Towards diagnostic differentiation between MTBC and NTM:

ELISA and Western blot antibody detection

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Abstract

In South Africa, bovine tuberculosis (BTB) occurs sporadically in cattle and is endemic in African buffaloes. Non-tuberculous mycobacteria (NTM) may induce cross-reactive immune responses which can interfere with the diagnosis of tuberculosis. This cross-reactive immune response has not yet clearly been established for the humoral branch of the immune system, but test performance has been suboptimal. Therefore, the aim of the study was to develop an NTM specific ELISA to better classify positive reactions to *Mycobacterium bovis* (*M. bovis*). Purified protein derivatives (PPDs) for the ELISA were made of the following NTM species: *M. fortuitum*, *M. malmesburiense* and *M. nonchromogenicum*. The results for the checkerboard titrations for the ELISA were not what was expected, due to a lot of background and no plateau height maximum. The plateau would have made it possible to identify a region of antigen and antibody excess so that the next step for the CBT could be performed. The semidry Western blot did not demonstrate presence of antibodies to the various PPDs. The absence of antibodies could be relating to experimental (Western blot optimisation, freeze-thaw cycles of sera, PPD production) or biological factors. The question arises whether the humoral immune system is triggered enough to induce a detectable response when an animal is infected with NTM. In line with what is known about (B)TB immunity, the cell-mediated immune response may primarily handle the bacterial load. More research is needed for more accurate (B)TB tests to be developed. For now, the best approach would be to join forces and test an animal with a cell-mediated immune response-based test supported by a serological assay.

Introduction

Bovine tuberculosis (BTB) is a disease that is endemic in different areas of the world and is mainly caused by *Mycobacterium bovis (M. bovis)* (Lyashchenko *et al.* 2008). *Mycobacterium bovis* is an acid-fast, gram-positive, rod-shaped bacterium (Karlson & Lessel, 1971). *Mycobacterium bovis* is part of the *Mycobacterium tuberculosis* complex (MTBC), which includes all mycobacterial species that can cause tuberculosis in humans or animals (Karlson & Lessel, 1971). Most species, including humans, are susceptible to *M. bovis*, the most susceptible species are cattle, goats and pigs (Radostits *et al.* 2006). Furthermore, several wildlife hosts are reservoirs of *M. bovis* infection for domestic livestock in different countries (K. P. Lyashchenko *et al.* 2008). Wildlife hosts are for example: red deer and wild boar in Spain, but also badgers in south-west England and in Ireland (Griffin *et al.* 2005; Infantes-Lorenzo *et al.* 2019), brush-tail possums in New-Zealand (Porphyre *et al.* 2007), mule deer, white-tailed deer, elk and bison in North America (O'Brien *et al.* 2006) and water buffaloes in Australia (de Lisle *et al.* 2001; Radostits *et al.* 2006). In South Africa, both cattle and African buffaloes are maintenance hosts of bovine tuberculosis (Michel *et al.* 2006). Due to wildlife reservoirs, control of tuberculosis is made difficult (Pollock & Neill, 2002).

In a large number of species, *M. bovis* causes a chronic, progressive and mostly respiratory disease (de la Rua-Domenech *et al.* 2006). Mycobacteria can be transmitted directly by exhaled air, through ingestion, and less frequently by contact with mucous membranes and broken skin. By inhaling the bacterium in an aerosol droplet an infection with *M. bovis* can be established. In the respiratory tract, the droplet attaches to the alveolar surface of the long. *Mycobacterium bovis* resides intracellularly in macrophages or other monocytic cells (de la Rua-Domenech *et al.* 2006; Pollock & Neill, 2002), which interact with cells from the innate and acquired immune responses (Pollock & Neill, 2002). *Mycobacterium bovis* is able to inhibit the fusion of the bacteria-containing macrophages with lysozymes, escaping killing (Pollock and Neill 2002; Rastogi *et al.* 2016). The predominant immunological response is effected by T-lymphocytes (T-cells) (de la Rua-Domenech *et al.* 2006). The infected macrophage presents secreted antigens or breakdown products of the bacterium to T-cells. The T-cells, which respond, will undergo clonal expansion and differentiation. Then followed by $CD4^+$ (T-helper cells) and $CD8^+$ T-cells (cytotoxic T-cells). Specific Th1-cells produce cytokines, like interferon-γ (INF-γ), which is believed to be one of the key players in (B)TB immunity and, amongst other functions, activates macrophages (Pollock & Neill, 2002). Natural killer cells (NK) are also part of the initial response to the mycobacteria, the cell-mediated immune response. The NK-cells also produce INF-γ, which can be cytolytic towards mycobacteria-infected target cells. The neutrophils are early-response cells and associated with developing granulomas (Pollock & Neill, 2002). But in the early stages of the disease, cattle may also excrete mycobacteria in nasal and tracheal mucus (de la Rua-Domenech *et al.* 2006; Radostits *et al.* 2006). In cattle, the infection is usually chronic but can remain subclinical for a long period of time. In most cases, clinically there are no signs or typical lesions of BTB readily detectable. Therefore, the ante-mortem surveillance primarily relies on the use of immunodiagnostic tests to detect infected cattle at an early stage (de la Rua-Domenech *et al.* 2006).

For more than a century, the tuberculin skin test is being used for the diagnosis of tuberculosis. The test is based on the measurement of delayed-type hypersensitivity (DTH) reaction of the skin after tuberculin injection. Tuberculins are purified protein derivates (PPDs), which are a crude mixture of proteins, lipids, and carbohydrates gained from for example *M. bovis* (PPD-B), but can also be produced from environmental mycobacteria, such as *M. avium* (PPD-A) (Infantes-Lorenzo *et al.* 2017; Yang *et al.* 2012) or others. The local reaction of the skin to

injection of tuberculin that can be observed in the skin test occurs due to the cell-mediated immune response (CMI). The skin test is assessed by examining the injection site for swelling before and approximately 72 hours after injection (plus or minus 4-6h). The injection site is evaluated for the presence of clinical signs of a DTH reaction and the skin fold thickness (SFT) is measured. The SFT before and after injection as well as the difference of the SFT between the site of injection of bovine versus that of avian tuberculin are compared (van der Heijden *et al.* 2016; de la Rua-Domenech *et al.* 2006; Monaghan *et al.* 1994). The test performance can be variable: the sensitivity ranges from 68-95%, and the specificity from 96-99%. The sensitivity is affected by the dose of tuberculin, the interval post-infection, desensitization, post-partum immunosuppression and observer variation. The specificity may be influenced by sensitization of the test by exposure to *M. avium*, *M. paratuberculosis* and nontuberculous mycobacteria (NTM) (Monaghan *et al.* 1994). Although the sensitivity and specificity can be influenced by a lot of factors, the skin test remains the primary ante-mortem diagnostic tool for BTB in cattle (de la Rua-Domenech *et al.* 2006)**.**

In 1985, the BOVIGAM™, which is an INF-γ release assay, was developed. The BOVIGAM™ is a rapid assay of the cell-mediated immune response used for the detection of BTB. Fresh heparinized whole blood samples are stimulated with PPDs and incubated at 37°C in the laboratory. After 20-24 hours, samples of the plasma supernatant are harvested and assayed (by a sandwich ELISA, commercially available kit) for the presence of IFN-γ (de la Rua-Domenech *et al.* 2006; Wood & Jones, 2001). The cytokine IFN-γ is used because it is released by T-cells in response to antigenic stimulation by *M. bovis* antigens if the animals were previously primed by infection with *M. bovis* (Wood & Jones, 2001). Like the tuberculin skin test, the BOVIGAM™ is also a CMI test, but it shows higher specificity and even sensitivity in BTB testing in cattle. The specificity in cattle varies from 96.2% to 98.1% depending on the cut-off point (Wood *et al.* 1991). In buffalo, a high sensitivity (92.1%) was reached, but at the cost of a greatly reduced specificity (68.3%). But by including environmental mycobacteria as *fortuitum* PPD (PPD-F) besides the standard avian PPD (PPD-A) the specificity increased to 95.1% and the sensitivity decreased only minimally to 83.3% at the 0.385 as the cut-off point for PPD-B (Michel *et al.* 2011).

The CMI response is most prominent in the early stages of BTB and tends to wane as the disease progresses and the animal may eventually become anergic, as shown in figure 1. An animal is anergic when they no longer exhibit a detectable CMI response, i.e. cannot be measured by the skintest or BOVIGAM™ assay, but may have a high level of circulating antibodies (de la Rua-Domenech *et al.* 2006; Pollock & Neill, 2002). When the bacterial load is high or in more advanced stages of the disease, animals may have an antibody response in the absence of a CMI response. Temporary anergy has also been described, if the animal is subjected to stress (de la Rua-Domenech *et al.* 2006; Monaghan *et al.* 1994). Tuberculous cows that calved within four to six weeks prior to the skintest sometimes fail to react (Monaghan *et al.* 1994; Pollock & Neill, 2002). Therefore, in order to detect infected animals that could otherwise be missed by the skin test and the IFN-γ assay, researchers have developed serological assays (Plackett *et al.* 1989; Pollock & Neill, 2002). Another consideration is that the skin test and the BOVIGAM™ assay require sophisticated laboratory infrastructure and are relatively expensive to use. In particular, when dealing with wildlife or in areas where finances are an issue or that are rural, serological tests provide a viable alternative.

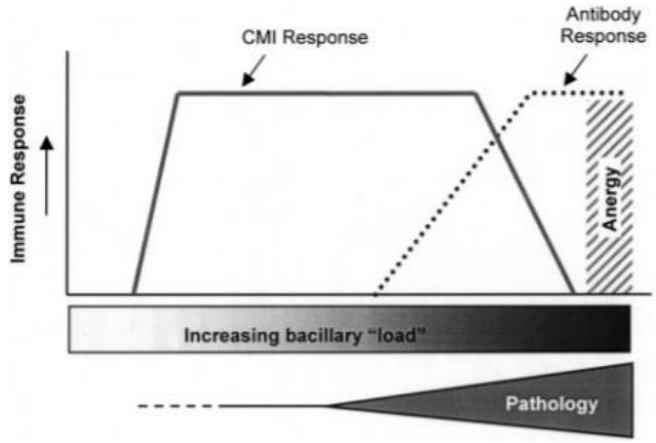

Figure 1: The spectrum of the immune response in cattle after infection with *M. bovis*. Initially, the cell-mediated immune (CMI) responses develop, while humoral antibody responses develop as the bacterial load increases. Anergy can occur in a more advanced stage of the disease, where CMI responses are no longer detected (Pollock & Neill, 2002).

The first TB ELISA was reported in 1972 and has been widely used for serodiagnosis of tuberculosis since (Engvall & Perlmann, 1972). It has been hypothesized, that due to cross-reactivity with NTM, the test has a problem with the specificity (Michel, 2008). The use of an ELISA with highly specific antigens could improve test specificity (Daniel & Debanne, 1987). Alternative serological tests, such as the lateral-flow-based rapid test (RT) and multi-antigen print immunoassay (MAPIA) have since been developed (Lyashchenko *et al.* 2008). Both tests showed satisfying results in an attempt to increase the specificity of (B)TB tests (Greenwald *et al.* 2009; Lyashchenko *et al.* 2008). In the RT, small numbers of wild boar and white-tailed deer exposed to *M. avium* did not demonstrate cross-reactivity with the detection of *M. bovis* specific antibodies. This suggested a higher specificity of the assay (Lyashchenko *et al.* 2008). In elephants, the MAPIA was used with a panel of 12 mycobacterial antigens, two protein fusions, and two native antigens, which showed a 100% sensitivity and 95%-100% specificity (Greenwald *et al.* 2009). In addition to these serological assays, an indirect PPD-B ELISA has been developed for use in wild boar to detect antibodies against *M. bovis* (Pérez de Val *et al.* 2017). The results showed a sensitivity of 86% and specificity of 100%, which supported the use of an indirect ELISA as an additional technique for the diagnosis of TB in wild boar (García-Bocanegra *et al.* 2012; Pérez de Val *et al.* 2017). In an ELISA for detection of bovine IgG anti-*M. bovis* antibodies in cattle the sensitivity was 73.6% and the specificity 94.1% (Ritacco *et al.* 1990). Unfortunately, false-positive results were obtained in 21/22 cattle infected with paratuberculosis, which demonstrates cross-reactivity between other mycobacteria (in this case *Mycobacterium avium* subsp. *paratuberculosis* or MAP) and *M. bovis* (Rastogi *et al.* 2016; Ritacco *et al.* 1990).

Serological tests such as an indirect ELISA are simple, inexpensive and can be used to screen many animals in a short time (Infantes-Lorenzo *et al.* 2017). A point of improvement of these tests for cattle and buffalo is the specificity. False-positive test results are hypothesized to arise due to the cross-reactivity of *M. bovis* and NTM (Gcebe *et al.* 2013). The NTM selected for the experiment in the present study have been shown to have an occurrence that is relatively widespread in South Africa (Gcebe *et al.* 2013). *Mycobacterium nonchromogenicum* and *M. fortuitum* have been isolated from nasal and pharyngeal swab samples from buffaloes and cattle, but mostly isolated from environmental sources like soil and water (Gcebe *et al.* 2013). *Mycobacterium malmesburiense* is a relatively recently discovered NTM. The bacterium is mainly found in environments with subtropical and Mediterranean climates in South Africa,

but has also been isolated from bovine nasal swabs (Gcebe, 2015; Gcebe *et al.* 2017). Genes that encode immunogenic proteins of *M. bovis*, like CFP10 and ESAT-6, have been demonstrated to be present in the genomes of several species of NTM (Gcebe *et al.* 2016), despite being widely used as highly specific *M. bovis* antigens. *Mycobacterium malmesburiense* includes the *esxB* gene encoding for CFP10. All three NTM have dnaK gene and mpb63 is present in *M. fortuitum* and *M. nonchromogenicum*. All these genes were detected in the PPD of these NTM and are thus expressed by the bacteria. Thus, exposure to the NTM expressing these immunogenic proteins, depending on the level of cross-recognition by T-cells, may lead to cross-reactive immune responses with *M. bovis* antigens (Gcebe, 2015).

Infection of buffaloes with *Mycobacterium fortuitum* have previously been shown to lead to 'false-positive' BTB diagnoses, illustrating cross-reactivity. *Mycobacterium fortuitum* is also described as the third most frequently encountered sensitization in herds with a history of non-specific reactors (Michel, 2008; Michel *et al.* 2011). For example, as found by Michel (2008), five African buffaloes were culled as a result of false-positive test results of the BOVIGAM™ assay: *M. fortuitum* was isolated from four lymph nodes of these buffaloes, but *M. bovis* could not be isolated (Michel, 2008). The false-positive diagnosis was further investigated with an experiment in which *M. fortuitum* was inoculated into 4 cattle that were subsequently monitored. In the first period, the cattle showed positive test results on 1 or 2 occasions. After boosting, one animal showed subsequent positive reactions that did not react previously, and it was concluded that nonspecific reactors were present (Michel, 2008). Another study showed that oral administration of *M. fortuitum* in cattle showed skin reactivity to mammalian and avian tuberculin as well as the PPD of *M. fortuitum* (Freerksen *et al.* 2013). Therefore, *M. fortuitum* is included in the BOVIGAM™ assay in South Africa to examine whether background responses are induced by NTM antigens (Michel *et al.* 2011). Therefore, a possibility to increase the specificity for the ELISA can be to distinguish between an animal that is truly infected with *M. bovis* or one that is rather infected with an NTM. The purpose of this study was to determine whether the specificity of an in-house ELISA for BTB can be increased through the inclusion of NTM PPDs.

Aim of the study

The aim of this study was to develop an NTM and MTBC specific ELISA to be able to differentiate between true *M. bovis* infections and infections with NTM, subsequently increasing the specificity of serological BTB diagnostics.

Hypothesis

H_0 - The inclusion of NTM specific antigens in an indirect ELISA for BTB will not increase the specificity of testing.

H_1 - The inclusion of NTM specific antigens in an indirect ELISA for BTB will increase the specificity of testing.

In order to detect or eliminate false-positive results, the specificity of the indirect ELISA must be increased. The purpose of this study was to achieve higher specificity through development, optimization of an indirect ELISA using the three different NTM PPDs as well as commercial PPD-B and PPD-A and to compare the test performance to that of other diagnostic approaches.

Materials and methods

Cattle and buffalo sera

Sera obtained during previous field studies and animal experiments were performed in the Department of Veterinary Tropical Diseases were used for this study. The following sera were available: i) Sera of animals experimentally infected with *M. nonchromogenicum* and *M. malmesburiense*. These animals were infected through intratracheal, oral and subcutaneous routes for a total of 9 times. Cell-mediated immune responses to PPDs of these mycobacteria were previously demonstrated in these animals (Jenkins *et al. in preparation*); ii) Sera of known BTB negative cattle as well as cattle with a well-defined *M. bovis* specific humoral response. These animals were part of a vaccination study described by van der Heijden *et al.* (2017); iii) Sera from known infected buffaloes. These sera were sourced from field studies in the Hluhluwe iMfolozi Park and Madikwe Game reserve (van der Heijden *et al.* 2019). Sera from animals that tested positive on the *M. bovis* specific ELISA (IDEXX TB ELISA) and were confirmed to be infected with *M. bovis* through culture were used; iv) Sera from known uninfected buffaloes were sourced from the section of Clinical Pathology of the Faculty of Veterinary Science, University of Pretoria.

NTM cultures and PPD preparation

Established cultures of *M. fortuitum*, *M. malmesburiense* and *M. nonchromogenicum* on Löwenstein-Jensen (LJ) medium containing PACT (Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim) were used for this purpose. Liquid cultures (10-20 ml) were prepared in Middlebrook 7H9 medium (Becton Dickinson, USA) supplemented with 0.1% OADC and 0.2% glycerol in a 250 ml bottle and inoculated with a loop full of bacteria. The cultures were incubated under continued shaking at 100 x *g* at 37°C. When turbid growth was observed, the cultures were screened for contamination. The screening was carried out by spread plating each culture onto two nutrient agar plates followed by incubation at 25°C and 37°C, respectively. The plates were monitored after 2 and 5 days of incubation for fungal or any bacterial growth not typical of mycobacteria. Ziehl Neelsen staining was done after 5 days to confirm the presence of acid-fast rods, and the absence of other organisms. If no contamination was detected, liquid cultures were upscaled to 750 ml in a 2 L Erlenmeyer flask with a cotton stop, and incubated for a further 4 weeks for the rapidly growing NTM (RGM) (*M. fortuitum* and *M. malmesburiense*) and 6 weeks for the slow-growing NTM (SGM) (*M. nonchromogenicum*) or until turbid growth was observed. All enlarged cultures showed turbid growth after approximately 2 weeks of incubation. At this point, the cultures were once again screened for contamination and if none was detected, used for the preparation of PPDs.

Purified protein derivatives were prepared from *M. malmesburiense* (PPD-M), *M. nonchromogenicum* (PPD-N) and *M. fortuitum* ATCC 6841 (PPD-F), following the protocol by Gcebe *et al.* 2016. The cultures were inactivated by steaming at 121°C for 20 minutes, followed by filter-sterilization using Whatman 40 filter paper (Sigma-Aldrich, South Africa) and a vacuum pump. The filtrates were then precipitated by the addition of 40% trichloroacetic acid (TCA) (Sigma-Aldrich, South Africa) (final concentration of 4% (v/v)) and left for a minimum of 12 hours at 4-8°C. The precipitates were then mixed by manual shaking and centrifuged for 20 minutes at 3900 x *g* at 4°C. The supernatants were discarded, and pellets were washed twice with 1% TCA, carefully mixed and centrifuged for 20 minutes at 3900 x *g* at 4°C. The supernatants were discarded, and the pellets resuspended in 10% NaCl and centrifuged for 20 minutes at 3900 x *g*. The supernatant was discarded, and the pellet was harvested after it was dried by turning the tube upside-down on sterile filter paper. Pellets were left to dry, weighed and subsequently diluted in 0.005% tuberculin buffer (0.005% Tween-80

in PBS: pH = 7.38). To every 100 mg wet pellet, 3.5 ml of tuberculin buffer is added, with a final concentration of 28.57 mg/ml. Prepared PPDs were stored at 4-8°C (Gcebe, 2015; Landi, 1963).

Checkerboard titrations and ELISA development

Indirect ELISA

The format of the ELISA assay that was to be developed in this study is that of an indirect ELISA, for the detection antibodies (IgG) against specific NTM as well as *M. bovis*. In this format, the ELISA plates are coated with antigens, in this case, PPDs, and detection of the antibodies is achieved by using a conjugated detection antibody directed at protein A/G of IgG molecules. Protein A/G is a non-species-specific conjugate regularly used in different setups of indirect ELISAs (Al-Adhami & Gajadhar, 2014). In order to optimize the assay, optimal concentrations of the various reagents were determined by checkerboard titrations (CBTs).

The general procedure for the NTM ELISAs was as follows: flat-bottomed 96-well maxisorp nunc-immuno plates (Thermo Fisher Scientific, Nunc A/S, Denmark) were coated with 50µl/well of the relevant PPD in 0.05M carbonate-bicarbonate buffer (Sigma-Aldrich, South Africa) with 0.01% sodium azide (coating buffer; CB) (Sigma-Aldrich, South Africa), overnight at 4ºC. Plates were decanted and washed 1x with 300µl/well of phosphate buffered saline (Sigma-Aldrich, South Africa) with 0.05% Tween-20 (PBS-T) (Sigma-Aldrich, South Africa). Blocking buffer (BB) was made up as 5% instant skimmed milk powder (Spar, South Africa) in PBS-T and plates were blocked with 300µl/well of BB for 1h at RT. Plates were washed as above with PBS-T. Serum samples were diluted in BB and 50µl/well was added and incubated for 1h at RT. Plates were washed 4x as above. The Pierce™ recombinant protein A/G-horseradish peroxidase (HRP) conjugate (Thermo Fisher Scientific, South Africa) was diluted in BB and 50µl/well was added and incubated for 1h at RT. Plates were washed as above and 50µl/well of 3,3',5,5'-tetramethylbenzine (TMB) (Sigma-Aldrich, South Africa) substrate was added and plates were incubated in the dark for 20 min at RT. The reaction was stopped with 50µl/well of 2M sulfuric acid (H_2SO_4) (Sigma-Aldrich, South Africa). The optical density (OD) was measured at 450ηm with an ELISA reader (Powerwave HT, Biotek, US).

Optimization indirect ELISA

The optimum concentrations of antigen, serum and conjugate, for the method described above, had to be determined with checkerboard titrations (CBTs). With CBTs, two components of the ELISA can simultaneously be titrated or optimized, as depicted in figure 2. During antigen optimization, different concentrations of PPD in CB were prepared in Eppendorf microcentrifuge tubes and titrated over the columns. In the initial experiment, the range of concentrations for NTM PPDs was 1280 IU/ml – 1,25 IU/ml; column 1 contained the highest concentrations, column 11 the lowest and column 12 was left uncoated to serve as a control (CB only). An equal volume of each concentration was coated on the plate. In the

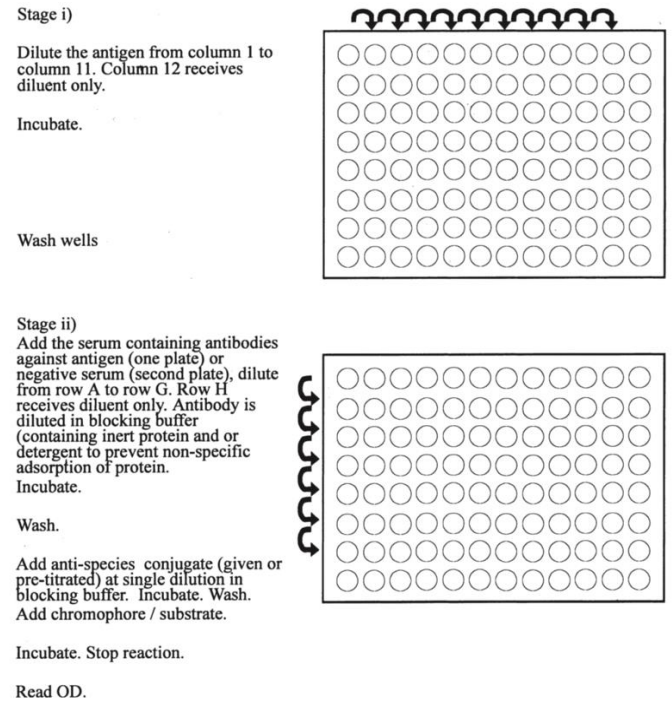

Figure 2: Checkerboard titration experiment to optimize two parameters in one experiment. In this example antigen and serum are titrated (Crowther, 2009).

same experiment, serum titration was done through preparation of different concentrations of serum diluted in BB on a separate serum dilution plate, which was on clear round-bottom immuno nonsterile 96-well plates (Thermo Fisher Scientific, Nunc A/S, Denmark). The initial range of serum concentrations was 1/100 – 1/6400, titrated over the rows; row A contained the highest concentration, row G the lowest and row H was left without serum to serve as a control (BB only). An equal volume of serum at each concentration was added to the plate in this manner. In experiments where antigen and serum were titrated, the conjugate was used at the concentration recommended by the manufacturer (1/10,000). The principal of the first CBT is depicted in figure 2.

A special CBT was set up during this study: in order to investigate the cause of the high background observed in both CBTs, the use of sodium azide (NaN_3) was evaluated, as it can interfere with the metal ions in HRP (Richardson et al, 1983). Therefore, a CBT was set up using the standard protocol using CB containing NaN_3 and an alternative protocol using CB without NaN_3, in order to assess its influence on the performance of the ELISA. In total, four plates were coated with PPD-B and ESAT-6/CFP10 as antigen (half a plate for each antigen) which was titrated versus serum (appendix 4 for the dilutions over the plates). In short, for 2 plates CB containing NaN_3 was used: one plate for positive serum and one plate for negative serum. Each plate was coated with PPD-B on the first 6 columns and ESAT-6/CFP10 on the latter 6 columns. For the remaining 2 plates, CB without NaN_3 was used, with the other conditions remaining the same.

The steps of titration of the conjugate and further optimization, validation, and statistics of the ELISA (Crowther 2009) could not be carried out as these initial CBTs were not successful. Due to the results, it was suspected that the expected antibody presence for the selected samples may not have been accurate, it was decided to try to establish antibody presence in each of the samples first. A Western blot was set up and used for this purpose, as described below.

SDS-PAGE and Semi-dry Western Blot

Gel preparation

The plates (Bio-rad) were assembled after the glasses were cleaned with soap water and ethanol. For making two 12% separating gels, 6ml of a 30% Acrylamide/Bisacrylamide mixture, 3.8ml of 1.5M Tris-HCl separating gel buffer (pH 8.8), 150µl of 10% SDS, 150µl of 10% ammoniumpersulfate and 5ml of H_2O were added together. As the last step 6µl of TEMED was added, because it makes the gel viscous. Therefore, after adding TEMED the liquid had to be put into the assembled glasses immediately. The glass plate construction was filled up with the separation gel mixture up to approximately one centimeter below the comb teeth mark. The gel was overlaid with 1ml ethanol to straighten the edges. The gel had to polymerize for 15-30 min.

The 5% stacking gel was prepared by adding gels 830µl of a 30% Acrylamide/Bisacrylamide mixture, 630µl of 1.5M Tris-HCl separating gel buffer (pH 8.8), 50µl of 10% SDS, 50µl of 10% ammoniumpersulfate and 3.4ml of H_2O were added together. Again, as the last step, 5µl of TEMED was added. The solution was mixed carefully to avoid generating air bubbles. Using a syringe, the separating gel solution was added into the glass plate on top of the stacking gel solution. The combs were inserted, making sure that no bubbles got trapped under the comb teeth ends. The stacking gel was polymerized for 30min.

PPD preparation

The different PPDs had different concentrations and were diluted in PBS to the following concentrations: 973µl/ml for PPD-B, PPD-F, PPD-M and PPD-N; and 753µl/ml for PPD-A.

For PPD-A the desired concentration was not possible to achieve, therefore it was a little bit lower. Ten microliters of loading dye was added to 10µl of PPD (1:1) in 0.5ml Eppendorff tubes and heated for 10min at 100ºC in a heating block in order to denature the proteins, in preparation for electrophoresis. After heating, the tubes were placed on ice. The samples then had to be loaded onto the SDS-PAGE gel using 10µl of each sample well and 4µl of ladder.

SDS-PAGE

The tank was placed into a container with ice. Gel holders were placed in the tanks, with largest glasses facing the outside. The tank and holders were filled with SDS-PAGE running buffer consisting of 0.025M Tris, 0.192M glycine and 0.1% SDS at a pH of 8.3. The combs were removed out of the gels and the samples loaded into the wells. The gel was electrophoresed for 1hr and 15min at 180V in a discontinuous buffer system using the Mini-PROTEAN Tetra cell electrophoresis apparatus (Bio-rad). The experiment was stopped as soon as the loading dye reached the bottom of the gel. The first gels were examined to check whether the protein of the PPDs successfully transferred to the gel, as well as the protein ladder. For this purpose, Coomassie blue staining solution was prepared using 1g Coomassie Blue, 250ml of 50% Methanol, 50ml of 10% acetic acid and 200ml of ddH_2O and the gel was stained with 25ml Coomassie blue in a plastic container for 30min. In order to destain the gel, the Coomassie blue was discarded and 25ml of 10% acetic acid solution was added. The other gels were prepared for the Western blot.

Western Blot (semi-dry)

If PPDs were successfully transferred to the gel, a western blot was carried out. A nitrocellulose membrane (0.45µm pore size; Thermo Fisher Scientific, Nunc A/S, Denmark) was cut into the same size as the gel (6.2cm by 8.4cm). The membrane was activated in 100% methanol for 1-3sec, washed for 1-2min with ddH_2O and soaked in transfer buffer consisting of 25mM Tris base, 192mM Glycine, 15% Methanol and made up to 1L with ddH_2O to a pH of 8.3-8.4, for 15min. Filter paper was cut into the same size as the nitrocellulose membrane and soaked for 15min in transfer buffer. The sandwich for in between the electrodes for the Western blot orientated from the negative pole to the positive pole consisted of four filter papers, the gel, the wet nitrocellulose membrane and another four filter papers put on top of each other. The sandwich was placed in the semi-dry blotting apparatus. The proteins on the gel were transferred to the membrane for 1hr and 20min at 20V using a semi-dry blotting method. The membranes were blocked with 20ml 5% instant skimmed milk powder in 10% PBS-Tween 20 (PBS-T), overnight at 4°C. The gel was stained with 25ml Coomassie blue to verify the transfer efficiency to the membrane.

Immuno-blotting

The next morning, the blocking buffer was discarded and the primary antibody (serum diluted to 1/100 in 0.5% instant skimmed milk powder in PBS-T) was added in a total volume of 5ml and incubated on the shaker, ensuring the membrane was submerged, for 20min at RT. This was followed by a short wash with PBS-T, after which the supernatant was discarded and another wash with PBS-T was carried out on the shaker for 3min. Next, the secondary antibody, the recombinant protein A/G conjugate (Pierce™, Thermo Fisher Scientific) (diluted to 1/10,000 in 0.5% milk powder in PBS-T) was added in a 5ml volume and incubated on the shaker for 15min. The membrane was washed as before. The TMB was added in a 5ml volume and incubated on the shaker for 10min. The reaction was stopped by the addition of tap water. The membranes were left to dry for 1hr, after which they were evaluated and stored.

Results

NTM cultures and PPD preparation

In the first attempts at growing liquid cultures of *M. fortuitum*, *M. malmesburiense* and *M. nonchromogenicum*, the growth rate was too rapid. It was discovered that 10% OADC was used instead of 0.1% as per the protocol, which had caused rapid overgrowth of the bacteria. After starting new cultures at the correct percentage of OADC, the cultures for both the SGM and the RGM showed sufficient growth after only 2 weeks. Tables 1-3 show the cultures of the NTM that were set up as well as the results of the screening for contamination through spread plating and ZN staining for each culture.

Table 1: Results of the *M. fortuitum* cultures for preparation for PPD-F.
Size small = 20ml, large = 750 ml; + = growth; - = no growth; C = contaminated; 1-6 = culture ID.

Culture ID & size	Growth	after 2 days		after 5 days		ZN-staining		Next step
		25ºC	37ºC	25ºC	37ºC	25ºC	37ºC	
1 - Large	+	+	+	+	+	+	+	Subculture Make PPD – failed
2 - Large	C	+	+	+	C	C	C	New small culture from LJ+PACT
3 - Small	+	+	+	+	+	+	+	Enlarge
3 - Large	+	+	+	+	+	C	C	Discard; tart over from LJ+PACT
4 -Small	+	+	+	+	+	+	+	Enlarge
5 – Small	+	+	+	+	+	+	+	Enlarge
6 – Small	+	+	+	+	+	+	+	Enlarge

The initial *M. fortuitum* culture was severely overgrown due to the high OADC content (Table 1). It had a white to grey cloudy appearance after steaming and was too concentrated. Consequently, it could not be filtered as the pores of the filter clogged up almost instantaneously, slowing down the process: after 2hrs only 10ml of the 750ml had been filtered. It was decided to stop the process, discard the culture, and new cultures were started from the stock. Finally, PPD-F could not be produced in-house as a culture was too thick to make PPD and the next culture got contaminated. As a result, it was decided to purchase PPD-F (30mg/ml) from the ARC-OVI for this project.

Table 2. Results of the *M. nonchromogenicum* cultures for preparation for PPD-N.
Size small = 20ml, large = 750 ml; + = growth; ± = questionable if contaminated or not; C = contaminated. Bold font = led to successful PPD preparation; 1-4 = culture ID.

Culture ID & size	Growth	after 2 days		after 5 days		ZN-staining		Next step
		25ºC	37ºC	25ºC	37ºC	25ºC	37ºC	
1 – Large	+	+	+	+	+	±	±	Subculture Make PPD – failed
2 – Large	C	+	+	+	+	C	C	New small culture from LJ+PACT
3 – Small	+	+	+	+	+	+	+	Enlarge
3 – Large	+	+	+	+	+	+	+	Subculture **Make PPD**
4 – Small	+	+	+	+	+	+	+	Enlarge

As can be seen from table 2, one large *M. nonchromogenicum* culture was used to prepare PPD-N. After the method described above, a total of 0.31g of wet pellet of PPD-N was obtained. The pellet was suspended in tuberculin buffer and brought to a concentration of 28.57mg/ml. A total of 11ml of PPD-N was prepared.

Table 3. Results of the *M. malmesburiense* cultures for preparation for PPD -M.
Size small = 20ml, large = 750 ml; + = growth; ± = questionable if contaminated or not; C = contaminated; 1-5 = culture ID. Bold font = led to successful PPD preparation.

Culture ID & size	Growth	after 2 days		after 5 days		ZN-staining		Next step
		25ºC	37ºC	25ºC	37ºC	25ºC	37ºC	
1 – Large	+	+	+	+	+	±	C	Discard; start over from LJ+PACT
2 – Small	+	+	+	+	+	+	C	Discard
3 – Small	+	+	+	+	+	+	+	Enlarge
4 – Small	+	+	+	+	+	+	+	Enlarge
5 – Small	+	+	+	+	+	+	+	Enlarge
3 – Large	+	+	+	+	+	+	+	Subculture **Make PPD**
4 – Large	+	+	+	+	+	+	+	Subculture **Make PPD**
5 – Large	+	+	+	+	+	+	+	Subculture **Make PPD**
3 – Small	+	+	+	+	+	+	+	Enlarge
4 – Small	+	+	+	+	+	+	+	Enlarge
5 – Small	+	+	+	+	+	+	+	Enlarge
3 – Large	+	+	+	+	+	+	+	Make PPD (not carried out)
4 – Large	+	+	+	+	+	+	+	Make PPD (not carried out)
5 -Large	+	+	+	+	+	+	+	Make PPD (not carried out)

Finally, as can be seen from table 3, the first three large *M. malmesburiense* cultures of 750ml each were used to prepare PPD-M. After the method described above, a total of 0.69g of wet pellet of PPD-M was obtained. The pellet was suspended in tuberculin buffer and brought to a concentration of 28.57mg/ml. A total of 24ml of PPD-M was prepared. The last three samples were ready to convert to PPD, but unfortunately there was no time left to prepare it.

Checkerboard titrations for ELISA development

CBT buffalo serum and PPD-B (appendix 1)

The first set of checkerboard titrations were carried out using buffalo serum (known positive and negative) and PPD-B as a coating antigen. For this experiment, two-fold titrations were used starting with 1280IU/ml for the antigen and 1/10 dilution for the sera. Graphical presentations of the outcome of the plates containing negative and positive serum of the first CBTs are depicted in figures 3 and 4. Figure 3 of the plate containing the positive serum shows a dilution of antigen until 5IU/ml for serum concentrations of 1/40 and higher. There is a slight increase in OD value after that. Column 11 with antigen at a concentration of 1.25IU/ml has a lower OD-value than column 12 without antigen. There was no plateau established on the plate and thus the optimal concentrations could not be determined. Figure 4 of the plate containing the negative serum shows relatively high OD-values for a negative serum, indicative of either

presence of antibodies (which should not be the case given the history and previous test results of the animal) or strong background reactions due to conditions on the plate.

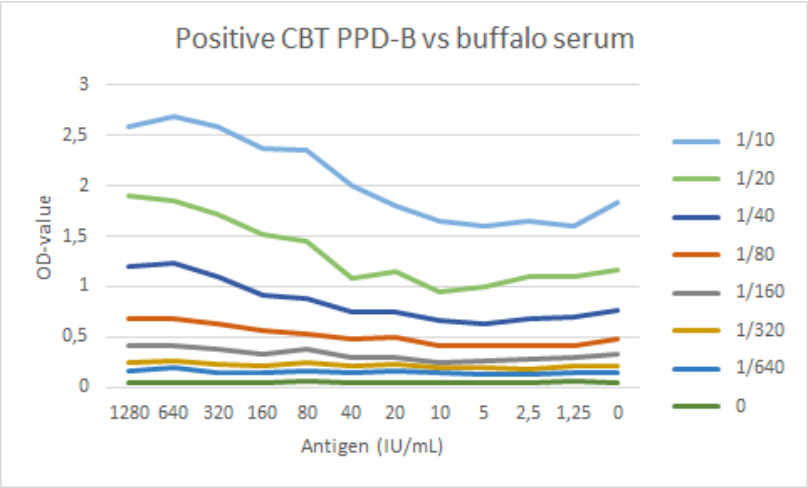

Figure 3. Checkerboard titration: PPD-B versus serum of positive tested buffalo for *M. bovis*.

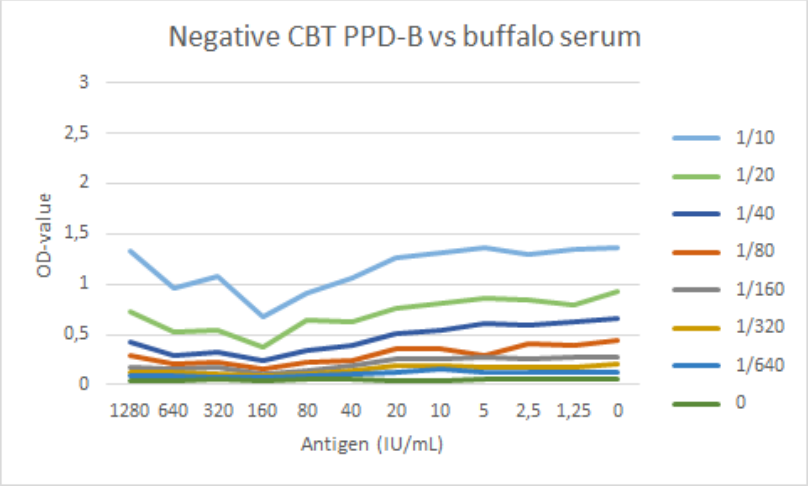

Figure 4. Checkerboard titration: PPD-B versus serum of a negative tested buffalo for *M. bovis*.

<u>CBT cattle serum and PPD-A</u> (appendix 2)

The second set of checkerboard titration was carried out with cattle serum, of BOVIGAM reactive *M. avium* serum and negative *M. avium* sample, and PPD-A as a coating antigen. The CBT was a two-fold titration starting with 1280IU/ml and a serum dilution starting at 1/100 dilution (appendix 2). The outcome of the CBT is depicted in figures 5 and 6. Figure 5 shows the CBT of the positive serum: a higher concentration of antigen (320IU/ml) had a lower OD-value, than the dilutions with less or no antigen. Furthermore, two abnormal peaks are shown in the figure at 640IU/ml with a 1/800 serum dilution and at 20IU/ml with a 1/6400 serum dilution. Again, the plateau was absent in this CBT, meaning that the optimal concentrations could not be determined. The negative cattle serum versus PPD-A shown in figure 6 again had a relatively high background. The negative showed colour development similar to that of the positive plate. On the negative plate, the OD-value initially also decreased at the start of the antigen dilution (1280IU/ml - 160IU/ml), but the OD-value subsequently increased at later antigen dilutions.

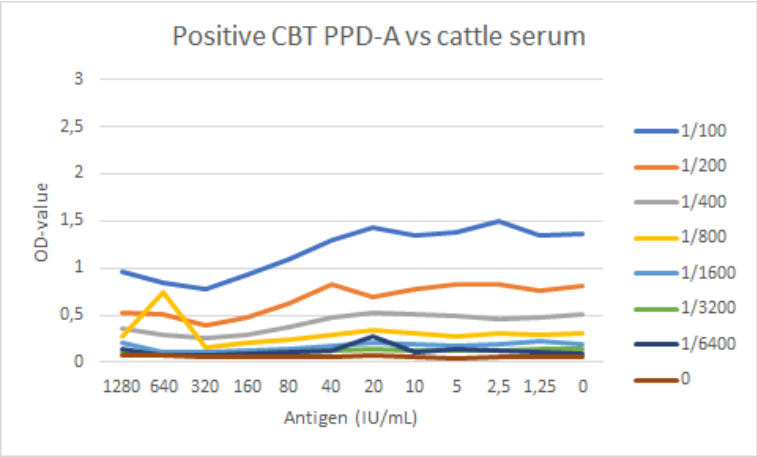

Figure 5. Checkerboard titration: PPD-A versus serum of a positive tested cow for *M. avium*.

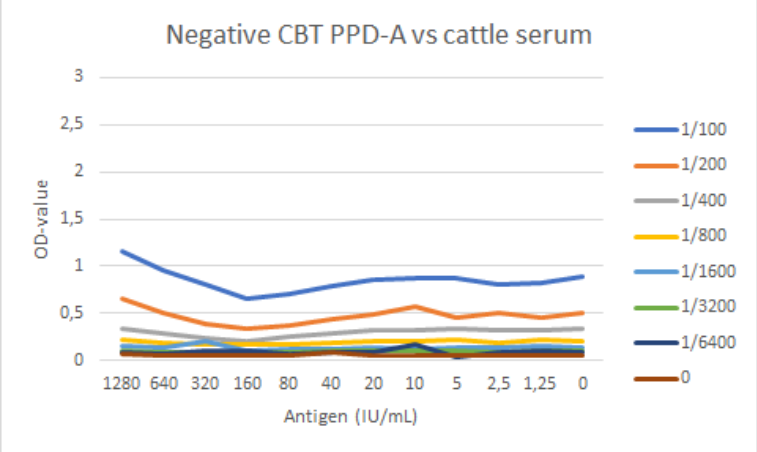

Figure 6. Checkerboard titration: PPD-A versus serum of a negative tested cow for *M. avium*.

CBT cattle serum with CFP10 and ESAT-6 (appendix 3)

The third set of checkerboard titrations were carried out using cattle serum (positive and negative tested sera for *M. bovis*) and CFP10/ESAT-6, highly immunogenic antigens of *M. bovis*, as coating antigen. For the experiment, a two-fold titration was used starting with 20µg/ml CFP10 and ESAT-6 and a 1/100 serum dilution (appendix 3). Graphical presentations of the outcome of the plates containing negative and positive serum are depicted in figures 7 and 8.

Figure 7 depicts the plate with a known positive *M. bovis* cattle serum. The plate showed erratic results somewhat increasing in OD-value for the 1/100-1/400 serum dilutions until 2.5-1.25µg/ml CFP10/ ESAT-6, and thereafter the OD-value decreased with decreasing antigen concentrations. Column 11 and 12 had in each serum dilution a very alike OD-value even though 11 had very low concentration antigen and 12 had no antigen coated. There was no plateau established on the plate.

Figure 8 depicts the plate with serum form a known negative animal, which showed relatively high OD-values, particularly for the 1/100 serum dilution. The OD-value decreased from a concentration of 20µg/ml of CFP10/ESAT-6 to 10µg/ml of CFP10/EASAT-6, whereas the OD-value slightly increased thereafter with decreasing antigen concentration. The trend could be seen for serum dilutions of 1/100-1/400. For further serum dilutions, the OD-value was fairly stable for the various antigen concentrations. An outlier can be observed on the plate at 1.25IU/ml and 1/6400 serum dilution, possibly due to insufficient blocking.

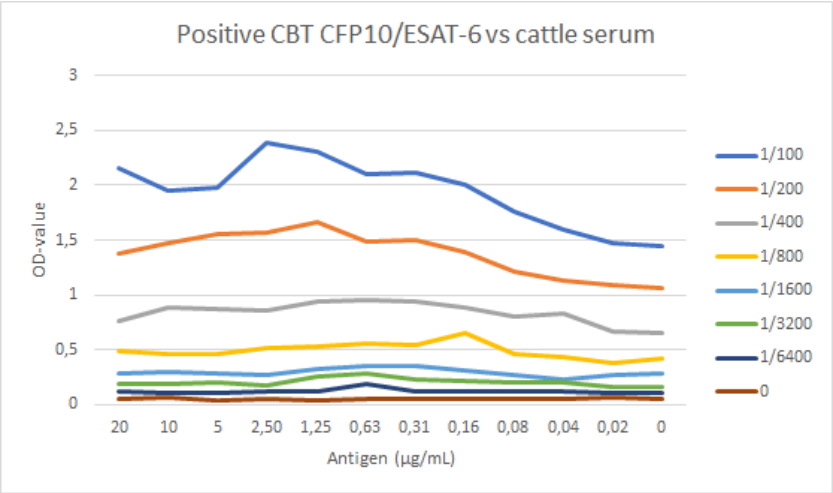

Figure 7. Checkerboard titration: CFP10 and ESAT-6 versus serum of a positive tested cow for *M. bovis*.

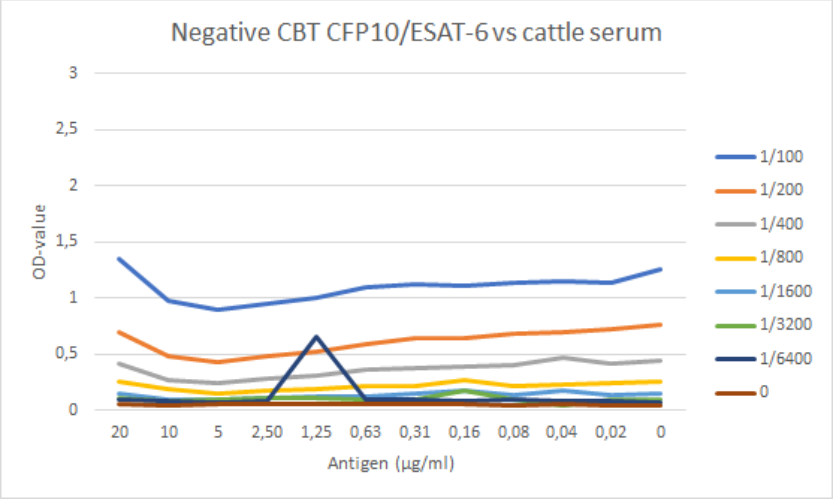

Figure 8. Checkerboard titration: CFP10 and ESAT-6 versus serum of a negative tested cow for *M. bovis*.

<u>CBT of cattle serum and PPD-B and CFP10/ ESAT-6 with or without NaN_3</u> (appendix 4)
In order to investigate the cause of the high background observed in the previous CBTs, the use of sodium azide (NaN_3) was evaluated as described in the materials and methods.

Halve of the four checkerboard titration plates was coated with CFP10 and ESAT-6, whereas the other halves were coated with PPD-B. For this experiment, two-fold titrations were used starting with 320IU/ml coated PPD-B and 10µg/ml CFP10/ESAT-6. On both halves of the plate 1/200 was the highest serum dilution (appendix 4). Figures 9 – 12 depict the influence of NaN_3 in 1/200 and 1/400 serum dilutions for PPD-B and CFP10/ESAT-6 with the known positive and negative sera. The other serum dilutions showed a similar course (appendix 4).

Starting with the CFP10/ESAT-6 coated parts of the plates, figures 9 and 10 show similar images. The positive plate with NaN_3 has a slightly higher OD-value than without NaN_3. The negative plate with NaN_3 had also a slightly higher OD-value than without NaN_3 until a 0.625µg/ml antigen dilution was reached, thereafter there was no difference seen between the two groups. The same trend was seen for both 1/200 and 1/400 serum dilutions. This indicated that NaN_3 has a small effect on the OD-value, but does not show a big enough difference to explain the background of the negative CBTs.

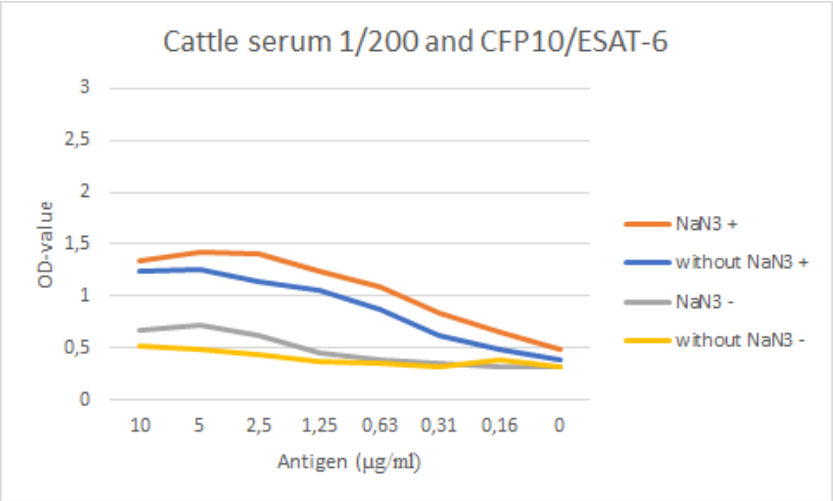

Figure 9. Checkerboard titration: CFP10 and ESAT-6 versus serum of a positive and negative tested cow for *M. bovis* with and without NaN_3 at a 1:200 serum dilution.

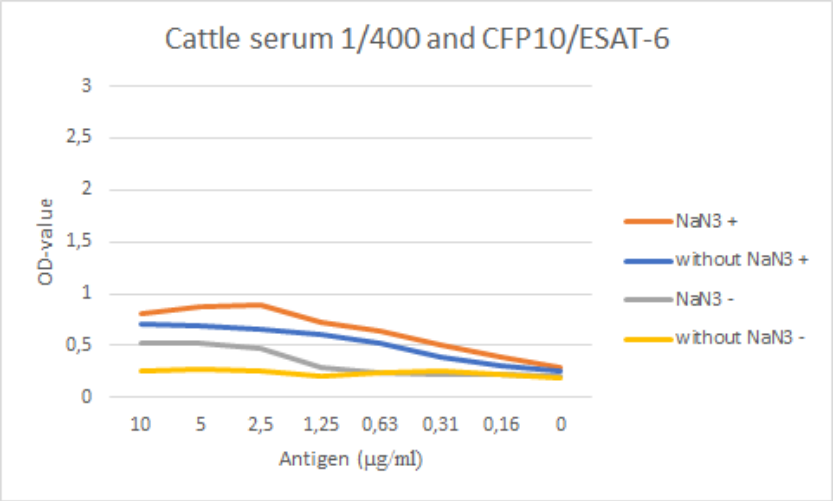

Figure 10. Checkerboard titration: CFP10 and ESAT-6 versus serum of a positive and negative tested cow for *M. bovis* with and without NaN_3 at a 1:400 serum dilution.

The PPD-B coated parts of the plates showed a different image. The reaction was stopped after 20 minutes as per the protocol, but unfortunately coagulation had occurred. Figures 11 and 12 depict the PPD-B coated parts for the 1/200 and 1/400 serum dilutions. The positive plate with NaN_3 has a slightly higher OD-value than without NaN_3 in figure 11 (1/200 serum dilution), where for 80 and 40IU/ml there was no difference in OD-value between with or without NaN_3. The 1/400 serum dilution the results were inconclusive, alternating between a higher OD-value for the protocol with or without NaN_3. The negative plate with NaN_3 was also inconclusive, due to the fact that the OD-value for the protocol with or without NaN_3 in both 1/200 and 1/400 serum dilutions was not consistently higher nor lower. As such, no clear NaN_3 interference could be demonstrated.

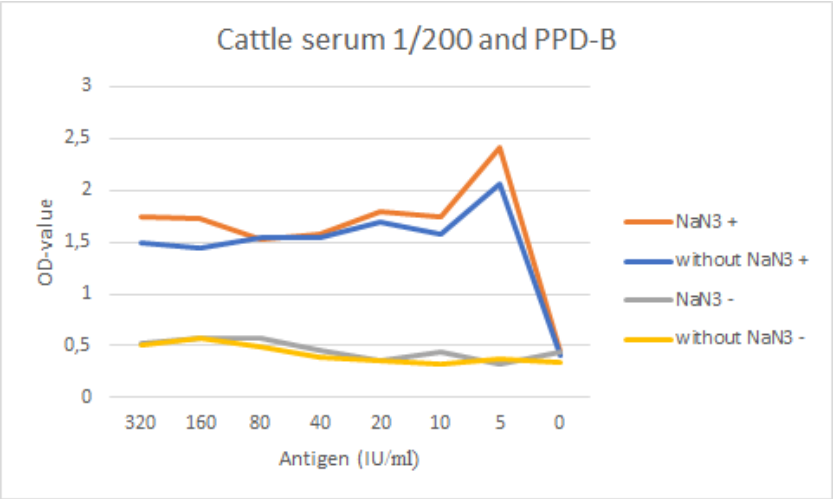

Figure 11. Checkerboard titration: PPD-B versus serum of a positive and negative tested cow for *M. bovis* with and without NaN_3 at a 1:200 serum dilution.

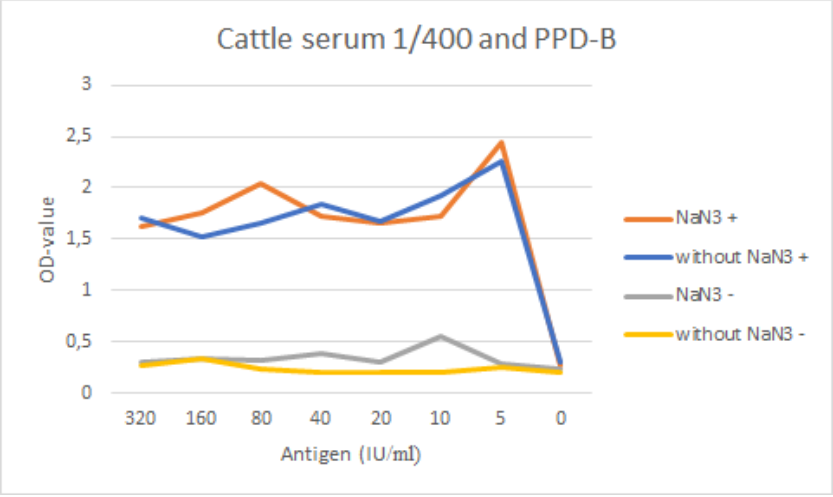

Figure 12. Checkerboard titration: PPD-B versus serum of a positive and negative tested cow for *M. bovis* with and without NaN_3 at a 1:400 serum dilution.

SDS-PAGE and Semi-dry Western blot

There were various difficulties encountered with the CBTs. The results showed that the control samples were far from ideal. For instance, the positive serum which originated from a known positive animal (and had previously tested positive on another serological assay), showed a weaker signal than expected. The opposite was found for negative samples; making the differentiation between positive and negative samples complicated. Rather than trying different dilutions or titrations to determine optimal concentrations through further CBTs, it was decided to first determine the presence or absence of antibodies in these serum samples through the use of Western blotting. Samples from animals with defined histories and previous test results were used to determine their suitability as controls in CBTs.

In total, 76 sera were tested as shown in tables 4-9. Some samples were tested up to three times, if the results were inconclusive during initial rounds of testing.

Table 4. Results of the western blots of known *M. bovis* reactive serum samples.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extremely intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control.

			Outcome Western blot					
Serum ID	**Species**	**Origin**	**PPD-A**	**PPD-B**	**PPD-F**	**PPD-M**	**PPD-N**	**Nil**
26	Cattle	Hluhluwe, KZN	0	2	0	n.d.	n.d.	0
26	Cattle	Hluhluwe, KZN	0	3	0	0	0	0
26	Cattle	Hluhluwe, KZN	0	2	0	0	0	0
28	Cattle	Hluhluwe, KZN	0	2	0	n.d.	n.d.	0
30	Cattle	Hluhluwe, KZN	0	1	0	n.d.	n.d.	0
231	Cattle	Hluhluwe, KZN	0	2	0	n.d.	n.d.	0
231	Cattle	Hluhluwe, KZN	0	3	0	0	0	0
162	Cattle	Hluhluwe, KZN	1	3	0	n.d.	n.d.	0
221	Cattle	Hluhluwe, KZN	(1)	1	0	n.d.	n.d.	0
242	Cattle	Hluhluwe, KZN	(1)	(1)	0	n.d.	n.d.	0
286	Cattle	Hluhluwe, KZN	3	3	0	n.d.	n.d.	0
28	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
28	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
28	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
44	Cattle	Hluhluwe, KZN	0	0	0	n.d.	n.d.	1
90	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
221	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
221	Cattle	Hluhluwe, KZN	0	0	0	0	0	1
268	Buffalo	Madikwe May	0	0	0	0	0	1
271	Buffalo	Madikwe May	0	0	0	0	0	1
289	Buffalo	Madikwe May	0	0	0	0	0	1
A14	Buffalo	HiP 2015	0	0	0	0	0	1
CP10219	Buffalo	Department of Clinical Pathology	0	0	0	0	0	1
CP18967	Buffalo	Department of Clinical Pathology	0	0	0	0	0	1
CP5946 C1	Buffalo	Department of Clinical Pathology	0	0	0	0	0	1
CP5946 G2	Buffalo	Department of Clinical Pathology	0	0	0	0	0	1
CP9891	Buffalo	Department of Clinical Pathology	0	0	0	0	0	1

Of the *M. bovis* tested samples, 4 of the 14 tested cattle samples were positive. Where some samples were tested in duplicate or even triplicate. Sample 28 was positive once and negative the three occasions. Eventually, only 3 samples were definitely positive for only PPD-B (samples 26, 30 and 231). Also, four samples were positive for PPD-B, but also PPD-A, while no other NTM responded. None of the 4 buffalo samples showed a positive PPD-B result on the nitrocellulose membrane.

Table 5. Results of the western blots of known *M. avium* reactive serum samples.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extreme intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control.

Serum ID	Species	Origin	Outcome Western Blot					
			PPD-A	PPD-B	PPD-F	PPD-M	PPD-N	Nil
T0 #16	Cattle	Cattle vaccine study	0	(1)	0	n.d.	n.d.	0
T9 #14	Cattle	Cattle vaccine study	0	(1)	0	n.d.	n.d.	0
T0 #11	Cattle	Cattle vaccine study	0	0	0	n.d.	n.d.	1
T9 #24	Cattle	Cattle vaccine study	0	0	0	0	0	1

Of the *M. avium* positive tested samples no samples tested positive for the PPD-A in the Western blot. Some were inconclusive (possible reaction to PPD-B), but none were positive for PPD-A

Table 6. Results of the western blots of known *M. fortuitum* reactive serum samples.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extreme intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control.

Serum ID	Species	Origin	Outcome Western Blot					
			PPD-A	PPD-B	PPD-F	PPD-M	PPD-N	Nil
T5 #24	Cattle	Cattle vaccine study	0	0	0	n.d.	n.d.	1
T5 #24	Cattle	Cattle vaccine study	0	0	0	0	0	1
T6 #24	Cattle	Cattle vaccine study	0	0	0	0	0	1
T6 #24	Cattle	Cattle vaccine study	0	0	0	0	0	1

For *M. Fortuitum* positive samples, none were positive, and all had no response of antibodies on the nitrocellulose membranes.

Table 7. Results of the western blots of known *M. malmesburiense* reactive serum samples.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extreme intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control.

Serum ID	Species	Origin	Outcome Western Blot					
			PPD-A	PPD-B	PPD-F	PPD-M	PPD-N	Nil
B1 #204	Cattle	NTM study	0	0	0	0	0	1
B3 #5	Cattle	NTM study	0	0	0	0	0	1
B5 #25	Cattle	NTM study	0	0	0	0	0	1
B5 #5	Cattle	NTM study	0	0	0	0	0	1

For *M. malmesburiense* positive samples, none were positive, and all had no response of antibodies on the nitrocellulose membranes.

Table 8. Results of the western blots known *M. nonchromogenicum* reactive serum samples.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extreme intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control.

			Outcome Western Blot					
Serum ID	**Species**	**Origin**	**PPD-A**	**PPD-B**	**PPD-F**	**PPD-M**	**PPD-N**	**Nil**
B1 #26	Cattle	NTM study	(1)	0	0	0	0	0
B3 #1	Cattle	NTM study	(1)	1	0	0	0	0
B1 #225	Cattle	NTM study	0	(1)	0	0	0	0
B1 #228	Cattle	NTM study	0	0	0	0	0	1
B1 #235	Cattle	NTM study	0	0	0	0	0	1
B1 #239	Cattle	NTM study	0	0	0	0	0	1
B1 #30	Cattle	NTM study	0	0	0	0	0	1
B2 #228	Cattle	NTM study	0	0	0	0	0	1
B2 #239	Cattle	NTM study	0	0	0	0	0	1
B2 #27	Cattle	NTM study	0	0	0	0	0	1
B2 #28	Cattle	NTM study	0	0	0	0	0	1
B3 #1104	Cattle	NTM study	0	0	0	0	0	1
B3 #235	Cattle	NTM study	0	0	0	0	0	1
B3 #239	Cattle	NTM study	0	0	0	0	0	1
B3 #30	Cattle	NTM study	0	0	0	0	0	1
B3 #H10	Cattle	NTM study	0	0	0	0	0	1
B4 #1104	Cattle	NTM study	0	0	0	0	0	1
B4 #12	Cattle	NTM study	0	0	0	0	0	1
B4 #228	Cattle	NTM study	0	0	0	0	0	1
B4 #235	Cattle	NTM study	0	0	0	0	0	1
B4 #239	Cattle	NTM study	0	0	0	0	0	1
B4 #26	Cattle	NTM study	0	0	0	0	0	1
B4 #27	Cattle	NTM study	0	0	0	0	0	1
B4 33	Cattle	NTM study	0	0	0	0	0	1
B5 #12	Cattle	NTM study	0	0	0	0	0	1
B5 #225	Cattle	NTM study	0	0	0	0	0	1
B5 #228	Cattle	NTM study	0	0	0	0	0	1
B5 #239	Cattle	NTM study	0	0	0	0	0	1
B5 #3	Cattle	NTM study	0	0	0	0	0	1
B5 #30	Cattle	NTM study	0	0	0	0	0	1
B5 #38	Cattle	NTM study	0	0	0	0	0	1

For *M. nonchromogenicum* positive samples, none were positive, and all had no response of antibodies on the nitrocellulose membranes. Three samples were positive for PPD-A of PPD-B, but all were negative for *M. nonchromogenicum*.

Table 9. Results of the western blots known negative controls.
0 = no bands; 1 = faint bands; 2 = intense bands; 3 = extreme intense bands; () = questionable result; n.d. = not done, PPD not included in the test; green: result matches the expectation based on earlier tests or history; orange: inconclusive result; red: result does not match the expectation based on earlier tests or history. PPD-A = purified protein derivative of *M. avium*; PPD-B = purified protein derivative of *M. bovis*; PPD-F = purified protein derivative of *M. fortuitum*; PPD-M = purified protein derivative of *M. malmesburiense*; PPD-N = purified protein derivative of *M. nonchromogenicum*; nil = negative control

Serum ID	Species	Origin	Outcome Western Blot					
			PPD-A	PPD-B	PPD-F	PPD-M	PPD-N	Nil
T3 #14	Cattle	Cattle vaccine study	0	0	0	n.d.	n.d.	1
T9 #24	Cattle	Cattle vaccine study	0	0	0	n.d.	n.d.	1
T1 #22	Cattle	Cattle vaccine study	0	0	(1)	n.d.	n.d.	0
T8 #15	Cattle	Cattle vaccine study	(1)	0	0	n.d.	n.d.	0
T3 #25	Cattle	Cattle vaccine study	0	1	0	n.d.	n.d.	0
T2 #12	Cattle	Cattle vaccine study	0	1	0	n.d.	n.d.	0

The known negative serum samples were two samples negative, but were not tested on PPD-M or PPD-N. There were three samples inconclusive and one sample was even positive on PPD-B, which was not expected.

Discussion

The development of an NTM specific ELISA for cattle and buffalo have been through some challenges and finally did not yet succeed. The possibility to differentiate between true *M. bovis* infections and infections with NTM by an indirect ELISA could not be finished. Although the differentiation has been established for CMI-response (Michel *et al.* 2011), but not yet for the humoral response. If the research would be repeated, the best approach would be to test the serum samples for the presence of antibodies before the CBTs are set up. Alternatively, positive and negative controls for the various PPDs and MTBC could be purchased, where available, or produced in-house. Particularly the latter was beyond the scope of this project, but is something that could be considered in future.

NTM cultures and PPD preparation

The volume of enrichment (OADC) adding according to the label of the media (7H9 broth) (10% of the total culture) made the culture overgrown. With the protocol of Gcebe (2015) 0.1% OADC was added and the culture grew as suspected. Different steps in the protocol of growing the culture went accordingly. The culture had good conditions while enlarging in the 2L Erlenmeyer's with cotton stop. Shaking the culture at 100 x g in the stove of 37 degrees made the culture grew fast. However, contamination of the culture was encountered during the process. A risk factor for contamination is the step of enlarging the small culture to the 2 L Erlenmeyer with cotton stop. Even though the Erlenmeyer flasks and pipet tips were sterilized, contamination was encountered and slowed down the process.

There are many human actions in the final steps of making PPD, which makes variation possible between the different PPD. For example, the last step in measuring the pellet. Some would maybe let the pellet dry longer, then it will be lighter. Or there could be a calibration error in the scales. The PPD concentration is calculated on the volume of buffer added and, therefore, can be incorrect.

Purified protein derivatives are a crude mix of proteins, which probably do not contain all the proteins that can cause an immune response in an animal. The biggest limitation of the study was that the PPD-M and PPD-N produced were not validated, due to time constraints and lack of control animals or known positive samples to do so. It is not known to what extent animals produce (measurable quantities of) antibodies in response to infection with NTM, and this is a key topic of further research in order to understand cross-reactive HI responses.

Checkerboard titration and ELISA development

The goal was to develop an ELISA because the method is relatively cheap and easy to perform. According to the method of Crowther (2009), the CBTs could not be completed due to no clear cut-off value of the various concentrations. In order to establish the optimal concentration, a plateau height maximum is needed, which was not visible on any of the plates. In the protocol the concentrations must be established before the next step, the concentration of the conjugate can be determined. This also meant that validation of the test could not yet be performed.

In addition, there was a problem with a lot of background in the CBTs, both the positive and negative plates. The OD-value in column 12 (no antigen) was more than in column 11 (with antigen, although in very low concentrations). It’s a possibility that the blocking buffer did not have the correct concentrations, or the blocking time was not long enough to work properly. Another possibility is that the serum could bind to the coating and maybe give a higher response, but this problem could also be attributed to the blocking step.

The CBT with PPD-B and buffalo serum was inconclusive, due to the high background of the negative plate. The serum sample was selected based on their response to the culture, skin test and IDEXX, which were originally developed for cattle. As were the ELISA originally set up for cattle, which could have interfered with the results. The concentration of the buffalo sera used, was 10-fold higher than cattle serum, which was based on previous results from the group. Ineffective blocking causing the serum to bind to the plate directly is still a possibility (Crowther, 2009). In the research of Tanner (2015), 62 buffaloes were sampled for antibody tests using the lateral flow technology (BovidTB Stat-Pak®), which showed 8% positive BTB reactors in the herd, while the IDEXX ELISA and the IFN-γ assay (BOVIGAM™) showed 0% positive reactors (Tanner *et al.* 2015). The indirect ELISA can also be less sensitive for BTB than other serological assays with buffalo serum.

The CBT with PPD-A and cattle serum was set up based on the known immunological background of the cattle serum. This was carried out to first establish the optimal concentrations for the general ELISA protocol using samples that were less valuable, to later adapt it for buffaloes. However, still no clear result was obtained, due to high background demonstrated on the negative serum plate. Similarly, these problems may be explained by what was mentioned above. The same applies to the CBT with the immunogenic antigens of PDD-B, namely CFP10/ESAT-6. One well (G5), was extremely positive, probably due to incorrect blocking. Furthermore, the diagnostic antigens used in the ELISA set-up can also be a point of improvement. CFP10/ESAT-6 are generally used for CMI based assays as they are known to be highly immunogenic, this may not be true in terms of the humoral response to BTB. The IDEXX for instance, which tests the humoral response, uses other antigens, namely MPB70 and MPB83 (Waters *et al.* 2011). As such, we may have seen other results if the CBT would have been done with these antigens, as this is something to consider in future.

Finally, sodium azide (NaN_3) was assessed as a possible factor for the problems encountered with the CBTs. Sodium azide was according to Richardson (1983), a possibility for higher background due to interference with the HRP. Therefore, a CBT was set up with and without NaN_3 in the coating buffer and one half with PPD-B and one with CFP10/ESAT-6. CFP10 and ESAT-6 with or without NaN_3 showed differences. The protocol using CB with NaN_3 almost always showed more background than without NaN_3. But the difference was not sufficient to be able to determine why column 12 shows a higher background than column 11. Shown in the results with the different concentrations of sera, all have a peak in the background at the second and third antigen dilution with NaN_3 added. The plates with PPD-B are not that representative, due to stopping the colour reaction to late. Precipitation had taken place. In conclusion, using coating buffer without NaN_3 resulted in slightly lower OD-values, but this is not enough to explain why the previous results were negative.

SDS-PAGE and Semi-dry Western blot

Given that we did not have clear sample controls for either positive or negative sera, a different approach was chosen, to first determine whether there are antibodies present in the different sera for the NTM and MTBC. The western blot is a technique that can detect antibodies to specific proteins, which allowed us to assess the suitability of the available sera as controls for the CBT. This was important, due to working with field study samples instead of known or commercial controls. The history of the samples is based on CMI responsiveness, instead of serology, giving uncertainty as to whether antibodies would be present. Except for the positive *M. bovis* samples, which have been tested on IDEXX, and therefore a humoral response has been established. The controls are crucial in the optimization of the assay, because

the assay will be based on these. Therefore it is of the utmost importance to know that the samples used, have the correct antibodies.

The protocol of the SDS-PAGE had to be optimized during the course of this study. Factors that were optimised included the concentration of protein (PPD) used in order to visibly show on the gel (which was demonstrated to be 10-fold higher than that used in the ELISA), the use of fresh Coomassie blue staining was shown to be important, the time of running the gel had to to ensure complete transfer of protein to the gel, in combination with the right percentage SDS of the gel, etc. Evidently, a lot of factors were at play in the one experiment, therefore any issue arising could have been multifactorial. Furthermore, every gel for the SDS-PAGE was handmade. Therefore, there is a possibility that an error has crept in as a result, although every time it was made by the same person, reducing some variability. Besides potential issues with the gels.

Western blotting is not a very sensitive method: a high concentration of protein and serum is needed in this assay. Again, the western blot had to be optimized for this study. The time required for the successful transfer of protein from the gel to the membrane proved particularly difficult to establish. In the end, an optimized protocol was established, but the processing of the results was difficult as the exact size of the different proteins present in the PPD were not known, and may be variable between preparations due to the crude nature of PPDs. For the NTM, the protein sizes have not been established yet. The molecular weights for PPD-B are known for a different % gel and different transfer time then used in this study (McNair *et al.* 2001). If proteomics would be performed on the various PPDs, it would give more clarity on what precisely is being measured. Previously, proteomics analyses performed on PPD-F, PPD-M and PPD-N (Gcebe *et al.* 2016), has provided insight into this. But it is important to realize that firstly, the composition may vary between batches, and secondly, only few proteins have been defined as being immunodominant for the humoral response in BTB (Lyashchenko *et al.* 2004).

According to the western blot, there was no positive antibody serum sample for NTM (table 5-8). This may suggest that the NTM may not trigger a detectable antibody (humoral) response in cattle, even in animals that did demonstrate clear CMI responsivity. As shown by Michel (2008) is it known that the NTM *M. fortuitum* activates a CMI response, and the study by Jenkins *et al.* (*in preparation*) demonstration this phenomenon in cattle infected with *M. malmesburiense* and *M. nonchromogenicum*. Considering that these NTM are not pathogenic, it is possible they do not activate a humoral response, as they may be cleared by the CMI and thus not circulate long enough for HI to be induced. Alternative explanations include that any antibodies induced, could have been undetected by the western blot, either due to low levels of circulating antibody or the crude nature of PPD preparations.

The western blots did occasionally show a response for PPD-A, but only if there was also a response for PPD-B detected in the same sample. Antibodies directed at PPD of *Mycobacterium avium* was only demonstrated in four samples (Table 4), but it was questionable. It was either faint or there was also a response seen towards PPD-B. This could point to cross-reactivity: A possibility is that infection with only *M. bovis* triggers antibodies that respond to the proteins in PPD-B, but possibly also the proteins that are present in PPD-A.

Finally, another possible explanation is that the serum samples could have been subjected to too many freeze-thaw cycles, which can harm the integrity of antibodies present in the sample and therefore interfere with further testing.

In order to assess whether the issues encountered during the study could have been biological in nature (i.e. absence of a humoral response to NTM) further studies would be required, and serum samples could be collected at several time points after inoculation of cattle with NTM to assess the presence and kinetics of the HI response. The use of recombinant antigens (known to be present/immunodominant in various NTM) species as opposed to PPD preparations should be considered as they may provide clearer evidence and would reduce the variability inherent to PPDs.

Altogether, further research is needed to increase the specificity and sensitivity of (B)TB testing. Animals should be tested by a CMI based test together with a serological assay to be able to detect animals in disparate stages of infection with *M. bovis* or NTM.

Conclusion

This study aimed to develop an NTM specific ELISA to better classify positive reactions to *M. bovis*, under the hypothesis that the inclusion of NTM in an indirect ELISA for BTB would increase the specificity of testing.

The specificity and sensitivity of the NTM inclusive ELISA test could not yet be established, as it is currently still under development. In any test development, the availability of true control samples is key in order to set up a novel test during optimisation and validation steps, and in subsequent use of the newly established assay. However, the Western blots carried out during this study did not demonstrate presence of antibodies to the various PPDs as expected, rendering our (clinical) samples unsuitable for further test development. The underlying reason for the absence of antibodies, could be experimental/artefactual (factors relating to Western blot optimisation, freeze-thaw cycles of sera, PPD production) or possibly biological (perhaps cattle don't mount a detectable antibody response against NTM). The latter may suggest that the cell-mediated immune response is the main actor against these mycobacteria, much like the response to MTBC, before the humoral response is induced.

More research is needed for the development of novel and improvement of existing (B)TB tests. Although, the assay could not be developed during this study, it remains a vital avenue to explore in order to broaden the scope and diagnostic window for BTB testing. For now, the best approach would be to test an animal with a serological assay, only in supplement to a well-established cell-mediated immune response-based test.

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Appendix

Appendix 1: CBT buffalo serum and PPD-B

Supplementary table 1: Negative serum. Raw data for the ELISA CBT titrating in twofold buffalo serum from 1/10 dilution over the rows and PPD-B starting 1280IU/ml over the columns using negative serum.

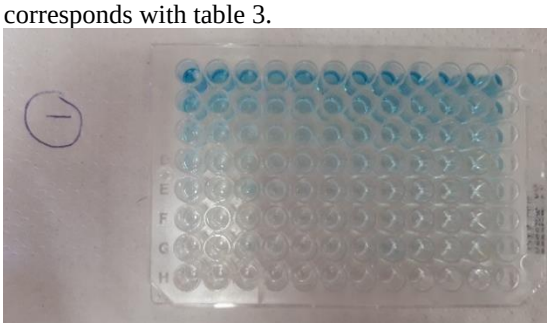
Negative	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1.331	0.970	1.077	0.676	0.918	1.063	1.266	1.319	1.360	1.289	1.345	1.368
B	0.726	0.524	0.546	0.378	0.647	0.630	0.760	0.809	0.867	0.841	0.790	0.928
C	0.428	0.296	0.325	0.243	0.335	0.388	0.518	0.544	0.606	0.593	0.633	0.656
D	0.285	0.210	0.228	0.153	0.224	0.242	0.36	0.358	0.293	0.405	0.387	0.446
E	0.183	0.153	0.169	0.113	0.148	0.192	0.261	0.267	0.279	0.267	0.273	0.282
F	0.125	0.119	0.105	0.099	0.110	0.142	0.192	0.188	0.182	0.178	0.179	0.217
G	0.093	0.098	0.077	0.070	0.096	0.103	0.130	0.160	0.125	0.120	0.127	0.132
H	0.047	0.049	0.053	0.049	0.051	0.051	0.050	0.046	0.057	0.053	0.051	0.064

Supplementary table 2: Positive serum. Raw data for the ELISA CBT titrating in twofold buffalo serum from 1/10 dilution over the rows and PPD-B starting 1280IU/ml over the columns using positive serum.

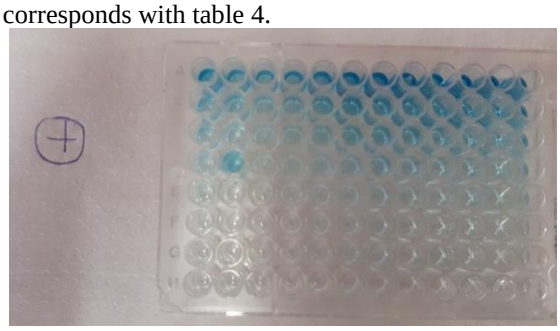
Positive	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	2.592	2.681	2.586	2.371	2.355	1.999	1.798	1.654	1.593	1.648	1.603	1.828
B	1.898	1.857	1.716	1.515	1.448	1.077	1.149	0.953	1.005	1.093	1.093	1.173
C	1.194	1.236	1.095	0.908	0.886	0.747	0.750	0.668	0.627	0.672	0.698	0.764
D	0.677	0.677	0.631	0.564	0.536	0.480	0.494	0.410	0.411	0.417	0.407	0.485
E	0.407	0.419	0.379	0.335	0.378	0.291	0.292	0.248	0.269	0.279	0.292	0.321
F	0.251	0.258	0.234	0.220	0.241	0.211	0.230	0.197	0.195	0.185	0.208	0.209
G	0.163	0.188	0.142	0.138	0.166	0.152	0.157	0.138	0.134	0.129	0.141	0.142
H	0.049	0.052	0.047	0.052	0.057	0.049	0.047	0.050	0.051	0.048	0.055	0.050

Appendix 2: CBT cow serum and PPD-A

Supplementary figure 1: Negative serum plate. Picture of the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and PPD-A starting 1280IU/ml over the columns using negative serum. The figure corresponds with table 3.



Supplementary figure 2: Positive serum plate. Picture of the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and PPD-A starting 1280IU/ml over the columns using positive serum. The figure corresponds with table 4.



Supplementary table 3: Raw data of negative serum plate. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and PPD-A starting 1280IU/ml over the columns using negative serum.

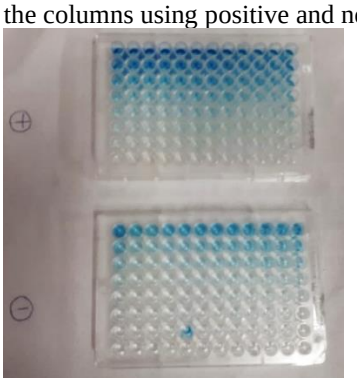
Negative	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1.154	0.952	0.799	0.650	0.701	0.791	0.856	0.877	0.881	0.807	0.816	0.899
B	0.663	0.500	0.389	0.341	0.376	0.442	0.482	0.570	0.456	0.510	0.464	0.504
C	0.338	0.283	0.242	0.213	0.258	0.294	0.316	0.320	0.335	0.325	0.319	0.337
D	0.224	0.193	0.169	0.164	0.168	0.186	0.211	0.209	0.226	0.186	0.229	0.212
E	0.152	0.132	0.207	0.111	0.116	0.128	0.141	0.126	0.145	0.137	0.150	0.145
F	0.111	0.094	0.085	0.080	0.094	0.106	0.108	0.109	0.104	0.096	0.106	0.102
G	0.087	0.069	0.109	0.112	0.075	0.085	0.085	0.167	0.043	0.081	0.103	0.093
H	0.065	0.055	0.057	0.061	0.057	0.082	0.057	0.056	0.058	0.057	0.051	0.047

Supplementary table 4: Raw data of positive serum. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and PPD-A starting 1280IU/ml over the columns using positive serum.

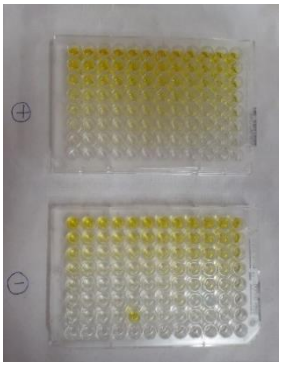
Positive	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.968	0.847	0.782	0.932	1.095	1.298	1.422	1.340	1.384	1.504	1.344	1.370
B	0.529	0.514	0.394	0.481	0.623	0.820	0.692	0.778	0.825	0.829	0.752	0.805
C	0.359	0.293	0.259	0.286	0.379	0.478	0.530	0.514	0.494	0.467	0.483	0.502
D	0.279	0.743	0.160	0.200	0.237	0.290	0.338	0.306	0.279	0.316	0.287	0.316
E	0.203	0.102	0.104	0.129	0.143	0.179	0.206	0.185	0.179	0.196	0.229	0.197
F	0.111	0.072	0.082	0.097	0.111	0.121	0.142	0.123	0.118	0.125	0.148	0.138
G	0.140	0.069	0.069	0.099	0.111	0.132	0.275	0.110	0.137	0.133	0.101	0.093
H	0.079	0.072	0.063	0.053	0.056	0.060	0.070	0.064	0.049	0.057	0.064	0.052

Appendix 3: CBT CFP10/ESAT-6 17/04/2018

Supplementary figure 3: Positive & negative serum plates before stopping solution. Picture of the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and CFP10/ESAT-6 starting 20µg/ml over the columns using positive and negative serum. This figure corresponds with table 5 and 6.



Supplementary figure 4: Positive and negative serum plates after stopping solution ready for reading. Picture of the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and CFP10/ESAT-6 starting 20µg/ml over the columns using positive and negative serum. This figure corresponds with table 5 and 6.



Supplementary table 5: Raw data of negative serum plate. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and CFP10/ESAT-6 starting 20µg/ml over the columns using negative serum.

Negative	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1.347	0.982	0.896	0.948	1.004	1.097	1.125	1.113	1.138	1.145	1.142	1.255
B	0.697	0.478	0.436	0.489	0.524	0.588	0.649	0.639	0.689	0.692	0.718	0.764
C	0.411	0.272	0.238	0.278	0.306	0.361	0.376	0.391	0.406	0.474	0.416	0.442
D	0.256	0.189	0.153	0.178	0.192	0.218	0.221	0.272	0.222	0.229	0.237	0.254
E	0.149	0.098	0.097	0.111	0.123	0.130	0.152	0.177	0.142	0.183	0.142	0.151
F	0.107	0.087	0.092	0.110	0.116	0.092	0.103	0.177	0.101	0.047	0.096	0.095
G	0.093	0.078	0.074	0.082	0.663	0.094	0.096	0.081	0.098	0.086	0.079	0.068
H	0.054	0.045	0.051	0.059	0.056	0.057	0.061	0.057	0.050	0.054	0.043	0.044

Supplementary table 6: Raw data of positive serum plate. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/100 dilution over the rows and CFP10/ESAT-6 starting 20µg/ml over the columns using positive serum.

Positive	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	2.150	1.944	1.981	2.385	2.309	2.096	2.119	2.000	1.753	1.591	1.476	1.450
B	1.372	1.475	1.560	1.562	1.670	1.481	1.497	1.389	1.218	1.135	1.085	1.057
C	0.759	0.885	0.874	0.855	0.941	0.955	0.947	0.880	0.804	0.825	0.662	0.648
D	0.488	0.460	0.459	0.519	0.535	0.563	0.551	0.659	0.462	0.436	0.381	0.422
E	0.289	0.292	0.282	0.267	0.326	0.358	0.358	0.318	0.271	0.237	0.269	0.291
F	0.185	0.188	0.200	0.173	0.255	0.282	0.236	0.214	0.204	0.205	0.161	0.162
G	0.119	0.112	0.110	0.117	0.125	0.188	0.121	0.125	0.120	0.116	0.101	0.105
H	0.049	0.067	0.045	0.051	0.045	0.050	0.049	0.054	0.059	0.054	0.062	0.050

Appendix 4: CBT with or without sodium azide in the coating buffer, for PPD-B and ESAT6/CFP10 vs cattle serum

Supplementary table 7: Experimental design and lay-out of the plates. Two plates with sodium azide in the CB (one using positive serum and one using negative serum). Two plates without sodium azide in the CB (one using positive serum and one using negative serum).

	PPD-B						ESAT-6/CFP-10					
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	320						10					
B	160						5					
C	80						2.5					
D	40						1.25					
E	20						0.625					
F	10						0.31					
G	5						0.16					
H	0 Serum 1/200	1/400	1/800	1/1600	1/3200	0	0 Serum 1/200	1/400	1/800	1/1600	1/3200	0

Supplementary table 8: Raw data of positive serum plate; without sodium azide. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/200 dilution over the rows and PPD-B starting 320IU/ml and CFP10/ESAT-6 starting 10µg/ml over the columns using positive serum.

	PPD-B						ESAT-6/CFP-10					
Positive	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1.492	1.700	2.033	2.116	2.238	0.172	1.247	0.705	0.356	0.209	0.132	0.048
B	1.446	1.520	2.287	2.467	2.489	0.088	1.251	0.687	0.391	0.215	0.143	0.048
C	1.552	1.662	2.001	2.442	2.731	0.056	1.141	0.666	0.366	0.227	0.151	0.047
D	1.543	1.834	2.129	2.549	2.590	0.060	1.055	0.610	0.366	0.216	0.144	0.050
E	1.697	1.672	1.939	2.215	2.549	0.054	0.871	0.530	0.320	0.225	0.131	0.050
F	1.573	1.917	2.728	2.546	2.230	0.055	0.628	0.385	0.242	0.162	0.119	0.051
G	2.070	2.264	2.655	2.337	1.779	0.076	0.492	0.315	0.187	0.131	0.096	0.053
H	1.492	1.700	2.033	2.116	2.238	0.172	1.247	0.705	0.356	0.209	0.132	0.048

Supplementary table 9: Raw data of negative serum plate; without sodium azide. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/200 dilution over the rows and PPD-B starting 320IU/ml and CFP10/ESAT-6 starting 10µg/ml over the columns using negative serum.

	PPD-B						ESAT-6/CFP-10					
Negative	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.506	0.267	0.155	0.116	0.075	0.044	0.517	0.264	0.156	0.098	0.194	0.046
B	0.569	0.328	0.163	0.108	0.077	0.084	0.479	0.278	0.178	0.107	0.079	0.250
C	0.486	0.241	0.162	0.112	0.086	0.051	0.435	0.263	0.177	0.120	0.104	0.046
D	0.390	0.205	0.151	0.107	0.086	0.044	0.363	0.207	0.156	0.109	0.064	0.043
E	0.351	0.199	0.125	0.091	0.096	0.069	0.354	0.244	0.136	0.084	0.066	0.043
F	0.329	0.204	0.135	0.085	0.076	0.065	0.320	0.256	0.123	0.099	0.084	0.047
G	0.370	0.249	0.141	0.125	0.101	0.103	0.390	0.232	0.130	0.085	0.108	0.049
H	0.346	0.208	0.143	0.104	0.081	0.061	0.318	0.190	0.136	0.124	0.072	0.045

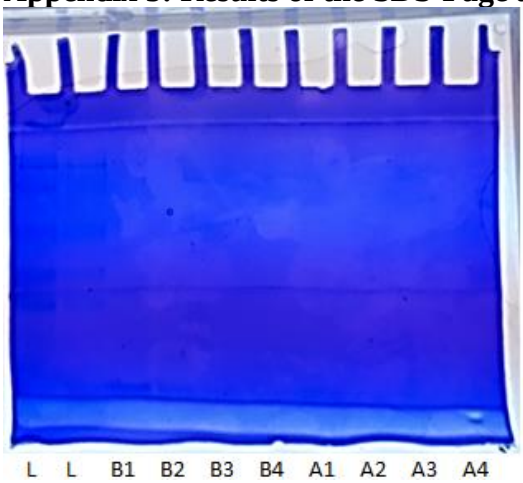
Supplementary table 10: Raw data of positive serum plate; with sodium azide. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/200 dilution over the rows and PPD-B starting 320IU/ml and CFP10/ESAT-6 starting 10µg/ml over the columns using positive serum.

	PPD-B						ESAT-6/CFP-10					
Positive	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1.744	1.624	2.042	2.312	2.705	0.051	1.335	0.810	0.524	0.380	0.276	0.205
B	1.734	1.752	2.088	2.519	2.754	0.049	1.431	0.883	0.613	0.460	0.368	0.261
C	1.533	2.036	2.015	2.153	2.485	0.048	1.403	0.892	0.621	0.447	0.360	0.275
D	1.571	1.720	1.918	2.442	2.639	0.061	1.238	0.727	0.463	0.291	0.188	0.093
E	1.788	1.663	2.348	2.646	2.656	0.052	1.083	0.634	0.386	0.238	0.151	0.079
F	1.742	1.724	2.538	2.877	2.140	0.046	0.845	0.503	0.298	0.183	0.119	0.056
G	2.409	2.436	2.808	2.592	1.731	0.043	0.656	0.386	0.239	0.154	0.099	0.053
H	0.439	0.273	0.182	0.115	0.084	0.046	0.491	0.290	0.182	0.122	0.083	0.045

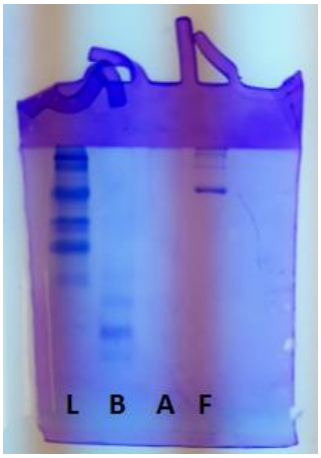
Supplementary table 11: Raw data of negative serum plate; with sodium azide. Raw data for the ELISA CBT titrating in twofold cattle serum from 1/200 dilution over the rows and PPD-B starting 320IU/ml and CFP10/ESAT-6 starting 10µg/ml over the columns using negative serum.

	PPD-B						ESAT-6/CFP-10					
Negative	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.519	0.293	0.206	0.139	0.126	0.079	0.679	0.520	0.296	0.321	0.229	0.200
B	0.577	0.326	0.165	0.101	0.075	0.034	0.729	0.531	0.491	0.349	0.297	0.267
C	0.566	0.322	0.150	0.097	0.069	0.040	0.626	0.472	0.375	0.319	0.286	0.236
D	0.462	0.377	0.139	0.093	0.069	0.047	0.450	0.293	0.209	0.146	0.115	0.087
E	0.357	0.299	0.151	0.089	0.067	0.044	0.382	0.241	0.158	0.107	0.084	0.055
F	0.442	0.544	0.133	0.134	0.064	0.043	0.349	0.223	0.138	0.104	0.073	0.048
G	0.315	0.283	0.132	0.085	0.082	0.076	0.324	0.218	0.139	0.247	0.070	0.047
H	0.443	0.240	0.155	0.099	0.073	0.046	0.328	0.199	0.132	0.095	0.067	0.044

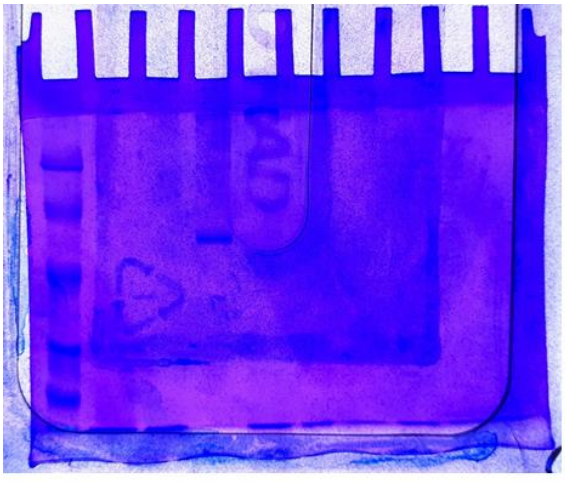
Appendix 5: Results of the SDS-Page and western blots



Supplementary figure 5: The gel to establish the right concentrations. L= Ladder, B = PPD-B at various concentrations (B1 = 937,5µg/mL, B2 = 468,75µg/mL, B3 = 234,37µg/mL, B4 = 117,19µg/mL), A = PPD-A at various concentrations (A1 = 781,25µg/mL, A2 = 390,62µg/mL, A3 = 195,31µg/mL, A4 = 97,66µg/mL).



Supplementary figure 6: Gel with correct concentrations for WB. L = Ladder; B = PPD-B 937,5µg/mL; A = PPD-A 781,25µg/mL; F = PPD -F 973,5µg/mL.



Supplementary figure 7: Gel with correct concentrations for WB. L = Ladder; B = PPD-B 937,5µg/mL; A = PPD-A 781,25µg/mL; F = PPD -F 973,5µg/mL,), M = PPD-M at various concentrations (M1 = 1875 µg/ml, M2 = 937.5 µg/ml, M3 = 468.75 µl/ml), N = PPD-N at various concentrations (N1 = 1875 µg/ml, N2 = 937.5 µg/ml, N3 = 468.75 µl/ml)

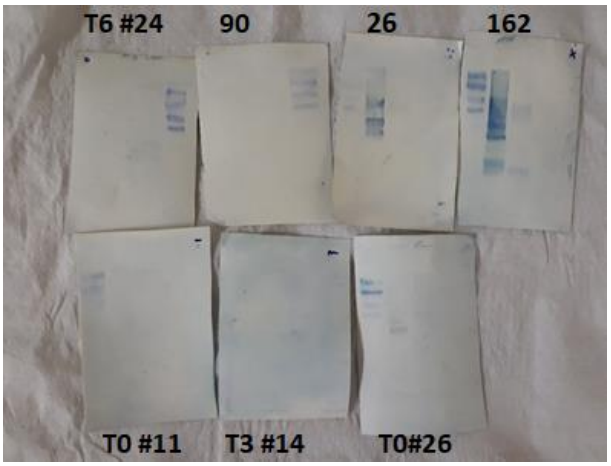
Supplementary table 12: Experimental design of the western blots. Four gels were made with PPD. After transfer the membrane were cut. In total 6 sera could be tested with the 5 different ppd. Table 12 shows gel 1 & 2.

Wells	1	2	3	4	5	6	7	8	9	10
	L	B	A	F	M	N		L	B	A

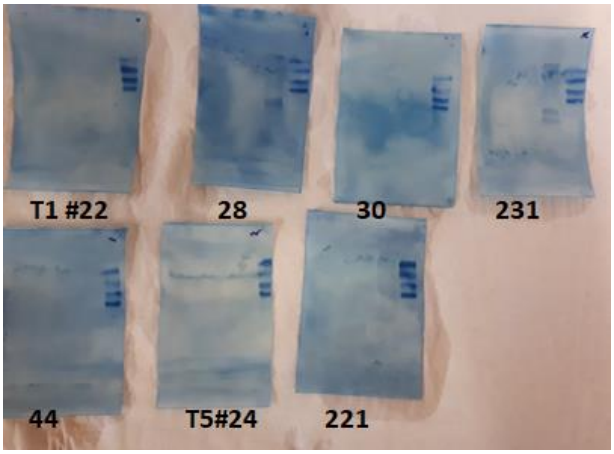
Supplementary table 13: Experimental design of the western blots. Four gels were made with PPD. After transfer the membrane were cut. In total 6 sera could be tested with the 5 different ppd. Table 13 shows gel 3 & 4.

Wells	1	2	3	4	5	6	7	8	9	10
	L	B	A	F	M	N	L	F	M	N

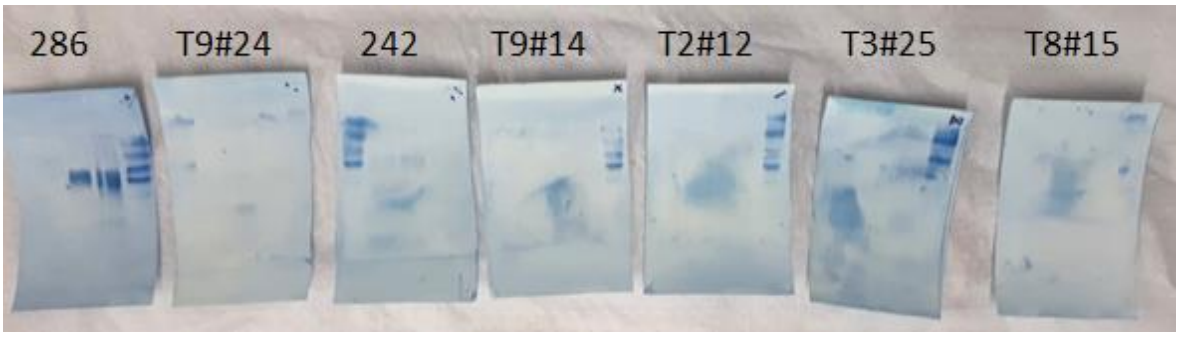
Supplementary figure 8: Results western blots 24 April 2018. First results of the western blot with no detectable antigens.



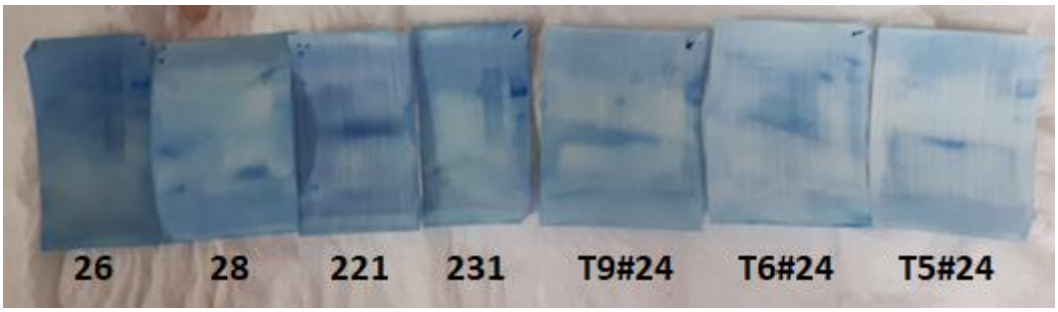
Supplementary figure 9: Results western blots 15 May 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots. T= timepoint; #=Animal ID.



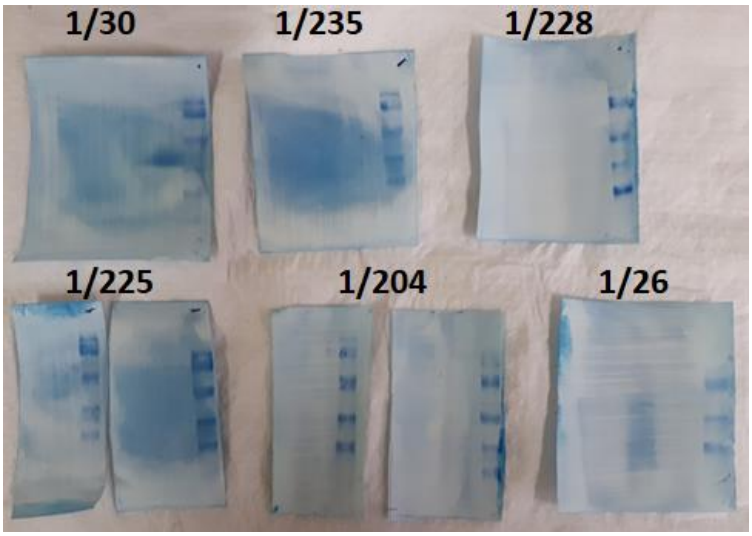
Supplementary figure 10: Results western blots 17 May 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots. T= timepoint; #=Animal ID.



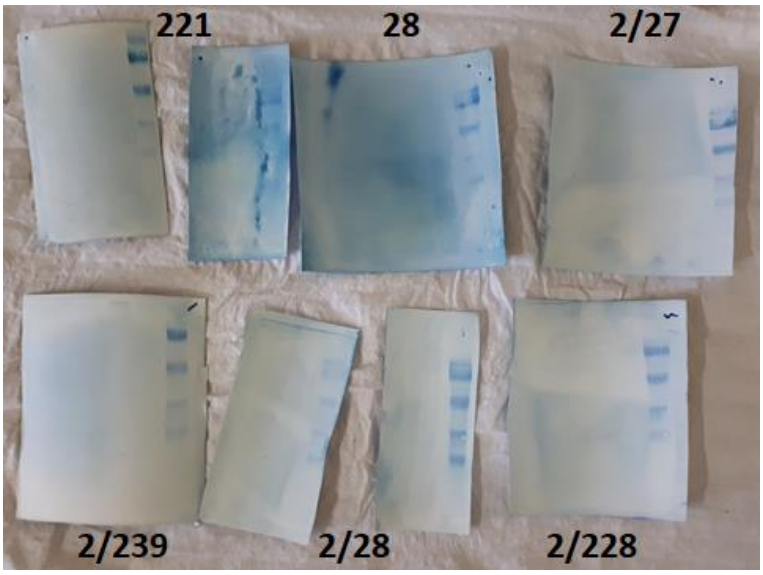
Supplementary figure 11: Results western blots 18 May 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots. T= timepoint; #=Animal ID.



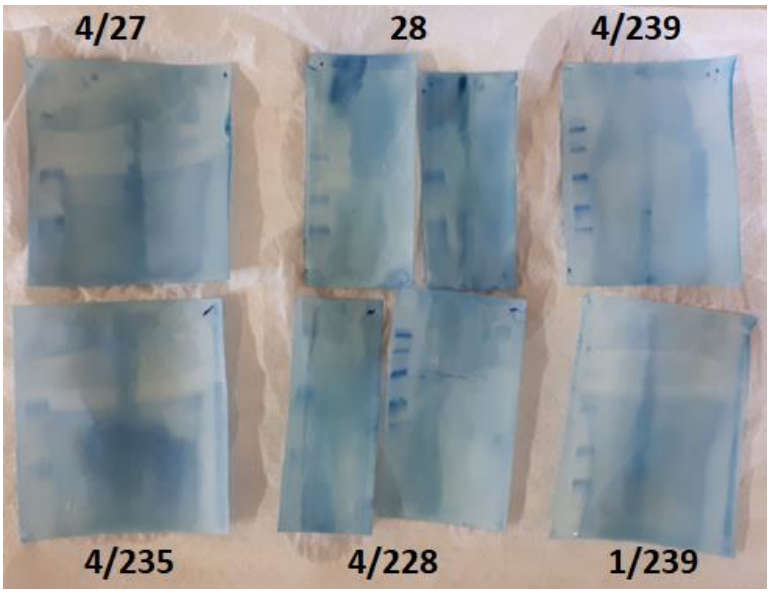
Supplementary figure 12: Results western blots 5 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots. T= timepoint; #=Animal ID.



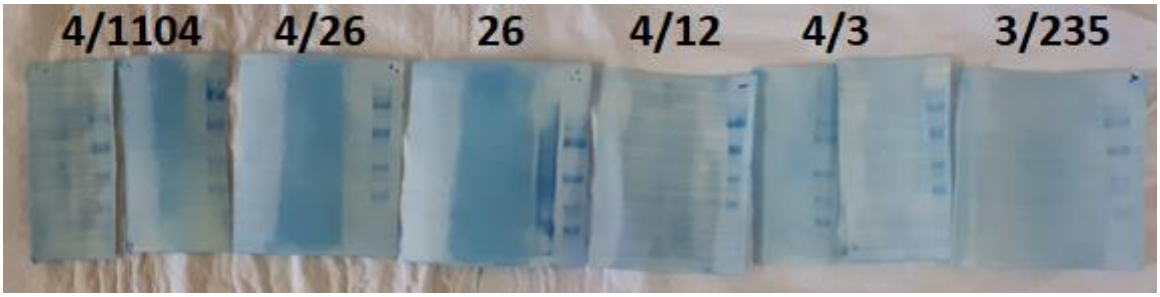
Supplementary figure 73: Results western blots 6 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



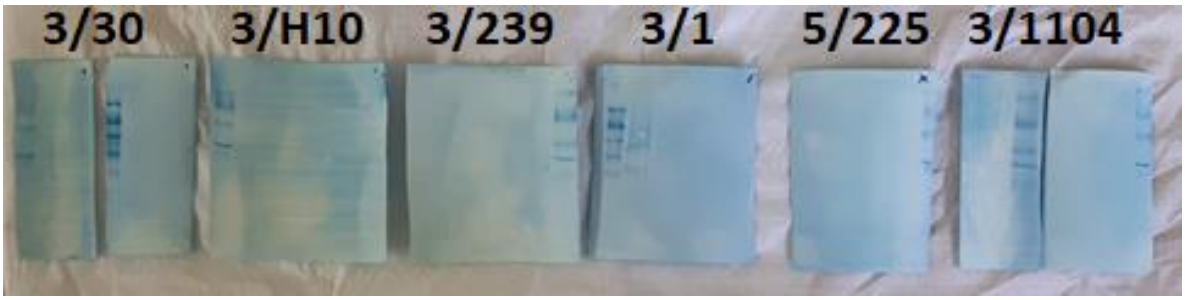
Supplementary figure 84: Second batch of results western blots of 6 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



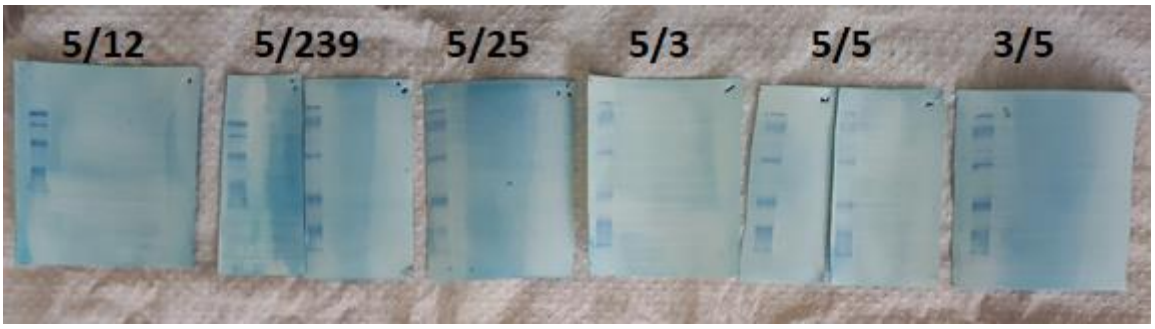
Supplementary figure 95: Results western blots 8June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



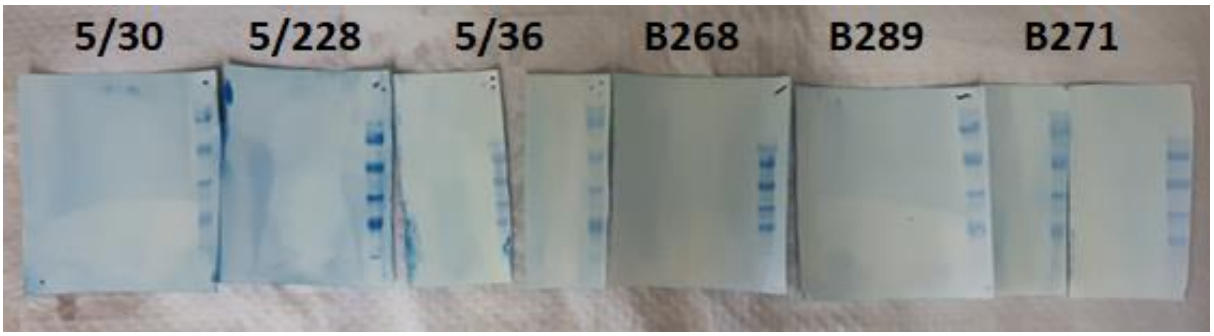
Supplementary figure 106: Results western blots 9 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



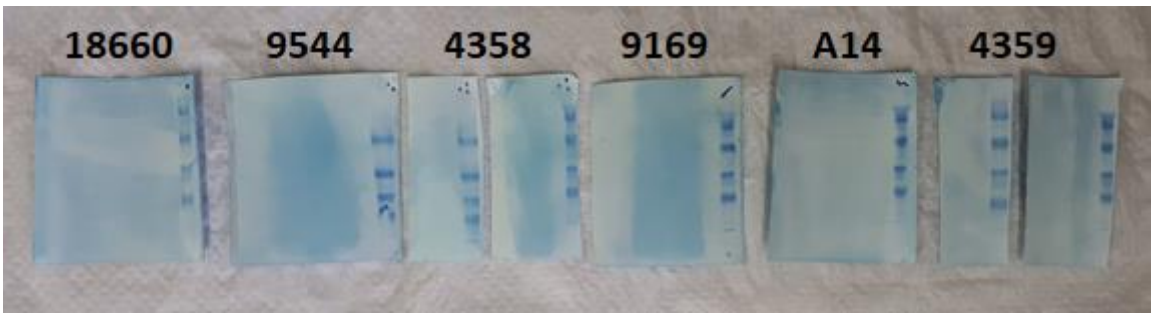
Supplementary figure 117: Results western blots 12 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



Supplementary figure 128: Results western blots 13 June 2018. Numbers reflect animal IDs of the cattle serum used for each of the blots; timepoint/animal ID.



Supplementary figure 139: Results western blots 14 June 2018. Numbers reflect animal IDs of the cattle and buffalo serum used for each of the blots; timepoint/animal ID.



Supplementary figure 20: Results western blots 15 June 2018. Numbers reflect animal IDs of the buffalo serum used for each of the blots; animal ID.