
<td>0.348</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.107</td>
<td>0.367</td>
<td>0.018</td>
<td>0.144</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.18</td>
<td>0.18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td><strong>DPP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T3)</td>
<td>0.305</td>
<td>0.141</td>
<td>0.506*</td>
<td>0.304</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.204</td>
<td>0.566</td>
<td>0.027</td>
<td>0.206</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.19</td>
<td>0.19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>N</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>(T4)</td>
<td>0.34</td>
<td>0.2</td>
<td>0.496*</td>
<td>0.703**</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.154</td>
<td>0.411</td>
<td>0.031</td>
<td>0.001</td>
</tr>
<tr>
<td>Sig. (2-tailed)</td>
<td>0.19</td>
<td>0.19</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>
16 **significant at the 0.01 level
17 *significant at the 0.05 level
18
19
20
21