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Evaluating the ability of non-tuberculous mycobacteria to induce non-specific reactions in bovine tuberculosis diagnostic tests in guinea pigs and cattle

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ABSTRACT

Limitations in diagnostic test performance are one of the major challenges hampering the eradication of bovine tuberculosis (bTB). Non-tuberculous mycobacteria (NTM) are considered one of the main causes of non-specific reactions in the intradermal tuberculin test, the most widely used bTB diagnostic test. To determine the role of NTMs in bTB misdiagnosis in Spain, an experimental study including the NTM species most commonly found in bTB-positive animals from bTB-free farms in the country (*M. avium* subsp. *avium* (*Maa*), "*Mycobacterium avium* subsp. *hominissuis*" (*Mah*), *M. bourgelatii*, *M. intermedium*, *M. kansasii* and *M. nonchromogenicum*) was carried out on guinea pigs and cattle. First, guinea pigs were sensitized with the selected NTMs, and six weeks post-sensitization four antigen mixtures (bovine-PPD, avian-PPD, P22 and ESAT6-CFP10) were inoculated intradermally and their effect was measured 24- and 48-h post-inoculation. Larger erythematous reactions were observed in guinea pigs sensitized with *Mah*, *M. kansasii*, and *Maa*, with significant differences in the reactions measured at the bovine-PPD inoculation site for the two first bacteria compared with other NTMs. The sensitization process was repeated in cattle, and five months post-sensitization the same antigen mixtures were inoculated in the cervical region and responses were measured at 48- and 72-h post-inoculation. A significantly higher increase in the skinfold thickness measured at the bovine-PPD inoculation site was observed in calves sensitized with *Mah*, *Maa*, *M. intermedium* and *M. kansasii*. These results demonstrate that certain NTM species may play a more significant role in bTB diagnostic interferences and show that results obtained in guinea pig and bovine models do not always coincide.

1. Introduction

Limitations in diagnostic tests for bovine tuberculosis (bTB) are often cited as one of the main obstacles preventing the success of eradication efforts worldwide (Schiller et al., 2010; Bezos et al., 2014a). These limitations include both reduced sensitivity (i.e., inability to detect infected cattle) and reduced specificity (i.e., inability to correctly classify non-infected animals). Among the latter, non-tuberculous mycobacteria (NTM), defined as mycobacteria not included in the *Mycobacterium tuberculosis* complex (MTC) in which all causative agents of bTB are included, are considered one of the main causes of non-specific reactions in the official bTB eradication tests in the European Union, the single and comparative tuberculin skin tests (SIT and SICCT) and the interferon (IFN)-γ assay (Jenkins et al., 2018; Michel, 2008). The term NTM comprises a very diverse group of bacteria, some of which can be found in soil and water (Biet and Boschiroli, 2014; Falkinham, 2021), and can be a risk for immunocompromised humans (Prevots and Marras, 2015; Wassilew et al., 2016). There are multiple references to the ability of NTMs to infect animals (Biet and Boschiroli, 2014; Lorente-Leal et al., 2022), and its isolation from TB-positive cows

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and abattoir samples has been previously reported (Ghielmetti et al., 2018; Rónai et al., 2016; Gomez-Buendia et al., 2024).

In order to minimize the impact of NTM on bTB diagnosis, tests based on the comparison of the response to both a member of the MTC (typically *M. bovis*) and a NTM (typically, *M. avium* subsp. *avium*, *Maa*) such as the comparative skin test (Goodchild et al., 2015) or the IFN-γ assay (Vordermeier et al., 2004) can be applied. In addition, highly specific antigens that can minimize the cross-reactivity induced by the presence of NTMs have been developed in the last few years (Casal et al., 2012; Middleton et al., 2021). However, establishing the effect of the different NTM species is difficult due to the large diversity of species and differences in the possible exposure of livestock to them depending on the region or production system among other factors.

The most widely studied NTM in terms of its influence on the performance of bTB diagnostic tests is *M. avium* subsp. *paratuberculosis* (*Map*), the causative agent of paratuberculosis or Johne's disease. Previous studies have demonstrated that *Map* infection could lead to both a decreased probability of detection of bTB-infected cattle (Aranaz et al., 2006; Álvarez et al., 2009) but also an increased probability of obtaining non-specific reactions in bTB-free cattle (Dunn et al., 2005; Brito et al., 2014).

There are several articles reporting the detection of NTMs in livestock and speculating on its effect on diagnostic tests in several regions including France, Hungary, Northern Ireland, and Spain (Biet and Boschiroli, 2014; Rónai et al., 2016; Gomez-Buendia et al., 2024; Varela-Castro et al., 2022; Hughes et al., 2005). These studies are a needed step in characterizing the landscape of NTMs isolated from cattle that might be hindering a correct bTB diagnostic but are descriptive by nature and mostly include samples from non-reactor animals and/or that belong to bTB-infected herds, leaving the specific role of NTMs in the occurrence of non-specific reactions undetermined.

At the experimental level, the guinea pig is one of the most widely used animal models in mycobacterial research (Li and Li, 2023), though its use has traditionally been focused on the study of the pathogenesis of *M. tuberculosis* and *M. bovis* infection for drug and vaccine development studies (Clark et al., 2015), and especially to assess the potency of the reagents used in the skin test, the tuberculins (WOAH, 2023). However, they have been seldom used in the frame of experimental challenges with NTMs (other than *Map*) aiming at evaluating the induction of cross-reactions to different mycobacterial antigens (Fernández-Veiga et al., 2023). Similarly, the literature regarding the assessment of the capacity of NTM other than *Map* to induce non-specific reactions to the official bTB tests in cattle under experimental conditions is scarce, with articles mainly focusing on *M. kansasii* (Waters et al., 2010, 2006; Vordermeier et al., 2007; Stabel et al., 2021), *M. avium* complex species, *M. scrofulaceum*, *M. flavescens* and *M. simiae* (Stabel et al., 2021; Corner, 1981; Corner and Pearson, 1978).

Here, in order to establish the causative role of NTM recovered from reactor cattle located in officially tuberculosis-free (OTF) herds in Spain in the occurrence of diagnostic interferences to the bTB diagnostic tests, an experimental study was carried out using a guinea pig and a bovine model.

2. Material and methods

Isolates belonging to six NTM species (*Maa*, "*M. avium* subsp. *hominissuis*" (*Mah*), *M. bourgelatii*, *M. intermedium*, *M. kansasii*, and *M. nonchromogenicum*), previously identified as among the most common in reactor cattle from OTF herds in Spain (Gomez-Buendia et al., 2024), were included in the study. All isolates were retrieved from cattle from OTF herds that tested positive to the single intradermal tuberculin (SIT) test but in which the presence of bTB in the herd of origin had been ruled out based on the lack of confirmation for at least three years after the detection of the SIT reactor yielding the NTM. More information about the strains is provided in Supplementary Table 1.

The experimental protocol was performed according to the European (86/609/EEC amended by the directive 2003/65/EC) and Spanish (RD 53/2013) legislation and was approved by the Ethics Committee of the Comunidad de Madrid and by the Ethics Committee for Animal Experiments of the Universidad Complutense de Madrid (PROEX 033/19).

2.1. Guinea pig assay

Forty specific pathogen-free Hartley (Crl:HA) female albino guinea pigs from Charles River Laboratoire France (Sain Bel, France) were divided into groups of five guinea pigs (except for a control group of two animals in the second trial) and housed in separate cages in the BSL-3 facilities. Group size was set to allow the inoculation of four antigen mixtures (hereinafter referred to as antigens) in one side of the animal using a Latin square design (i.e., a minimum of four animals) plus an extra animal to account for possible losses during the 6–7-week period between sensitization with the live NTMs and inoculation of the antigens. A 6–7-week period was selected as this is the recommended duration for the evaluation of PPD potency in guinea pigs (OIE, 2017). Two trials, including 20 and 17 animals, were conducted.

In the first trial, three groups of guinea pigs were inoculated with *Mah*, *Maa,* and *M. nonchromogenicum* (one strain per group). An additional group of five guinea pigs was added as a negative control group.

In the second trial, *M. bourgelatii*, *M. intermedium*, and *M. kansasii* were inoculated to three additional groups. In this second trial, two guinea pigs were used as a negative control group.

All inocula were prepared during the exponential growth phase. The NTMs were cultured in Middlebrook 7H9 medium (Becton, Dickinson and Company, East Rutherford, NJ, USA) supplemented with 0.36 % sodium pyruvate, 0.09 % casein, and 10 % OADC (oleic albumin dextrose catalase), using the "pelleted wet weight method" as described elsewhere (Hines et al., 2007). In brief, each NTM was centrifuged at 2500 g for 30 min, the supernatant was discarded, and the pellet was weighed. Finally, dilutions were made to reach the final concentration of 0.0002 mg/ml. Viable bacteria were counted on Middlebrook 7H11 plates.

Each animal was inoculated with 0.0001 mg of wet mass living NTM suspended in 0.5 ml of 9 g/L of sodium chloride in the right hind limb via intramuscular route using 1 ml syringes fitted with 25 G 5/8'' as recommended by the Manual of Terrestrial Animals of the WOAH (World Organisation for Animal Health, 2023) and the European Pharmacopoeia (EDQM Council of Europe, 2019). Negative control groups were inoculated with 0.5 ml of phosphate-buffered saline (PBS).

Six to seven weeks after the experimental infection, thoracic and abdominal regions of both sides of the animal were shaved and 0.1 ml of four different antigens were inoculated intradermally on each side, for a total of eight inoculations per guinea pig. Scars, wounds, or marks present before the inoculation were recorded and, if necessary, the location was modified to avoid areas of the skin where these were observed. Four antigenic products were inoculated: a purified protein derivate (PPD) of *Mycobacterium bovis* strain AN-5 (PPD-B), a PPD of *Maa* strain D4 ER (PPD-A) (CZ Veterinaria, Porriño, Spain), an immunopurified antigenic product of PPD-B known as P22 (Infantes-Lorenzo et al., 2017) (Instituto de Salud Carlos III, Madrid, Spain), and the ESAT-6/CFP-10 cocktail (E/C) (Lionex GmbH, Braunschweig, Germany). The bovine and avian PPDs were the same PPDs used in the TB eradication program in Spain. Inoculations for PPDs were performed at a concentration of 500 IU/ml, while P22 and E/C at 20 μg/ml. Inoculation sites for each antigen were rotated in different animals using a Latin square design to avoid a location effect.

After 24- and 48-h post-inoculation, the presence and area of the erythema were measured with a digital caliper and evaluated together with the presence of local clinical signs (exudate, oedema, and pain) in all experimental and control groups. Two diameters were measured at each location to increase the accuracy of the area calculation.

2.2. Cattle assay

Sixty-two mixed-breed calves of approximately six months of age from two historically OTF beef farms of about 250 animals each, located in central Spain with no history of non-specific reactions were selected for the cattle assay. All animals were tested with the SIT and IFN-γ tests prior to the start of the study to verify their negative TB status.

Animals were then divided into groups of 10 animals and a control group of 12. The sample size was determined considering that reactions in the control group may follow a Poisson distribution, with animals expected to show an increase of 1 mm (a possible but negative skin test result) and the sensitized groups expected to show an increase of 3 mm (considered a biologically significant increase since it would lead to an inconclusive or positive result in the SIT test).

One of the species included in the guinea pig model (*M. bourgelatii*) was not considered here due to the lack of response observed in this model (see results), and thus five experimental groups receiving the following NTM were investigated: *Maa*, *Mah*, *M. intermedium*, *M. kansasii* and *M. nonchromogenicum*.

All inocula were prepared during the exponential growth phase as explained before, but here the pellet obtained after the centrifugation was transferred to a sterile tube containing 8–10 glass beads and sterile distilled water. The suspension was mixed in a vortex to disaggregate the bacteria and was allowed to stand for 30 min. Then, the supernatant was adjusted to 1.0 McFarland standard. Finally, CFU was calculated by seeding dilutions on duplicate Middlebrook 7H11 plates.

Each animal was inoculated subcutaneously in the right prescapular region with 1 ml of suspension containing 10^6 CFU of a given strain (10 animals per strain). Another group of 12 animals included as a negative control group was inoculated with the same volume of PBS.

After five months, in line with previous work (Roy et al., 2019; Bezos et al., 2015), the same four antigens evaluated in the guinea pig assay were used to perform a skin test. PPD-B and PPD-A were inoculated at the concentration used in the Spanish eradication TB program (25,000 IU/ml), and P22 and E/C were used at 500 μg/ml. Inoculations of 0.1 ml were performed using a Dermojet syringe (AkraDermojet, Pau, France) on the left side of the neck, with locations per antigen rotating in different animals following a Latin-squared design to take account of any position-dependent effects.

The skinfold thickness of each animal was measured before and 48 and 72 h after inoculation by the same veterinarian using the same caliper to exclude potential biases.

Also, blood samples from all animals were collected in heparinised tubes at the time of antigen inoculation. Samples were transported directly to the laboratory and processed within four hours after collection. Samples were distributed on a CELLSTAR® (Greiner Bio-One International GmbH, Österreich, Austria) 24-well cell culture plate (1 ml per well) and stimulated with 100 μl of PBS, and 20 μg/ml of PPD-B, PPD-A, P22 and E/C. Another aliquot of all samples was also stimulated using pokeweed mitogen (Lectin from *Phytolacca americana*, Sigma, Merck KGaA, Darmstadt, Germany) at 2 μg/ml as a measure of lymphocyte viability (Estes et al., 1994; Horii et al., 1998). The plates were incubated in a humid atmosphere (i.e., with a basin of distilled water to avoid drying) at 37ºC for 24 h and centrifuged at 600 g for 12 min to collect the stimulated plasma (SOP/006/EURL). Then, plasma samples were analysed using the IDvet kit (ID Screen® Ruminant IFN-γ, IDvet, Innovative Diagnostics, Gravels, France) according to the manufacturer instructions (using 25 μL of each sample + 25 μL of dilution buffer 1). Results were expressed as optical densities (OD) by measuring the absorbance of each well at 450 nm. All samples were validated as they showed high mitogen-stimulated OD values (OD > 0.5). For interpretation, results were transformed to a sample-to-positive (S/P) ratio considering the values of the positive and negative controls included in each plate as follows:

$$S/P = \left(\frac{OD\ bovis - OD\ avium}{OD\ mean\ positive\ control - OD\ mean\ negative\ control} \right) * 100$$

2.3. Postmortem examination, bacteriological culture and PCR

After the experiments, guinea pigs and cattle were culled and subjected to a postmortem examination to identify macroscopical lesions. Lungs, inguinal lymph node, liver, spleen, and kidney samples (guinea pig) and lungs and lymph nodes (mediastinal, retropharyngeal, tracheobronchial and prescapular) (cattle) were collected for bacteriology.

Samples were then subjected to bacteriological culture as previously described (Lorente-Leal et al., 2019). Briefly, samples from the same animal were pooled and homogenized. After, samples were decontaminated with a 0.75 % hexadecyl pyridinium chloride solution (Sigma-Aldrich, St. Louis, MO, USA) and inoculated in a solid Löwenstein-Jensen and Coletsos media supplied with 1 % sodium pyruvate (Difco, Madrid, Spain) at 37ºC and incubated for up to three months.

If bacterial growth was observed, DNA from a loopful of colony-forming units was extracted, resuspended in 200 μl of HPLC-grade water (Sigma-Aldrich, St. Louis, MO, USA) and heat-inactivated at 100ºC for 15 min. Then, DNA was analysed using a set of real-time PCRs targeting the Internally Transcribed Spacer (ITS) (Sevilla et al., 2015) for identification of microorganisms belonging to the genus *Mycobacterium*, IS*6110* (Lorente-Leal et al., 2021) to exclude the presence of MTC members, and IS*1311* (Sevilla et al., 2015) for identification of *M. avium* complex members. In the case of detection of the IS*1311*, another PCR targeting the IS*901* (Kunze et al., 1992) and IS*1245* (Guerrero et al., 1995) sequences were applied to distinguish between *Maa* and *Mah* species. In case the presence of MTC or MAC members was discarded, DNA samples were subjected to Sanger sequencing targeting a 16S rRNA fragment (Wilton and Cousins, 1992).

2.4. Statistical analysis

To determine whether, depending on the NTM to which each guinea pig was sensitized, there were differences in the erythema (in mm^2) induced by each antigen, we conducted a Kruskal-Wallis (K-W) test followed by a Dunn post-hoc test with a Holm-Bonferroni correction. Additionally, differences in the erythema induced by the inoculated antigens depending on the NTM to which the guinea pigs were sensitized were compared using a Friedman test followed by a Nemenyi-Wilcoxon-Wilcox (N-W-W) all-pairs post-hoc test for pairwise comparisons considering the guinea pig as a block. For the cattle assay, we performed the same analyses using the increase in the skinfold thickness (in mm) as the outcome variable. Analysis was performed in R version 4.3.1 (R Core Team, 2023) using the PMCMRplus R package (Pohlert, 2023). Figures displaying the responses in guinea pigs/cattle were obtained using the geom_jitter function from ggplot2, which adds a small amount of random variation to the location of each point, to allow visualization of multiple points with the same values (Wickham, 2016).

3. Results

3.1. Guinea pig assay

Guinea pigs sensitized with *Mah* experienced the largest reactions (mm^2) at the locations inoculated with PPD-B (median = 103 mm^2) and PPD-A (208 mm^2) at 24 h post-inoculation (Fig. 1). A lower reaction to PPD-B was observed in guinea pigs sensitized with *Maa* (25 mm^2) while response to PPD-A was similar to the one observed for *Mah* (185 mm^2). In the case of guinea pigs in the *M. kansasii* group, large reactions in response to the inoculation of PPD-B were observed (95 mm^2), while

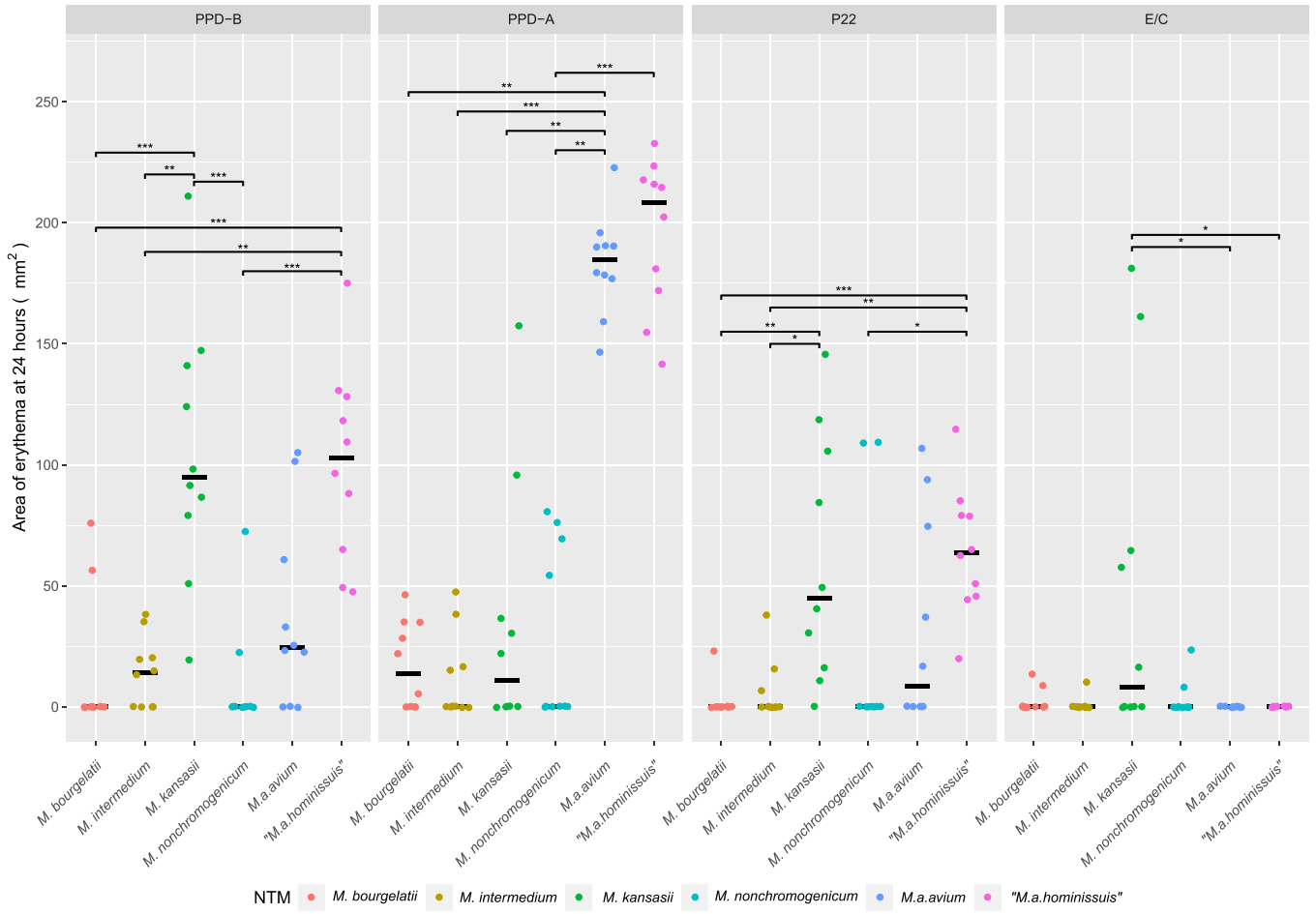

Fig. 1. Area of erythema observed (in mm^2) in the guinea pigs at 24-h post-inoculation. Black lines represent the median of the response reaction for each antigen. Brackets represent significant differences according to the Dunn post-hoc test. Stars denotes the *p*-value as follows: '*': $0.05 < p\text{-value} < 0.01$; '**': $0.01 < p\text{-value} < 0.001$; and '***': $p\text{-value} < 0.001$.

responses to PPD-A were limited (Fig. 1). These three species also induced a limited response to P22, while only some animals sensitized with *M. kansasii* reacted to E/C (8 mm^2). No reactions were observed in guinea pigs inoculated with any other mycobacteria or in the negative control group. Measurements were lower at 48 h compared with 24 h post-inoculation (Supplementary Figure 1B and 1C).

There were significant differences in the response depending on the NTM to which the guinea pigs were sensitized for all antigens at 24 and 48 h (K-W test; $p < 0.001$). In the case of PPD-B, guinea pigs sensitized with *Mah* and *M. kansasii* exhibited significantly higher responses compared to the other NTM and were not significantly different from each other. A similar result was observed for PPD-A, with *Mah*- and *Maa*-sensitized guinea pigs showing significant differences compared with the others. Results for P22 were identical to the ones observed for PPD-B but no statistically significant differences were observed between guinea pigs sensitized with *Maa* and *Mah*. Lastly, for E/C, only sensitized guinea pigs to *M. kansasii* showed significant differences to certain other NTMs (Fig. 1).

Furthermore, significant differences between the erythema area observed at each antigen inoculation site after 24 h were only observed in the *Maa* (Friedman test; $p = 0.005$) and *Mah* ($p = 0.002$) groups, in both cases due to the differences between PPD-A and E/C (N-W-W test; $p < 0.001$). Additionally, at 48 h there were also significant differences between PPD-A and P22 for *Mah*-sensitized guinea pigs ($p = 0.04$) (Supplementary Figure 1C).

No macroscopic lesions were observed in any of the animals in the postmortem analyses. After performing the bacteriological culture, we were able to recover the inoculated NTM in five animals (two *M. nonchromogenicum*, two *Mah* and one *Maa*).

3.2. Cattle assay

One calf included in the control group died during the experiment due to a pulmonary disease not related to the experiment.

Several cattle sensitized with *Maa*, *Mah*, *M. intermedium* and *M. kansasii* showed an increase in skinfold thickness of more than 2 mm in PPD-B and PPD-A inoculated areas (Fig. 2). In general, reactions were larger at 72 than at 48 h post-inoculation (Supplementary Figure 2). Considering the equivalent of a severe interpretation of the SIT test based on the response to PPD-B (animals with > 2 mm would be considered reactors), 18 cattle (36 % of all cattle excluding the control group) would be positive, with at least one reactor observed in all groups except the *M. nonchromogenicum* one. The results of the SIT and SICCT tests under different criteria for each NTM are shown in Table 1.

Increases in skinfold thickness > 2 mm at the P22 and E/C inoculation sites at 48 h post-inoculation were also observed in 6 and 2 animals (Supplementary Fig. 2), respectively, and 5 and 3 animals at 72 h (Fig. 2). Applying the equivalent of a standard interpretation in the SIT (≥ 4 mm increase in the absence of clinical signs), P22 and E/C elicited reactions in only one (*M. kansasii*) and two animals (*Mah* and *M. kansasii*) at 48 h, and two (*M. kansasii*) and three animals (*Maa*, *Mah*, and *M. kansasii*) at 72 h, respectively (Fig. 2 and Supplementary Fig. 2).

There were significant differences (K-W test; $p < 0.001$) in the skinfold thickness increase produced by the inoculation of each antigen

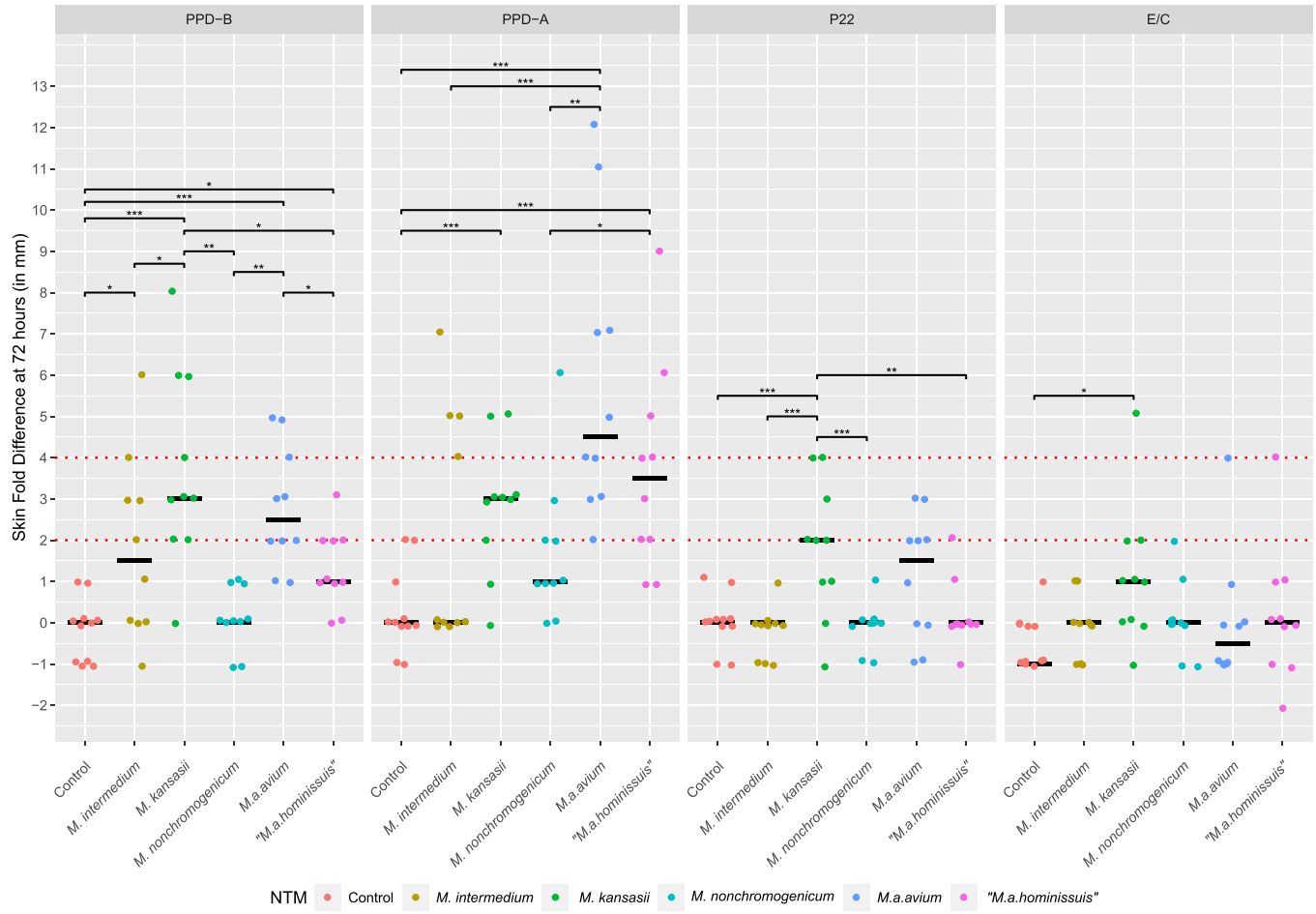

Fig. 2. Skinfold thickness increase (in mm) in the calves at 72-h post-inoculation. Black lines represent the median. Red dotted lines represent the limits considered for qualitative interpretation of the skin test (skin fold increase > 2 mm & ≥ 4 mm). Brackets represent significant differences according to the Dunn post-hoc test. Stars denotes the *p*-value as follows: '*': $0.05 < p$-value < 0.01; '**': $0.01 < p$-value < 0.001; and '***': p-value < 0.001.

Table 1
Positive animals to the skin test in cattle considering different interpretation criteria at 48- and 72-h post-inoculation.

Criteria	*M. intermedium*	*M. kansasii*	*M. nonchromogenicum*	*Maa*[6]	*Mah*[7]	Total
48 h						
SIT severe interpretation[1]	3/10	7/10	0/10	5/10	1/10[5]	16/50
SIT standard interpretation[2]	2/10	3/10	0/10	3/10	1/10[5]	9/50
SICCT severe interpretation[3]	1/10	5/10	0/10	2/10	1/10[5]	9/50
SICCT standard interpretation[4]	1/10	2/10	0/10	1/10	1/10[5]	5/50
72 h						
SIT severe interpretation[1]	4/10	7/10	0/10	5/10	2/10[5]	18/50
SIT standard interpretation[2]	2/10	5/10	0/10	3/10	1/10[5]	11/50
SICCT severe interpretation[3]	2/10	5/10	0/10	3/10	1/10[5]	11/50
SICCT standard interpretation[4]	1/10	3/10	0/10	1/10	1/10[5]	6/50

1. PPD-B $>$ 2 mm; 2. PPD-B $\geq$ 4 mm; 3. PPD-B $>$ 2 mm & PPD-B $>$ PPD-A; 4. PPD-B – PPD-A $>$ 4 mm; 5. The animal was considered positive due to the presence of a crust produce by the PPD-B; 6. *M. avium* subsp. *avium*; 7. "*M. avium* subsp. *hominissuis*".

depending on the NTM group for all antigens and times except for E/C at 48 h post-inoculation (Fig. 2 and Supplementary Fig. 2A). All calves except those sensitized with *M. nonchromogenicum* exhibited significantly higher responses at the PPD-B inoculation site compared to the control group. Additionally, *M. kansasii*-sensitized calves' responses were significantly higher than those of the other groups (Fig. 2). Results for PPD-A were similar to those for PPD-B except for the control and *M. intermedium* groups between which no significant differences were observed. Furthermore, *Maa*-sensitized calves showed a significantly higher increase compared to the other groups (Fig. 2). In contrast, for P22 and E/C, only *M. kansasii* differed from the control group and, in the case of P22, from other NTM groups except *Maa* (Fig. 2).

Besides, significant differences between the skinfold thickness increases induced depending on the antigen were observed in all NTM groups (Friedman test; $p < 0.001$) except in the control and *M. intermedium* groups at 72 h post-inoculation (Supplementary Figure 2 C), while no statistically significant differences were observed in the *M. nonchromogenicum* and control groups at 48 h post-inoculation (Supplementary Figure 2B). In the *Maa* and *Mah* groups, the reactions to PPD-A were significantly higher than those to P22 and E/C. Additionally, a significantly larger increase in the PPD-A inoculation site compared to E/C was observed in *M. intermedium* (48 h),

M. nonchromogenicum (72 h) and *M. kansasii* (48 and 72 h) post-inoculation (Supplementary Figure 2B and 2 C). Moreover, PPD-B yielded significantly higher reactions in *Maa* (48 h) and *M. kansasii* (48 and 72 h)-sensitized calves compared to E/C (N-W-W test; $p < 0.05$) (Supplementary Figure 2B and 2C).

Cattle also reacted to antigens when performing the IFN-γ test. According to the manufacturer instructions, applying a more sensitive cut-off (S/P ≥ 16 %, use in bTB infected herds only), 11 animals yielded a positive result, seven sensitized with *M. kansasii*, two with *M. intermedium*, and one with *Mah* and *M. nonchromogenicum*. Applying a less sensitive cut-off (S/P ≥ 35 %), only three animals, all of them sensitized with *M. kansasii*, were considered positive.

Cattle sensitized with *M. kansasii* had the highest ODs at the PPD-B (median = 1.55), P22 (0.77) and E/C (0.31) stimulated wells. For the PPD-A, cattle sensitized with *Maa* had the highest ODs (1.65), similar to *Mah* (1.48). All the results are shown in Fig. 3.

One animal inoculated with *Maa* showed granulomatous TB-like lesions on postmortem examination. Lesions were observed in the prescapular and mediastinal lymph nodes. Following bacteriological culture, we were able to recover the inoculated NTM in two animals (one *Maa* and one *Mah*).

4. Discussion

One of the major obstacles to bTB eradication is the limited diagnostic accuracy of the tests used. NTM has been pointed out as an important source of non-specific reactions in the official eradication tests, hampering efforts to eradicate the disease and diminishing stakeholder confidence in eradication programs (Jenkins et al., 2018; Biet and Boschiroli, 2014). Considering the results obtained in a previous study in which 373 isolates of NTM from skin test-positive cows originating from OTF herds in Spain were identified (Gomez-Buendia et al., 2024) we conducted an experimental assay using a guinea pig and a bovine model to elucidate whether the most prevalent NTM species found in these animals were indeed the likely cause of such non-specific reactions.

Significant differences in the magnitude of the reactions depending on the specific NTM strain in both the guinea pig and cattle models were observed for all antigens inoculated, but particularly for the avian and bovine PPDs (Fig. 1 and Fig. 2). Not surprisingly, the largest reactions at the PPD-A inoculation site were recorded in guinea pig and cattle sensitized with *Mah* and *Maa*; this was expected given that PPD-A is derived from *M. avium* strain D4 ER, and therefore animals sensitized with *M. avium* subspecies should have the strongest reactions. Nevertheless, the problem that MAC members can cause in bTB diagnosis was also exemplified by the reactions observed at the PPD-B inoculation site in these animals, though in guinea pig *Mah* induced the largest PPD-B reactions while for cattle it was *Maa* (Fig. 1 and Fig. 2). Considering that PPD-A and PPD-B share approximately 146 proteins (Infantes-Lorenzo et al., 2017), the cross-reactivity induced by MAC members can be expected, but the reasons for the differences in the response induced by each of the two *M. avium* subspecies here (which differed depending on the animal model considered) are unclear. *Mah* and *Maa* are two closely related *M. avium* subspecies. *Mah* is more genomically diverse due to its adaptation to different environments, while *Maa* is more clonal (Uchiya et al., 2017; Mizzi et al., 2022). Their main genetic differences are related to certain regions that encode virulence and host adaptation genes, particularly observed among the mycobactin synthesis cluster, even though most of the disparate genes are poorly characterized (Mizzi et al., 2022).

The other NTM inducing large reactions at the PPD-B site in both the guinea pig and cattle models was *M. kansasii,* leading to the occurrence of positive SIT reactions in 7/10 cattle if a severe interpretation (i.e., considering positive any animal with a reaction > 2 mm) was applied (Table 1, Fig. 2). Moreover, if a standard interpretation of the IDvet test (S/P ≥ 35 %) is applied, the three negative calves in the SIT would have been considered reactors in the IFN-γ assay, further highlighting the diagnostic problem that this NTM species can cause. Unlike other NTMs, *M. kansasii* is known to harbor certain genes that encode proteins among the most abundant identified in PPD-B (Infantes-Lorenzo et al., 2017),

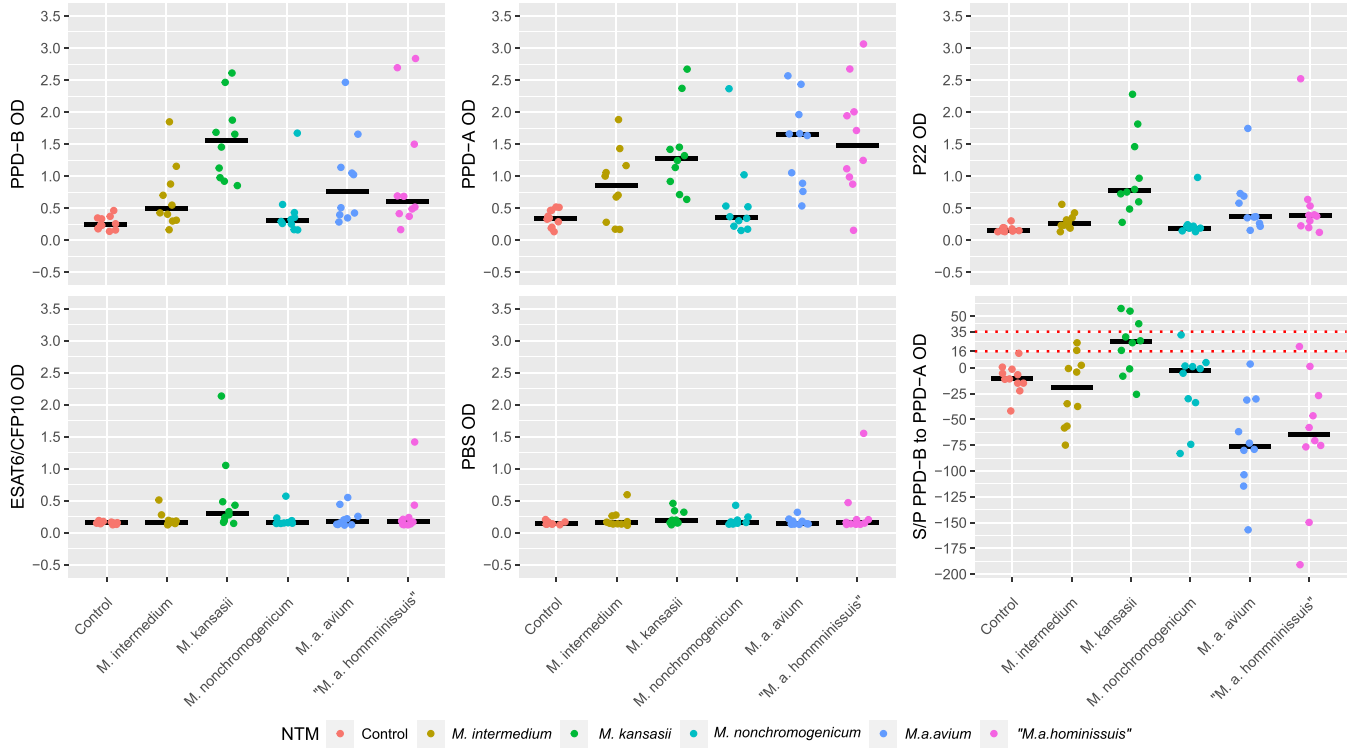

Fig. 3. Optical densities observed at the IFN-γ test depending on the antigen used for the stimulation. Black lines represent the median. Red dotted lines indicate diagnostic cut-off points for IDvet (S/P ≥ 16 % & S/P ≥ 35 %).

such as ESAT-6 and CFP-10 (Renshaw et al., 2005), MPB70, MPB83 and molecular chaperones GroES and GroEL (Waters et al., 2006; Gcebe et al., 2016). Therefore, the use of more specific antigens such as the E/C complex in the skin test would theoretically have a limited effect minimizing the cross-reacting responses elicited by *M. kansasii*. However, only one bovine had a reaction of > 2 mm at the E/C inoculation site (Fig. 2), what could reflect the lower immunogenicity of this antigen compared to bovine PPD, as also evidenced in *M. bovis* infected cattle. The use of comparative tests, such as the SICCT test, that also consider the response to PPD-A to identify reactors, has demonstrated to mitigate the diagnostic interference caused by *M. kansasii* in cattle (Waters et al., 2010, 2006). However, in our study two or five out of the seven SIT reactors would be still considered positive by applying a severe or standard cut-off in the SICCT test (Table 1).

Regarding the effect of *M. intermedium*, while limited responses were observed in guinea pigs, cattle sensitized with this NTM displayed significantly larger responses to PPD-B and PPD-A compared to the control group suggesting a higher reactivity in this host species. Notably, for PPD-B, four animals exceeded the 2 mm threshold and two had an increase of 4 mm or more while for PPD-A four animals had a response $\geq$ 4 mm. The literature related to this NTM is scarce and is mostly focused on its importance as an opportunistic pathogen in human patients linked to contaminated water sources (Edson et al., 2006). *M. bourgelatii*, which is closely related to *M. intermedium* (Guérin-Faublée et al., 2013), was not included in the cattle assay due to the lack of response to all antigens observed in the guinea pig assay. However, in hindsight and given the differences in immunogenicity of *M. intermedium* and other NTMs based on the results obtained in the guinea pig or the cattle models for, this may not have been a sensible choice, and therefore additional studies should be conducted to evaluate the ability of *M. bourgelatii* to induce positive responses to certain antigens in cattle.

Considering the number of articles mentioning *M. nonchromogenicum* as one of the most common NTMs found in cattle (Biet and Boschiroli, 2014; Rónai et al., 2016; Gomez-Buendia et al., 2024; Hughes et al., 2005) and the reports describing its isolation from lesioned samples from abattoirs (Ghielmetti et al., 2018; Gcebe et al., 2013; Berg et al., 2009; Nuru et al., 2017), the lack of response to most antigens observed in animals sensitized with this NTM was surprising (Fig. 1 and Fig. 2). These results are in agreement with a previous study (Fernández-Veiga et al., 2023) in which limited responses after the inoculation of both avian and bovine PPDs and P22 were observed in guinea pigs even when inoculated with a higher dose of *M. nonchromogenicum* (10^6 CFU compared with 10^3 in this study) (Fernández-Veiga et al., 2023). This raises several questions regarding the external validity of the results obtained in experimental models, particularly when involving guinea pigs, for this NTM species. *M. nonchromogenicum* can be found in the environment, both in soil and water sources (Gcebe et al., 2013), so naturally sensitized animals may be exposed to high concentrations of the bacteria, potentially over long periods of time, which could lead to an increased immune response (Falkinham, 2021; Pavlik et al., 2022). Research conducted in Ireland revealed the presence of *M. nonchromogenicum* in the nasal mucosa of cows (McCorry et al., 2004), suggesting that the respiratory route could potentially be an effective means for this species to induce sensitization in animals, though it is not clear how common this exposure may be.

The combined consideration of the four antigens evaluated here highlighted the usefulness of comparative tests based on the inclusion of PPD-A and of tests based on more specific antigens such as P22 and E/C compared with single tests only based on PPD-B for discriminating between responses induced by NTMs and those due to *M. tuberculosis* complex members. Interestingly, no animals were positive on both skin tests and IFN-γ assay if the standard cut-offs were applied, which also highlights the potential of the combined use of these techniques to maximize the specificity of the tests. However, comparative tests or tests based on more specific antigens must be used carefully in settings where bTB may be present since their application is known to lead to a decrease in diagnostic sensitivity (Lahuerta-Marin et al., 2018; Bezos et al., 2014b), what could have disastrous consequences from an eradication stand-point.

Our results should be interpreted with care since our experimental models were based on a relatively high dose administered through the intramuscular route and thus the responses observed in sensitized animals may not adequately reflect what animals experience when exposed naturally through other routes to these NTMs in the field. Still, the relative differences observed here when exposed to different NTM species and/or antigens can help to understand the potential impact that these bacteria may have on the performance of the bTB diagnostic tests and the potential usefulness of the available antigens.

5. Conclusions

MAC members and *M. kansasii* induced the largest reactions in both a guinea pig and a cattle model particularly when considering the PPDs traditionally used in bTB diagnosis. Nonetheless, the use of comparative tests that compare the response to PPD-B with that elicited to PPD-A would help minimizing diagnostic interference in cattle. The use of alternative antigens decreased the number of reacting animals though even in the small groups evaluated here some animals still had significantly larger reactions when exposed to certain NTMs. Differences in the results obtained in the guinea pig and cattle models for certain NTMs including *M. intermedium*, *Maa* and *Mah*, highlight the limitations of relying exclusively on a guinea pig model for the evaluation of the reactivity induced by NTMs but also for assessing the usefulness of antigens for bTB diagnosis.

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Aaron Rendahl: Writing – review & editing, Formal analysis. **Alberto Diez-Guerrier:** Writing – review & editing, Resources, Investigation. **Javier Ortega:** Writing – review & editing, Investigation. **Alberto Gomez-Buendia:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Julio Alvarez:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Beatriz Romero:** Writing – review & editing, Supervision, Resources, Conceptualization. **Javier Bezos:** Writing – review & editing, Supervision, Conceptualization. **Jose Luis Saez:** Writing – review & editing, Resources, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.110250.

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