Performance of two candidate bovine tuberculins in the intradermal tuberculin test in South African cattle naturally infected with *Mycobacterium bovis*

By

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List of Abbreviations

ANOVA- Analysis of variance

ISBT -International Bovine Standard Tuberculin

PPD-Purified Protein Derivative

WHO-World Health Organisation

OIE-International Organisation for Animal Health

bTB-Bovine Tuberculosis

CA-1-Candidate bovine tuberculin one-0.02mg,

CA-2- Candidate bovine tuberculin one-0.1mg,

CB-1-Candidate bovine tuberculin two-0.02mg,

CB-2-Candidate bovine tuberculin two-0.1mg,

M.bovis-Mycobacterium bovis

NIBSC- National Institute for Biological Standards and Control

Thesis Summary

Performance of two candidate bovine tuberculins in the intradermal tuberculin test in South African cattle naturally infected with *Mycobacterium bovis*

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Department: Veterinary Tropical diseases

The International Standard Bovine Tuberculin (ISBT) was confirmed for use in 1986 and serves as an internationally accepted reference for calibration and quality control. The World Health Organization has been involved in the production of a standard bovine tuberculin since 1976. More recently, the OIE in collaboration with the WHO coordinated a project for the development and testing of a new ISBT. By means of the international collaborative study, the performance of the ISBT against two new candidate tuberculins A and B, at two different concentrations in different regions of the world was tested. This study formed part of this international collaboration and tested the performance of the candidate tuberculins A (CA-1; 0.02mg, CA-2; 0.1mg) and B (CB-1; 0.02mg, CB-2; 0.1mg) in naturally infected cattle in South Africa.

In order to test the performance of the candidate tuberculins A and B and the ISBT, 130 animals from known bovine tuberculosis positive herds were sampled. The BOVIGAM^{TM} assay was used to identify 24 animals from the 130 animals, that were bovine tuberculosis positive and these animals were selected for the intradermal tuberculin testing. Evaluation of the performance of candidate tuberculins was done by comparing the skin test reactions of the tuberculins with the skin test reactions of the current ISBT. The means of the skin fold increases in the ISBT was compared to the means of the skin fold increases in candidates A and B. These were compared using ANOVA in excel analysis package, and the results indicated that there was no significant difference between the ISBT and the candidates A and B. The different concentrations in the tuberculins also did not register any significant difference in reactions and there was also no significant difference in the performance of the tuberculins on the different sides of the bovine neck. The clinical signs at the injection sites of both the ISBT and the candidate tuberculins were compared using ANOVA in excel analysis package and the p values were also >0.05 which indicated a lack of a significant difference between them. These results suggest that the candidate tuberculins A and B worked as effectively and gave similar skin responses to the current ISBT and may be considered for field diagnosis of bovine tuberculosis.

Chapter 1

Introduction

Tuberculin testing has been used in diagnosing tuberculosis since the 1930s (Yang et al., 2012). The purified protein derivatives of *Mycobacterium bovis* and *Mycobacterium avium* are used in the diagnosis of bovine tuberculosis. The work of producing a Bovine International Standard tuberculin has been carried out since 1976 when the World Health Organisation (WHO) began an evaluation of candidate bovine PPDs in order to choose a new Bovine International Standard Tuberculin PPD (BIS) (Haagsma J, 1982). The current International Standard Bovine Tuberculin (ISBT) was developed in 1986 and its main aim and function is to serve as an internationally accepted reference for calibration and for use in quality control, to supply tuberculin manufacturing companies and also to ensure uniformity of the Purified Protein Derivative (PPD) on an international level (Frankena et al., 2018). The ISBT plays a very important role in the control of *Mycobacterium bovis* (*M. bovis*). In many countries, tuberculin testing is mandatory. It is important to have a standard that has been tested to check the performance because low potency of a PPD can make it less efficient for bovine tuberculosis (bTB) diagnosis (Duignan et al., 2019a). Tuberculin PPDs need to be evaluated in the target species and in the geographical areas where they are applied. That is why a global standardization for the current standard is needed. The stock of the current ISBT is declining, and because of this the OIE and WHO have proposed the production of a new ISBT to meet the global requirement. The subject of this thesis is part of this global study whose aim is to evaluate the performance of the new ISBT. In the global study the performance of two candidate tuberculins will be tested in different areas of the world.

1.1.1 Objectives of the Study

In our study the objective was to evaluate the performance of two candidate bovine tuberculins produced by the National Institute for Biological Standards and Control (NIBSC) (United Kingdom), in the intradermal tuberculin test in naturally infected cattle in South Africa, and compare the performance of these to the ISBT.

This main objective will be achieved by a) comparing the performance of the standard and candidate tuberculins at different concentrations, and b) comparing the performance of the standard and candidate tuberculins by the clinical signs produced at the site of injection, and c) the skin tests responses were evaluated and compared for the different sides of bovine neck.

1.2 Literature Review

Mycobacterium bovis is a bacterium that is responsible for causing bovine tuberculosis (bTB). *M.bovis* can affect both animals and humans. *M.bovis* is found worldwide, but the prevalence is less in some of the more developed countries due to the prevailing control measures, in contrast to developing countries

(Musoke et al., 2015). Although cattle are the domestic reservoir host, *M.bovis* is also endemic in some African buffalo (*Syncerus cafer*) populations, which then act as reservoir for the disease (Michel et al., 2009). Inhalation of aerosols containing *M. bovis* containing particles from the lungs of infected animals is the usual route of infection. In humans, it can also be contracted by drinking infected milk from bTB positive animals (Jemal, 2016). Inhaled bacilli are phagocytosed by macrophages that may either clear the infection or allow the mycobacteria to grow. A primary focus will be formed which is surrounded by epithelioid cells, granulocytes, lymphocytes and later giant cells. This necrotic centre may calcify and the lesion will then be surrounded by granulation tissue with a capsule around it forming a tubercle. These lesions form primarily in the lungs but may also be produced elsewhere. The infection can spread through the lymphatic channels to the lymph nodes or haematologically to other organs of the body such as, pleura, peritoneum, liver and also kidneys, a condition known as miliary tuberculosis. (Cynthia m Kahn, 2005, Jemal, 2016). The clinical signs in affected animals include emaciation, lethargy, fluctuating fever, anorexia and the animal will be coughing. There may be lymphadenopathy (Cynthia m Kahn, 2005, Jemal, 2016).

In the late 1800s, experiments commenced in cattle on the use of tuberculin, which was injected into the chest wall of clinical tuberculosis cases (Good et al., 2018). Through the years the tuberculin testing has been refined and standardized in its usage and dosage (Goldstein et al., 2002). In 1909, a tuberculin test was done in the neck region and the results in cattle were based upon measurements of the skin thickness increases at the site of the injection (Good et al., 2018). The intradermal tuberculin test has continued to be an of important tool in the control of bTB.

The standard diagnostic tests for *M. bovis* are the intradermal tuberculin skin test, either in the form of the single intradermal skin test or the comparative intradermal cervical tuberculin test (Nayak S, 2012)- and the bovine gamma interferon assay (Wood et al., 1991, Lahuerta-Marin et al., 2015). Bovine tuberculosis has been better controlled in most developed countries due to the policies of testing the cattle by means of intradermal tuberculin test, and slaughtering animals that test positive. This, in combination with movement restrictions has seen bTB becoming better controlled in many parts of the developed world. A major complication arises when cattle come into contact with infected wildlife populations such as infected badgers, white tailed deer, brushtail possum or African buffalo (Woodroffe et al., 2005, White et al., 2008, O'Brien et al., 2011, Carstensen and Doncarlos, 2011). Studies have shown that the intradermal tuberculin test has a specificity ranging from about 88%-99%, which makes it suitable for herd level testing (De la Rua-Domenech R, 2006, M.L. Monaghana, 1994, Álvarez et al., 2012, Schiller et al., 2010, Ameni et al., 2008). The high specificity means that the animals found to be negative are truly negative. In addition, several studies have shown that the sensitivity of the intradermal tuberculin test has ranges varying from 68% - 95%, 55.1% - 93.5% and 40.1% - 92.2% (Alvarez et al, 2012, M.L.Monaghana, 1994, De la Rua-Domenech R, 2006 and Schiller et al, 2010), with an average of 80%, meaning that 80% of animals that have been found to be reactors are definitely infected with *M. bovis*.

The tuberculin test has also been used in other bovine and non-bovine species. In the deer (white tailed deer, red deer and reindeer) the single intradermal tuberculin test has been used in the mid cervical region with a sensitivity of 80% (Cousins and Florisson, 2005). In pigs, the intradermal tuberculin test is performed on the dorsal surface of the ear and in some studies the intradermal tuberculin skin test done in pigs has proved to be reliable (Alfredsen and Saxegaard, 1992). In non-human primates the tuberculin skin test is applied on the upper eyelid and the sensitivity in most cases is poor (Cousins and Florisson, 2005). In dogs the tuberculin skin test is performed on the medial surface of the pinna and in both cats and dogs the tuberculin skin test has proved unreliable (Sykes and Gunn-Moore, 2014).

1.2.1 Intradermal Tuberculin Test

The intradermal tuberculin test skin uses a purified protein derivative (PPD). These are proteins obtained from the lysis of *Mycobacterium bovis* and *Mycobacterium avium* respectively (Duignan et al., 2019a). The injection of this PPD into the skin of an animal that has been exposed to *Mycobacterium bovis* or *Mycobacterium avium*, will result in the *Mycobacterium bovis* or *Mycobacterium avium* specific memory T- cells being attracted to the site of injection and multiplying in reaction to the PPD antigen, thus producing a local delayed hypersensitivity reaction at that site.

The single intradermal test is especially used on known bTB negative herds as a routine test in bTB control programmes (Good et al., 2011). Positive herds may also be tested with the single intradermal test, for the purpose of faster elimination of test positive cattle. The single intradermal test may be used in combination to the additional tests such as gamma interferon test (DAFF, 2016). In the single intradermal test, the injection site is prepared by shaving a small area in the neck region. The area is usually free of any swelling that may interfere with the results. The bovine tuberculin is injected into the skin area, the thickness of the skin at site of injection is measured before and 72 hours after the injection.

The single intradermal comparative tuberculin skin test compares the animals' immune response to two different PPDs, bovine tuberculin and avian tuberculin. These PPDs are injected into the skin on the cervical region and the thickness at the site of injection is measured before the injection and 72 hours after the injection. This is used to differentiate between animals infected with *M.bovis* and animals that had been exposed to or infected with other types of non-tuberculous *Mycobacterium* species found in the environment which do not cause bTB (Poirier et al., 2019). Cattle that are infected with *M. bovis* will show a greater reaction to bovine tuberculin than avian tuberculin. According to Awah Ndukum, (Awah-Ndukum et al., 2016) the World Organization for Animal Health (OIE) has recommended that a difference of 4mm or more in skin fold increase after 72 hours is considered positive for bTB. Test sites used in the single intradermal test may vary in sensitivity and between countries or areas. One advantage of using this test is that it increases the rate of finding true positives because it differentiates

between cattle infected with *M. bovis* and those exposed to other *Mycobacterium* species and therefore sensitized to tuberculin. One major disadvantage is that cross reactions may occur in animals infected with other non-tuberculous *Mycobacterium* species found in the environment. The results may be classified as either negative result; positive result; or inconclusive result which are animals that show a reaction to the bovine tuberculin which is more than the avian, but with the difference not being 4mm or more and cannot be classified as a positive reactor (DAFF, 2016). The comparative test is used for herds where the bTB status or the history of the herd in relation to bTB is unknown or unclear and where non-specific reactions have occurred and there is a need to reach a definitive diagnosis.

The intradermal tuberculin test has some disadvantages in that the test requires two herd visits. This is disadvantageous because some animals may not be available for the second herd visit rendering the tests incomplete. Challenges with the tuberculin also arise if the tuberculin is not kept at an optimum temperature constantly, this could lead to the protein being denatured. Technical errors arise from incorrect injection, for example into the sub cutaneous tissue as opposed to injecting into the skin. Other errors could arise from erroneous reading of the callipers and erroneous placement of the PPD. Failure of the owners to present the cattle for the second measurement after 72 hours is another challenge with this test.

1.2.2 Bovine Gamma Interferon Assay

Another diagnostic test that can be used for tuberculosis diagnosis in bovines is the bovine gamma interferon assay (BOVIGAM) (Rothel J J, 1992). This *in vitro* interferon-gamma (IFN-γ) assay was developed in Australia in the 1980s (Wood, 2001) and has been recommended by the OIE since 1996 (OIE Terrestrial Manual) as a test additional to the tuberculin intradermal test. Most bTB control programmes depend on the use of the IFN-γ assay as an additional test to the intradermal test to fully achieve the detection of infected animals. In this test, whole blood is stimulated using bovine PPD and avian PPD for about 24 hours. The test also has a positive control such as pokeweed mitogen and a negative control which is an unstimulated aliquot of blood. The antigens stimulate the release of the cytokine IFN-γ by the sensitised T-lymphocytes. The plasma is then harvested and assayed for IFN-γ production. A commercial enzyme sandwich immunoassay is used. The animal is considered positive if the optical density of the bovine PPD is greater than the optical density of the avian PPD (Van der Heijden 2016, Whipple D L, 2001). The BOVIGAM is advantageous because it is a sensitive and quick test. It requires only one herd visit which improves compliance and eliminates the risk of farmers failing to avail the animals after 72 hours. The bovine gamma interferon assay has a high sensitivity ranging from 91.3-99.2%, but a very low specificity ranging from 3.6-11.1% (Pucken et al., 2017). It also has an advantage of detecting *M. bovis* earlier than the skin test (Wood and Jones, 2001).

As the intradermal tuberculin testing has gone through modifications over the years, in 1986 an ISBT was chosen (Holzman, 2002). The (World Health Organization) WHO, OIE and European Union (EU)

have set the required standards for tuberculin production, performance, and testing in bovines (Good et al., 2018). The WHO and OIE continues to work to ensure that ISBT continues to be available. As such they undertook a recent project to replace the ISBT in a three-phase project, selection of candidate tuberculin, preliminary evaluation and international collaboration. In the international collaboration, the candidate tuberculins chosen are tested in guinea-pigs sensitized with live *M. bovis,* and they are also tested in guinea-pigs which have been sensitized with heat-inactivated *M. bovis*. They will also be tested in naturally infected cattle as well as cattle sensitized by experimental infection. These studies were carried out in different regions of the world to test the performance of the tuberculin under the different conditions mentioned. This was the first time that such an evaluation was carried out in Southern Africa. Our research was part of the third phase, the International collaboration, where the performance of the two candidate tuberculin was assessed against the ISBT in cattle that have been naturally infected with *M.bovis* in South Africa (WHO, 2018)

Chapter 2

Materials and Methods

2.1 Study area

The study was carried out in the UMkhanyakude district of northern Kwazulu/Natal Province in South Africa. A communal farming area where cattle are registered under the Nibela dip tank register were selected because previous research confirmed the presence of bovine tuberculosis in these communal cattle herds (Sichewo et al., 2019).

2.2 Animals and Sampling

Blood was drawn from 130 animals from herds with known bTB infections. The blood was collected in heparin tubes and then tested using the BOVIGAM™ commercial assay (Michel et al., 2011). The animals that were bovine reactors in the BOVIGAM™ were used in the intradermal tuberculin testing to meet the OIE protocol requirement of exclusively recruiting *M. bovis* infected cattle. Twenty-four animals were selected to be used in the intradermal tuberculin testing, based on their reaction in BOVIGAM™ considered indicative of *M. bovis* infection.

2.3 Materials

The materials included the BOVIGAM™ test kit, Heparin vacutainers, 18G needles, sterile 24 well tissue culture plates, bovine PPD, avian PPD, the ISBT which was 1.8mg in each ampoule which was about 58 500 IU, and the PPDs of the two candidate tuberculins which were procured from the National Institute for Biological standards and Control (NIBSC). Other materials included insulin syringes, needles, callipers, hair clippers, clipboard, tool box and a cool box (DAFF, 2016).

2.4 Methods

2.4.1 BOVIGAM assay

Blood was collected from each animal into a heparinized tube, 1ml of blood was aliquoted into each of the wells of the 24 well tissue culture plate. Each animal had a total of 4mls of blood aliquoted, 1ml of blood for each antigen as shown in the figure below. This was done for all blood samples. Then stimulating agents were added to the blood. The bovine PPD at 30µl, avian PPD at 60µl, Pokeweed Mitogen (Positive control) at 5µl/ml of blood, and an unstimulated measure of blood as a negative control. The culture trays were appropriately labelled according to the animal numbers. The culture trays were incubated at 37ºC for 24 hours. Plasma was then harvested (approximately 250µl) and pipetted into 96 tube storage racks. The plasma was assayed following the manufacturers' instructions for the BOVIGAM™. The blood samples that showed reactivity to bovine PPD were the animals recruited for the intradermal tuberculin test.

Table 2.1 Placements of Reagents in 24 Well Culture Plate

Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6
Bovine PPD	Bovine PPD	Bovine PPD	Bovine PPD	Bovine PPD	Bovine PPD
Avian PPD	Avian PPD	Avian PPD	Avian PPD	Avian PPD	Avian PPD
Pokeweed Mitogen	Pokeweed Mitogen	Pokeweed Mitogen	Pokeweed Mitogen	Pokeweed Mitogen	Pokeweed Mitogen
Nil	Nil	Nil	Nil	Nil	Nil

Bovigam testing was performed in two stages. Stage 1 comprised screening of all plasma samples in the field laboratory. In the absence of an ELISA reader the ELISA plates were evaluated visually based on the colour intensity at the end of the conjugate incubation step (blue colour development) for classifying animals into positive, negative, avian and suspect bovine reactors.

Stage 2 testing involved repeat testing of all plasma samples in the ELISA reader allowing for OD measurement for confirmation of final reactor status. Samples were classified positive (bovine reactors) if OD (Optical Density) of PPD-b (Purified Protein Derivative Bovine) minus OD of PPD-a (Purified Protein Derivative Avian) = >0.1. Samples were classified as avian if OD for PPD-a was more than OD for PPD-b. Additionally, criteria of test validity were met when: OD of (Pokeweed Mitogen) PWM > 0.35 (Michel et al. 2011) and OD of PPD-b minus OD of Nil = >0.1

2.4.2 Preparation of ISBT and Candidate Tuberculins A and B

The current International Standard and the pre-lyophilised and freeze-dried preparations of the two candidate bovine tuberculin preparations were supplied by the National Institute for Biological Standards and Control (NIBSC). For the ISBT the freeze-dried preparations were dissolved in sterile double distilled water to 0.1mg and 0.02mg per dose which corresponded to 3250 and 650 IU, respectively. The candidate tuberculins were diluted at five-fold intervals maintaining the weights of 3250 and 650 IU, which was 0.1mg and 0.02mg in concentration. The injection volume was 0.1ml which was delivered in a 1 ml insulin syringe.

2.4.3 Intradermal Tuberculin Test

This was done as a single intradermal test, which compared the ISBT PPDs and the PPDs of the two candidate tuberculins. Each animal was injected with the ISBT in two concentrations of 0.1mg and 0.02mg and also the candidate tuberculins in equivalent concentrations.

2.4.3.1 Preparation of the tuberculin injection sites

Using hand-held double-sided razors, four areas were shaved on each side of the neck, in the middle cervical region with a distance of about 15cm between the injection sites. Before shaving, the areas were palpated for any swellings or lumps that could interfere with the results.

2.4.3.2 Tuberculin injection sites and administration of tuberculin preparations

Initially the candidate tuberculins were compared with the ISBT in eight reactor cattle and this was repeated with two more sets of eight reactor cattle, a total of 24 cattle was used. Four intradermal injections were applied on each animal on each side of the neck in a Latin square design as depicted in figure 2. The thickness of the skin fold at each injection site was measured with callipers, as accurately as possible before application of the PPDs and 72 hours after.

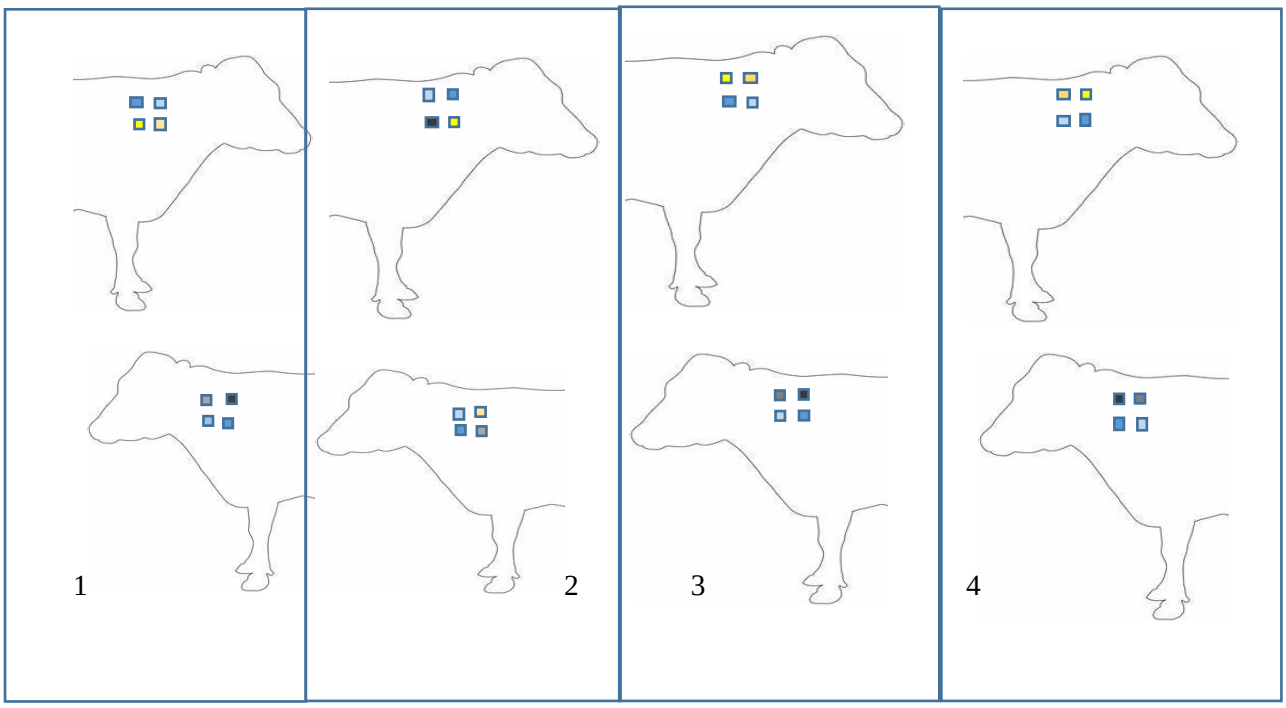

Figure 2.1 Latin square-type layout of tuberculin injection sites1

2.5 Statistical Analysis

2.5.1 Skin Fold Measurements

The skin fold increase measurements for all the different tuberculins were entered into a table, Table 2.2. This information was transferred to an excel sheet, and using the Microsoft excel analysis package, the variance in the means of the skin fold increases was calculated by using a one factor ANOVA. This was done to test if there was a significant difference between the means of the skin fold increases on the undiluted ISBT and the diluted ISBT. The skin increases for the tuberculins A and B were compared to the skin fold increases of the ISBT. The skin fold increase of the candidate tuberculin A was compared to the skin fold increase of candidate tuberculin B. These comparisons were carried out using one factor ANOVA in the excel analysis add in.

[1] Key: ISBT-2 (0.1mg), ISBT-1 (0.02mg) , CA-2(0.1mg) , CA-1 (0.02mg) , CB-2(0.1mg) , CB-2(0.02mg) .

2.5.2 Clinical Signs

The injected sites were assessed for clinical signs by visual inspection and palpation. The clinical signs which were observed at the injection sites were, diffuse (D), oedema (O), adhesions (A), circumscribed (C), flat (F), hard (H) and necrosis (N). These clinical signs were coded according to increasing severity from zero to twenty as depicted in table 2.2. The clinical signs were ranked arbitrarily and allocated a score according to their indication of *M. bovis* infection whether they were a weak, moderate or strong indicator. Zero indicating clinical signs for non-specific reactions such as circumscribed, one and two for a weak indication such as slight diffuse and diffuse reaction, three for moderate indication such as slight oedema, 15 for strong indication such as oedema and 20 for very strong indication of *M. bovis* infection such necrosis, adhesion and lymph nodes. The total score for the clinical signs was calculated by adding up the values of the reactions at a given site and the data was entered into a table, Table 3.3. A one factor ANOVA was carried out on this data using the Microsoft excel data analysis add in, to compare the severity of the clinical signs elicited by the different tuberculins, ISBT, candidate A and candidate B.

Table 2.2 Coding of clinical signs with increasing severity

Clinical Sign or appearance	Description	Score
Flat	If a reaction is flat and without other accompanying clinical signs, *M. bovis* infection unlikely	0
Hard	A hard reaction with no other signs, the reaction is likely caused by non-specific infection	0
Circumscribed	A circumscribed reaction with no other signs may be caused by non-specific infection	0
Slight diffuse	A reaction showing a slight diffusion, that is when the area of the reaction progressively runs into the surrounding tissue.	1
Diffuse	A diffuse reaction is mostly associated other clinical signs which are indicative of *M. bovis* infection usually indicates towards a positive *M. bovis* infection	2
Slight oedema	A reaction showing some slight oedema. An oedematous reaction is one that has accumulation of fluid in tissue.	3
Oedema	Strong indicator of *M. bovis* infection	15
Necrosis, Adhesions and Lymph nodes	Lymph nodes which are enlarged and painful to touch as well as necrosis, and adhesions, which are felt between the skin and subcutaneous tissue are very strong indicators of *M. bovis* infection.	20

2.5.3 Test Agreements

Test agreements between the Bovigam and the skin test were determined by means of a two-by-two table, by comparing the positive and negative results of both the Bovigam and the skin test. The diagnosis for each animal was evaluated as positive, at the specific cut-off of 4 mm or less in the case of a severe clinical sign such as oedema, necrosis or adhesion, in the intradermal skin testing. Animals reading less than 4mm and not displaying severe clinical signs were recorded as negative. The positives were recorded in a table as one, while the negatives as zero (Appendix D) the summary is shown in Table 3.4. Two by two tables were thus created (Appendix E) to determine the test agreements by means of Cohens Kappa coefficient. The test agreements for the various tuberculins were determined. The formula for calculating test agreement percentage was (a+d)/(a+b+c+d). Where (a+d) is the observed agreement divided by the total observations.

Chapter 3

Results

3.1 Animals

A total 130 animal from nine herds with known bTB infection were selected to collect blood samples for gamma interferon IFN-γ assay (BOVIGAM^{TM}), which was used to identify animals that were bTB positive. Some of the cattle in this group had previously been T- branded to indicate that they had tested bTB positive in a previous study (Sichewo et al., 2019). These were targeted for blood sampling.

3.2 Bovigam Assay to Recruit Infected Cattle

In the Bovigam screening assay, 21.5% (28/130) animals sampled were classified as either bovine or suspect reactors. Twenty-four of these cattle were confirmed as bovine reactors in the full Bovigam assay in stage 2. Two animals (Nos 23 and 119), were classified as avian reactors and one animal (No. 25) was negative on full Bovigam testing (stage 2). Data from these latter three animals were excluded from the analysis because their immune responses were not specific to M. bovis. The animals that were bovine reactors were recruited for the intradermal tuberculin test. Table 3.1 shows a summary of the results obtained during the screening the and full Bovigam testing. Appendix B shows the detailed results obtained during the screening and in the full Bovigam testing.

Table 3.1 Summary Results of BOVIGAMTM Screening

Number of Animals	Screening	Full Bovigam Testing		
		Avian	bTB Positive	bTB Negative
4	Suspect		3	1
24	Positive	2	22	

3.3 Intradermal Tuberculin Test (skin test)

The 24 animals that were classified as bovine reactors underwent the intradermal tuberculin testing to compare the performance of the candidate bovine tuberculin A (CA-1, CA-2) and candidate bovine tuberculin B (CB-1, CB-2) against the International Standard Bovine Tuberculin (ISBT) in two different concentrations of 0.1mg and 0.02mg for all the three tuberculins. The skin test responses to the candidates and the standard bovine tuberculins were evaluated under eight different regimens as shown in Figure 2.1. The increases in skin fold thickness were noted and entered in Table 3.2. The skin

measurements before the application of the different PPDs and 72 hours after the PPD application, and the clinical signs elicited on the different injection sites, are shown in the Appendix.

3.4 Performance of two candidate bovine tuberculins in comparison with the International Standard bovine tuberculin in the tuberculin skin test

3.4.1. Tuberculin Concentration

The increase in skin fold thickness for the different tuberculins at different concentrations are outlined in Appendix C and a summary of these skin fold increases is shown in Table 3.2. The increase in skin fold thickness for the diluted (0.02mg) and undiluted (0.1mg) ISBT was evaluated using ANOVA and the p-value was 0.307. These values at 95% confidence interval showed that there was no significant difference in the means of the skin fold increases as a result of the concentration of the ISBT.

The increase in skin fold for the candidate A-1 and candidate A-2 was analysed using the ANOVA and the p-value obtained was 0.08, indicating that there was no significant difference in the increase in skin fold thickness for the candidate A either diluted or undiluted. The skin test responses for the undiluted and diluted candidate B were analysed using an ANOVA to verify if there was a significant difference in the increase in skin fold thickness of the aforementioned, and the p-value obtained was 0.26 indicating that there was no significant difference in the increase in skin fold thickness in the candidate B-1 and candidate B-2. The skin test responses to ISBT were compared to the skin test responses of the candidate tuberculins A and B, and the p-value obtained from the ANOVA comparing undiluted bovine tuberculins A and ISBT was 0.26, and the p-value obtained from comparing the undiluted bovine tuberculins B and ISBT was 0.76. The p-value obtained from ANOVA analysis of skin test responses to the undiluted bovine candidates A and B was 0.13. These P values demonstrated that there was no significant difference on the skin test responses in all the three PPDs at the two concentrations.

Table 3.2 Summary of skin fold thickness increases at different concentrations and locations

PPDs at different concentrations	Skinfold thickness Increases between 0-2mm	Skinfold thickness Increases between 2-4mm	Skinfold thickness increases >4mm	Total number of animals
ISBT-1 Left	9	7	8	24
ISBT-1 Right	6	8	10	24
ISBT-2 Left	5	10	9	24
ISBT-2-Right	7	6	11	24
CA-1 Left	4	1	1	24
CA-1 Right	6	6	6	
CA-2 Left	1	1		24
CA-2 Right	2	9	11	
CB-1 Left	4	6	12	24
CB-1 Right		2		
CB-2 Left	9	4	5	24
CB-2 Right	2	1	3	

3.4.2 Location of Tuberculin Injection Sites

The skin tests responses were evaluated according to the different locations of the cervical region.

(a) Left versus right cervical region

The skin test responses for the ISBT were combined for both diluted and undiluted because there was no significant difference between the skin folds increases of the diluted and undiluted ISBT. The P values obtained from the comparison between the increase in skin fold thickness on the left side of the neck and the right side was 0.57.

3.4.3 Clinical Signs

Most of the 24 animals displayed diffuse or slightly diffuse responses on one or more injection sites. These reactions, ran into the surrounding tissue without a clear boundary as depicted in Figure 3.2. 20 animals displayed oedema or slight oedema at one or more injection sites. The oedematous swellings had abnormal fluid accumulation in the tissue around the injection sites as depicted in Figure 3.1 which shows a diffused oedematous swelling. Four animals displayed circumscribed skin responses at least on one injection site. This type of swelling had a clear demarcation between the reaction area and the normal skin as depicted in Figure 3.3 which shows a circumscribed swelling. Adhesions were noted in

three animals. While four animals also presented with necrosis at one or more injection site, this was seen as a small dark area around the centre of the reaction.

Figure 3.1 Bovine showing oedematous skin response to injection of M. bovis PPD

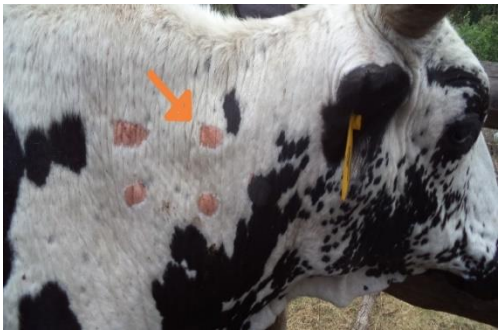

Figure 3.2 Bovine showing diffuse skin response to injection of M. bovis PPD

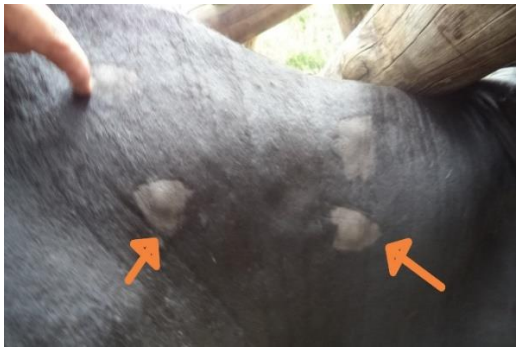

Figure 3.3 Bovine showing circumscribed skin response to injection of M. bovis PPD

The clinical signs were assigned a value to indicate the likelihood of *M. bovis* infection as is shown in Table 2.2. All clinical signs observed at the injection sites were tallied according to the coding, and entered into a table, which indicated how the animals reacted to the different tuberculins by the clinical signs as shown in Table 3.3. Table 3.4 shows the summary of the total number of animals per outcome

for the different tuberculins. The p-value obtained by comparing the combined clinical signs elicited by the ISBT was 0.34, indicating that there was no significant difference in the clinical signs on the left and right sides of the neck.

The p-values obtained from comparing the means of clinical signs were as follows; comparison between the undiluted standard bovine tuberculin and candidate bovine tuberculin A was 0.14; comparing the means of clinical signs between the undiluted standard bovine tuberculin and candidate bovine tuberculin B undiluted, p-value was 0.72; comparing the means of the clinical signs between the diluted standard bovine tuberculin and candidate bovine tuberculin A diluted, p-value was 0.85; comparing the means between diluted standard bovine tuberculin and candidate B diluted, p-value was 0.14; comparing the means between candidate A undiluted and candidate B undiluted, p-value was 0.12 and comparing the means between candidate A diluted and candidate B diluted, the p-value was 0.28. The p-values obtained were all greater than 0.05, this indicated that there was no significant difference in the clinical signs between the different tuberculins used. Although there was no significant difference in all of the comparisons some comparisons showed greater difference between them. The p value for the comparison between the undiluted standard bovine tuberculin and candidate bovine tuberculin A diluted, showed a greater difference as the difference was closer to 1. Other comparisons such as the comparisons between the diluted, and undiluted candidate tuberculins A and B, diluted standard and candidate B bovine tuberculin had difference of less 0.5 p-value.

The animals all reacted differently to the PPDs as shown in Table 3.3 from the total score of the reactions. The total scores were arranged in three groups; the low score=<45, intermediate = 46 to 90; high score = >90. These scores were compared to the full Bovigam testing results (PPDb - PPDa), and the results showed that score of the animals did not in any way reflect on their PDDb-PPDa score. The animals in the low segment had both low, intermediate and high readings in the full Bovigam testing. The observation for intermediate and high scores was similar, with the reading for full Bovigam testing varying between low, intermediate and high.

Table 3.3 Clinical signs following injection with the standard and candidate bovine tuberculin preparations

Animal ID	ISBT -1	ISBT -2	CB-1	CB-2	CA-1	CA-2	Total score
9	2	2	1	2	2	2	11
12	9.5	17	2	17	17	17	79.5
20	9.5	2	2	17	2	17	49.5
24	17	17	5	2	17	17	75
32	0	0.5	0	1	0	0	1.5
33	0	1	17	2	5	17	42
38	8.5	17	5	0	17	17	64.5
43	3	2.5	17	0	0	0	22.5
50	17	17	0	0	17	17	68
55	9	17	17	17	17	17	94
63	17	9.5	17	17	0	17	77.5
65	5	9.5	5	0	0	17	36.5
67	11	21	37	5	0	5	79
74	1	8.5	17	17	17	2	62.5
75	0	0	5	1	0	0	6
77	0	2.5	17	5	5	5	34.5
86	11	11	0	5	2	5	34
97	4	8.5	23	17	17	16	85.5
99	11	11	17	0	17	25	81
122	17	17	17	0	37	37	125
124	8.5	9.5	0	17	17	2	54
125	17	17	17	17	0	17	85
128	8.5	9.5	22	37	2	22	101
131	12.5	11.5	25	22	2	42	115

3.5 Test Agreements

The Kappa coefficient for the comparison between the undiluted ISBT and the Bovigam was calculated and the value obtained was 0.67. The Kappa coefficient for the test agreement between the Bovigam and the candidates' tuberculin A and B were 0.79 and 0.54 respectively. The candidate bovine tuberculins and the ISBT were further analysed to verify the test agreements between them and the following were the Kappa coefficients derived, ISBT-1 compared with candidates A and B undiluted were 0.79 and 0.71 respectively. These results are summarised in Table 3.5.

Table 3.4 Summary of the total number of animals per outcome for the different tuberculins2

Test outcomes	ISBT-1	ISBT-2	CB-1	CB-2	CA-1	CA -2	Bovigam results
0	6	8	6	11	13	5	0
1	18	16	18	13	11	19	24

Table 3.5 Summary of test agreement percentages between the Bovigam test and the intradermal skin test using the standard and candidate bovine tuberculins

	ISBT-2	ISBT-1	CA-2	CB-2
Bovigam	66.67%	75.00%	79.16%	54.16%
ISBT-2		83.33%	79.16%	70.83%

[2] Where the variables, '0' represents the negative test outcomes and '1' positive test outcomes

Chapter 4

4.1 Discussion

The intradermal tuberculin skin testing with Purified Protein Derivative is the recommended test by the OIE for screening against TB in bovines (Thakur et al., 2016), therefore antigen standards are important to ensure that disease control programmes are effective (Duignan et al., 2019b) on a world wide scale, and are fit for the intended purpose (Good M, 2011). Candidate tuberculin A and candidate tuberculin B were tested in comparison to the current International Standard Bovine Tuberculin (ISBT), and this was the first time that international collaboration also tested the performance of the ISBT in the African region particularly South Africa as well as other continents outside Europe. Data from skin tests in naturally infected bTB positive animals was collected and analysed.

The performance of the ISBT was evaluated and the results of the skin test showed that there was no significant difference in the skin fold thickness increases between the undiluted (0.1mg) and diluted (0.02mg) ISBT on the left and right sides of the neck. The clinical signs also indicated that there was no significant difference ($p= 0.34$) in clinical signs on the left and right sides of the neck. This indicates that for practical purposes the skin test can be performed on either the right or the left side of the bovine neck. This is supported by the absence of statistically significant differences in the responses to the ISBT between the two anatomical sites.

The performance of two candidate bovine tuberculins in the skin test, was evaluated and the results showed that there was no significant difference ($p=0.26$) between the means of the increase in skinfold thickness, in the candidate tuberculin A and candidate tuberculin B, against the ISBT. This was further shown by the clinical signs produced by the different PPDs. The clinical signs produced by the different bovine tuberculins were compared, and the results showed that there was no significant difference in clinical signs between the ISBT and candidate tuberculins A and B. This study thus demonstrated the correlation between increase in skin fold and clinical signs at a given site. The greater the skin fold increase the more severe the clinical signs observed at the injection site. This also became evident when analysing the data from the total clinical sign score. For example, animal number 122 had the highest total clinical sign score observed, and it also had higher values for skin fold increase. Whilst animal number 32 which had recorded the lowest clinical sign score, also had lower skin fold increases. This difference in skin responses could be attributed to immunological responses, the pre-allergic phase and the anergic phase. An animal that is newly infected (between 3-6 weeks) will show no skin response to the bovine tuberculin, this is the pre-allergic phase while the anergic phase the is the chronic or advanced stage where the immune system of the animal changes from a cell-mediated response to a humoral antibody response (tbhub.co.uk, 2020, DAFF, 2016) and does not show a skin response after inoculation with bovine tuberculin.

Although there was no significant difference in clinical signs displayed at the site of inoculation, there were some tuberculins that had greater differences in terms of p-values than others. This indicates that there was still considerable variation in the clinical signs at the injection site, elicited by different tuberculins.

Several animals had been branded T in a previous study (Sichewo et al., 2019) to show that they were positive for bTB in the intradermal skin test, and these were animals numbered 65, 122, 124, 125, 128 and 131. The clinical signs showed that these animals still had strong positive reactions to all the PPDs and this is in contrast with previous studies that demonstrated that, especially animals that have been recently infected with *M. bovis*, display strong reactions at the site of injection (Good et al., 2018).

There was a substantial test agreement between the Bovigam and the ISBT intradermal skin test, according to the rating of Landis and Koch and other scholars (Landis and Koch, 1977, Watson and Petrie, 2010). This indicates that Bovigam test results compared to the results from the skin test with the standard bovine tuberculin would be considerably similar. The test agreements between the Bovigam and the candidate tuberculins A and B were also measured and the results indicated the agreements to be substantial and moderate respectively. This indicates that the results obtained from the Bovigam would be considerably similar to results obtained from candidate bovine tuberculin A, and would be relatively similar to the results obtained from the intradermal skin test using bovine candidate B. The analysis showed that the percentage agreement between standard and the candidate bovine tuberculin A both were substantial (Cicchetti and Sparrow, 1981, Watson and Petrie, 2010). This indicates that there was considerable similarity in the results obtained from the different bovine tuberculins. The undiluted ISBT and the diluted ISBT had an excellent test agreement in the results with the Kappa coefficient of 0.83, as noted in other studies where the Kappa coefficient of more than 0.8 is rated as excellent (Kottner et al., 2011, Waldner et al., 2004), indicating that the concentration of the standard tuberculin did not affect its performance.

The sensitivity of the tuberculin test is one of its limitations, some studies have shown that the sensitivity of this test can be low, ranging from 63% to 95%, with an average of 80%. This means that about 20% of the infected animals can be missed (Singhla et al., 2019, M.L. Monaghana, 1994, De la Rua-Domenech R, 2006). In our study however, the animals used were all known positives from a high-risk area, the sensitivity and positive predictive value in such a study would be high as shown by other studies (Goodchild et al., 2015). The sensitivity of the tuberculin test can be affected by the stage of the disease as well as the severity, causing a positive animal to read as negative. Other limitations with the tuberculin skin test include the presence of environmental mycobacteria, suppression of immune system of the animal either by drugs or other disease and errors due to testing personnel.

Chapter 5

5.1 Conclusion

The intradermal tuberculin test remains an OIE recommended test for bTB in cattle worldwide, and in the interest of using a standardized and fit for purpose approach, this study was undertaken. It was part of a global study to evaluate two candidate tuberculins as potential replacements for the current International Standard Bovine Tuberculin. This study is important because it contributes to the control of bTB by ensuring that the tuberculin chosen as the next ISBT will be able to produce good results in cattle on a worldwide platform including Southern Africa. In South Africa, the performance of the candidate tuberculins in cattle naturally infected with *M. bovis* indicated that there was no significant difference in their performance. This was established from the increases in skinfold thickness and the clinical signs elicited at the injection sites, which were not significantly different among the three types of tuberculins used.

It was beyond the scope of this study to recommend which of the two candidate tuberculins A or B should be retained as a future replacement for the current ISBT because our study covered the performance of these tuberculins only in the region of South Africa. For that conclusion to be made, the data from all the collaborating partners that participated in the international collaborative study will be subjected to a meta-analysis

5.2 Recommendations

It is recommended that similar evaluations for the performance of current or future standard tuberculins be performed to include more diverse climatic conditions and management practises. Future studies for the African region could include more countries in the different climatic zones and use different breeds of cattle including the local breeds which are managed in a more traditional way.

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Appendices

Appendix A

		Site1 - Left neck				Site 1 - Right neck				Site 2 - Left neck				Site 2 - Right neck				Site 3 - Left neck				Site 3 - Right neck				Site 4 - Left neck				Site 4 - Right neck			
Anim	Regin	1-0h	1-72h	Difference	1- Clinical signs	1-0h	1-72h	Differenc	1- Clin :	2-0h	2-72h	Difference	2-Clin :	2-0h	2-72h	Difference	2-Clin sig	3-0h	3-72h	Differenc	3-Clin sig	3-0h	3-72h	Difference	3-Clin si	4-0h	4-72h	Differenc	4-Clin sign	4-0h	4-72h	Differen	4-Clin signs
124	1	7.5	11	3.50	D, [O]	8.7	11.5	2.80	D	8	13.2	5.20	F, H	6.8	10.6	3.80	D, O	6.9	10.9	4.00	D, O	6.4	10.3	3.90	C, [O]	7.8	7.3	(0.50)	NR	6	10.5	4.50	D, O
74	1	6.3	7.8	1.50	D, O	5.1	6	0.90	NR	7.4	11.8	4.40	D, O	5.1	8.3	3.20	D	6.5	8.7	2.20	D, O	4.4	7.6	3.20	D, O	6.2	6.5	0.30	NR	5.3	8.2	2.90	D
9	1	11.4	14.5	3.10	D	6.2	6.7	0.50	D	10	14	4.00	[D]	10.1	13.3	3.20	[D]	11	13.3	2.40	D	9.2	13.5	4.30	D	9.8	15	5.20	D	9.4	12.1	2.70	D
12	1	7.2	12.7	5.50	D, O	7.1	11.7	4.60	F, D, O	7.7	12.5	4.80	F, D	6.7	11.2	4.50	D, O	7.4	13	5.60	D, O	6.4	11.9	5.50	D, O	8.4	11.7	3.30	F, D	6.8	11.8	5.00	D, O
125	2	7.5	8.2	0.70	NR	6.6	10.2	3.60	D, O	7.5	8.2	0.70	D, O	7	13.3	6.30	D, O	6.6	23.6	17.00	D, O	5.4	8.6	3.20	D, O	7.7	12.2	4.50	D, O	3.7	11.3	7.60	D, O
67	2	8.7	9.9	1.20	NR	6.7	12.3	5.60	D, [O]	7.8	15	7.20	D, O	8.1	18.1	10.00	D, [O]	8.2	20.9	12.70	D, O, A	7.9	14.2	6.30	D, [O]	8.2	16.6	8.40	D, O, [A]	6.8	17.6	10.80	D, [O]
20	2	6.5	10.4	3.90	D	6.2	6.7	0.50	D, O	8.2	11.3	3.10	D	6.5	8.5	2.00	D	7	9.9	2.90	D	7.2	7.4	0.20	D, O	8	10.4	2.40	D	7.4	9.8	2.40	D, O
19	2	5.8	5.5	(0.30)	NR	5.9	6	0.10	NR	6.7	6.3	(0.40)	NR	7	6.4	(0.60)	NR	6	6.2	0.20	NR	6.5	6	(0.50)	NR	6.6	6.8	0.20	NR	5.4	6.4	1.00	NR
75	2	7.6	8.3	0.70	NR	8.1	7.6	(0.50)	NR	7.7	8.5	0.80	NR	7.6	8.5	0.90	NR	8.3	10.2	1.70	D, [O]	7.2	7.4	0.20	[D]	8.2	8.9	0.70	NR	9.2	9.9	0.70	NR
128	3	8	24.9	16.90	D, O, A	5	15.3	10.30	D, N	5.5	19.1	13.60	D, A	4.8	16.5	11.70	D	6	15.8	9.80	D, O	5.7	14.5	8.80	D	6	5.9	(0.10)	NR	6.2	13.2	7.00	D, O
77	3	7.7	9.6	1.90	D, [O]	8.2	11.3	3.10	D, [O]	7.2	10.7	3.50	D, O	8.5	10.5	2.00	D, [O]	8.3	9.7	1.40	D, [O]	6.6	6.5	(0.10)	NR	8.3	8.8	0.50	NR	8.5	10.1	1.60	F
86	4	7.2	8.7	1.50	NR	8	11	3.00	D	8.4	12.3	3.90	D, [O]	7.5	10.3	2.80	D, [O]	9.7	12	2.30	D, O	9.6	10.4	0.80	D, [O]	10.3	12.9	2.60	D, O	9.5	13.3	3.80	D, [O]
131	4	9	18.3	9.30	D, [O], L	7.4	13.3	5.90	D	7.7	14.7	7.00	D, N	7.6	20	12.40	D, A, L	9.1	9.4	0.30	NR	7.6	14.3	6.70	D, [O], L	9.2	14.7	5.50	C, O, N	7.5	8.4	0.90	NR
24	4	11.9	18.3	6.40	D, [O]	9	12.5	3.50	D, O	10.5	15.5	5.00	D	8.1	15	6.90	D, O	8.2	15	6.80	D, O	8.4	13.2	4.80	D, O	8	10.4	2.40	D , O	8.6	16.1	7.50	O, D
33	5	8.3	12.1	3.80	D	9.6	13.5	3.90	D, O	8.2	12	3.80	F	8.4	11.9	3.50	D	8.7	11.9	3.20	D, O	8.1	9.8	1.70	D, [O]	9.7	11.8	2.10	F	10.2	13.3	3.10	F, [D]
97	5	9.4	18.3	8.90	D, O	7.6	19.3	11.70	D, O	10.4	11.5	1.10	NR	7.7	19.5	11.80	D, O	10	16.4	6.40	C, N, O	8	13.2	5.20	D, O	9.8	17.2	7.40	C, O	9	15.6	6.60	D, O
32	5	10.3	10.7	0.40	[D]	10.1	9.5	(0.60)	NR	10.5	12.5	2.00	[D]	9.9	10.4	0.50	NR	9.8	9.1	(0.70)	NR	8.6	10.1	1.50	NR	9.8	9.5	(0.30)	NR	10.1	10.4	0.30	NR
43	6	7.2	7.7	0.50	NR	5.6	8.5	2.90	F	6.1	10	3.90	F	6.4	9.8	3.40	D, [O]	6.1	8	1.90	D, [O]	5.9	7.2	1.30	[D]	7.3	9.3	2.00	D, O	6.4	8.3	1.90	NR
50	6	7.3	7.5	0.20	NR	6.6	9.5	2.90	D, O	8.2	12	3.80	D, O	6.6	9.8	3.20	D, O	7	7.8	0.80	D, O	5.7	7.4	1.70	D, O	7	9.8	2.80	C	7.7	9.2	1.50	D, O
38	6	9	9.7	0.70	NR	7.5	12.5	5.00	D, O	9.1	14	4.90	D, O	8	12.1	4.10	D, O	8.7	12.9	4.20	D, O	8	9.2	1.20	F	9.7	14.2	4.50	D, [O]	7.6	10.5	2.90	D, O
99	6	8.5	8.6	0.10	NR	7.4	13	5.60	D, [O],	8.4	13.3	4.90	D, O	7.4	13	5.60	D, [O]	8.9	11.8	2.90	C, D, [O]	7.4	12.5	5.10	D, O	8.1	13.1	5.00	D, O	7.8	10	2.20	D, O
55	7	9.9	15.9	6.00	D, O	9.5	14.6	5.10	D , O	9.2	15.1	5.90	D, O	12.9	17.3	4.40	D, O	11	13.4	2.90	D, O	9.2	11.6	2.40	[D]	9.8	12.8	3.00	D, O	13.1	15.7	2.60	D, O
63	7	10.2	16.2	6.00	D, O	8.7	13.5	4.80	D	9.8	10.2	0.40	NR	9.4	15.1	5.70	D, O	10	11.7	1.70	D, O	7.5	10.3	2.80	D, O	10.1	11.6	1.50	D, O	8.1	10.7	2.60	D, O
122	8	5.6	14	8.40	D, O	5.9	10.5	4.60	D, O	5.4	5.6	0.20	NR	5.7	14.3	8.60	D, O, N	5.2	12.1	6.90	D, O	4.6	9.2	4.60	D, O, N	5.3	11.2	5.90	D, O	5.3	11	5.70	D, O
65	8	10.2	14.7	4.50	D, O	8.2	11.2	3.00	D	8.5	8.6	0.10	NR	8.5	12.5	4.00	D, O	9.4	12.6	3.20	D, [O]	6.8	8	1.20	NR	8.6	11.3	2.70	D , [O]	9.5	11.8	2.30	D, [O]

D=Diffuse O- Oedema, [O] slight oedema, NR No reaction N- Necrosis A -Adhesic L- Lymp C- Circumscribed , F- Flat, H-Hard

Bovine tuberculins applied

- ISBT-1
- ISBT-2
- CA-1
- CA-2
- CB-1
- CB-2

Appendix B

Results of full BOVIGAM™ Screening

	Screening	Full Bovigam test					Interpretation
Sample ID		Nil	PWM	PPDa	PPDb	PPDb - PPDa	
9	Suspect	0.047	3.029	1.021	1.127	0.106	Bovine reactor
12	Positive	0.069	2.818	0.134	0.583	0.449	Bovine reactor
19	Suspect	0.013	2.37	0.41	0.559	0.149	Bovine reactor
20	Positive	0.034	0.851	0.048	0.39	0.342	Bovine reactor
23	Positive	0.224	3.492	0.749	0.45	-0.299	Avian reactor
24	Suspect	0.03	1.941	0.164	0.585	0.421	Bovine reactor
25	Suspect	0.073	1.344	0.095	0.074	-0.021	Negative
32	Positive	0.034	3.173	0.183	2.063	1.88	Bovine reactor
33	Positive	0.446	2.966	1.843	3.43	1.587	Bovine reactor
38	Positive	0.087	1.746	0.336	2.879	2.543	Bovine reactor
43	Positive	0.033	1.141	0.16	0.88	0.72	Bovine reactor
50	Positive	0.083	3.334	0.874	1.68	0.806	Bovine reactor
55	Positive	0.03	1.184	NS	NS	0	Bovine reactor
63	Positive	0.034	2.866	2.117	2.282	0.165	Bovine reactor
65	Positive	0.129	3.286	2.428	3.399	0.971	Bovine reactor
67	Positive	0.028	3.337	0.135	1.264	1.129	Bovine reactor
74	Positive	0.151	2.307	0.408	0.795	0.387	Bovine reactor
75	Positive	0.043	2.529	0.11	2.715	2.605	Bovine reactor

77	Positive	0.035	1.005	0.154	0.761	0.607	Bovine reactor
86	Positive	0.065	2.834	0.145	1.045	0.9	Bovine reactor
97	Positive	0.078	2.019	0.151	2.61	2.459	Bovine reactor
99	Positive	0.03	3.502	0.065	0.63	0.565	Bovine reactor
119	Positive	NS	0.475	1.127	0.453	-0.674	Avian reactor
122	Positive	0.008	2.449	0.815	2.405	1.59	Bovine reactor
124	Positive	0.067	3.065	0.234	1.348	1.114	Bovine reactor
125	Positive	0.178	2.903	0.405	1.5	1.095	Bovine reactor
128	Positive	0.064	1.797	0.339	1.472	1.133	Bovine reactor
131	Positive	0.037	1.154	0.733	2.632	1.899	Bovine reactor

PWM - Pokeweed Mitogen; PPDa - PPD avian; PPDb - PPD bovine.; NS - No sample

Appendix C

Skin fold thickness increases at different concentrations and locations

ID	Side of Neck	ISBT-1	ISBT-2	CA-1	CA-2	CB-1	CB-2
124	L	-0.5	4.0			5.2	3.5
	R	3.8	2.8	4.5	3.9		
74	L	0.3	2.2			4.4	1.5
	R	3.2	0.9	2.9	3.2		
09	L	5.2	2.4			4.0	3.1
	R	3.2	0.5	2.7	4.3		
12	L	3.3	5.6			4.8	5.5
	R	4.5	4.6	5.0	5.5		
125	L	0.7	4.5	0.7		17.0	
	R	3.6	6.3		5.6		3.2
67	L	7.2	8.4	1.2		12.7	
	R	5.6	10		10.8		6.3
75	L	3.1	0.7	0.7		1.9	
	R	0.5	0.9		0.7		0.2
20	L	7.2	2.4	3.9		2.9	
	R	5.6	2.0		2.4		0.2
128	L	-0.1	9.8			13.6	16.9
	R	7.0	8.8	11.7	10.3		
77	L	0.5	1.4			3.5	1.9
	R	1.6	-0.1	2.0	3.1		
86	L	2.3	2.6			1.5	3.9
	R	0.8	3.8	3.0	2.8		
131	L	0.3	5.5			9.3	7.0
	R	6.7	0.9	5.9	12.4		
24	L	6.8	2.4			6.4	5.0
	R	4.8	7.5	3.5	6.9		
97	L	7.4	1.1			6.4	8.9
	R	6.6	11.8	5.2	11.7		
32	L	-0.3	2.0			-0.7	0.4
	R	0.3	0.5	1.5	-0.6		
33	L	2.1	3.8			3.2	3.8
	R	3.1	3.5	1.7	3.9		
43	L	1.9	3.9			2.0	0.5
	R	1.3	3.4	1.9	2.9		
50	L	0.8	3.8			2.8	0.2
	R	1.7	3.2	1.5	2.9		
38	L	2.9	4.9			4.5	0.7
	R	5.1	4.1	2.9	5.0		
99	L	2.9	4.9			5.0	0.1
	R	5.1	5.6	2.2	5.6		
63	L	6.0	1.5	0.4	1.7		
	R	2.6	4.8			2.6	5.7
55	L	6.0	3.0	5.9	2.9		
	R	2.4	5.1			2.6	4.4
122	L	5.9	8.4			6.9	0.2
	R	5.7	4.6	4.6	8.6		
65	L	2.7	4.5			3.2	0.1
	R	2.3	3.0	1.2	4.0		

Appendix D

Intradermal tuberculin test outcomes using standard and candidate bovine tuberculin[3]

Animal ID	ISBT-1	ISBT-2	CB-1	CB-2	CA -1	CA-2	Bovigam results
9	1	0	1	0	0	1	1
12	1	1	1	1	1	1	1
20	1	0	0	1	0	1	1
24	1	1	1	1	1	1	1
32	0	0	0	0	0	0	1
33	0	0	1	0	0	1	1
38	1	1	1	0	1	1	1
43	0	0	1	0	0	0	1
50	1	1	0	0	1	1	1
55	1	1	1	1	1	1	1
63	1	1	1	1	0	1	1
65	0	1	0	0	0	1	1
67	1	1	1	1	0	1	1
74	1	0	1	1	0	1	1
75	0	0	0	0	0	0	1
77	0	0	1	0	0	0	1
86	1	1	0	1	0	0	1
97	1	1	1	1	1	1	1
99	1	1	1	0	1	1	1
122	1	1	1	0	1	1	1
124	1	1	1	1	1	1	1
125	1	1	1	1	0	1	1
128	1	1	1	1	1	1	1
131	1	1	1	1	1	1	1

[3] Where the variables, '0' represents the negative test outcomes and '1' positive test outcomes

Appendix E

Two by two tables for test agreement calculation[4]

(a)		Bovigam	Bovigam	
		0	1	
ISBT-2	0	0	8	8
ISBT-2	1	0	16	16
		0	24	24
Percentage of agreement=66.67%				

(b)		Bovigam	Bovigam	
		0	1	
CA-2	0	0	5	5
CA-2	1	0	19	19
		0	24	24
Percentage of agreement=79.16%				

(c)		Bovigam	Bovigam	
		0	1	
CB-2	0	0	11	11
CB-2	1	0	13	13
		0	24	24
Percentage of agreement=54.16%				

(d)		CA-2	CA-2	
		0	1	
ISBT-2	0	4	4	5
ISBT-2	1	1	15	17
		5	19	24
Percentage of agreement=79.16%				

(e)		CB-2	CB-2	
		0	1	
ISBT-2	0	6	2	8
ISBT-2	1	5	11	16
		11	13	24
Percentage of agreement=70.83%				

(f)		ISBT-1	ISBT-1	
		0	1	
ISBT-2	0	5	3	8
ISBT-2	1	1	15	16
		6	18	
Percentage of agreement= 83.33%				

(g)		Bovigam	Bovigam	
		0	1	
ISBT-1	0	0	6	6
ISBT-1	1	0	18	18
		0	24	24

75.00%

[4] Where the variables, '0' represents the negative test outcomes and '1' positive test outcomes

Appendix F

Animal Ethics Committee Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

5 March 2020

Approval Certificate
New Application

AEC Reference No.:	REC230-19
Title:	Evaluation of the performance of two candidate bovine tuberculins in the intradermal tuberculin test in South African cattle naturally infected with Mycobacterium bovis
Researcher:	Dr CP Sikalonzo
Student's Supervisor:	Prof AL Michel

Dear Dr CP Sikalonzo,

The **New Application** as supported by documents received between 2020-01-15 and 2020-02-24 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2020-02-24.

Please note the following about your ethics approval:

1. The use of species is approved:

Species and Samples	Number
Cattle	30
Blood collection	30 (10 ml each)

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-03-05.
3. Please remember to use your protocol number (REC230-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix G

Research Ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research Ethics Committee 20 January 2020

CONDITIONALLY APPROVAL

Ethics Reference No	**REC230-19**
Protocol Title	**Evaluation of the performance of two candidate bovine tuberculins in the intradermal tuberculin test in South African cattle naturally infected with Mycobacterium bovis**
Principal Investigator	**Dr CP Sikalonzo**
Supervisors	**Prof AL Michel**

Dear Dr CP Sikalonzo,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC230-19) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Conditionally approved (pending obtaining other relevant approvals)

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

Appendix H

Section 20 Approval

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Prof Anita Michel
Department Veterinary Tropical Diseases
Faculty of Veterinary Sciences
University of Pretoria

Email: Anita.michel@up.ac.za

Dear Prof. Michel,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your application, submitted on 12 February 2019, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study "*International Collaborative Study to evaluate candidate tuberculin in comparison with the current international standard*", refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;

3. Samples to be transported must be packaged in compliance with the Regulations of the National Road Traffic Act, 1996 (Act No 93 of 1996) or IATA requirements;
4. A veterinary import permit must be obtained prior to the importation of the candidate tuberculin and all conditions stated therein must be adhered to;
5. All blood samples must be destroyed at the end of the study;
6. This section 20 expires on 1 June 2019.

Title of research/study: "International Collaborative Study to evaluate candidate tuberculin in comparison with the current international standard."
Researcher (s): Prof Michel
Institution: Department Veterinary Tropical Diseases; Faculty of Veterinary Sciences
University of Pretoria
Your Ref./ Project Number: 12/11/1/1/6
Our ref Number:

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019 -04- 15

Appendix I

Section 21 Approval

SAHPRA
SOUTH AFRICAN
HEALTH PRODUCTS
REGULATORY AUTHORITY

REQUEST FOR THE USE OF AN UNREGISTERED MEDICINE IN TERMS OF SECTION 21 OF ACT 101 OF 1965

Tel (012) 842 7587
Fax

Enquiries: Dr A.T Sigobodhla
References: 26/2/2 (VCT/02/2019)
09 April 2019

Faculty of Veteinary Science
100 Old South Road
ONDERSTEPOORT
0110

Att: Prof. A. Michel
Tel: 012 529 8426
Fax: 012 529 8312
Email: anita.michel@up.ac.za

PERMISSION FOR USE OF UNREGISTERED MEDICINES IN TERMS OF THE PROVISIONS SECTION 21OF ACT 101 OF 1965.

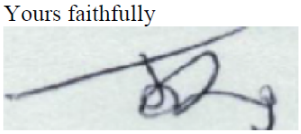
Your application refers:

Authorization is hereby granted for the import / purchase of the following unregistered product on condition that:

Name of product:	Bovine Tuberculin PPD x 60 ml
Species:	Bovine
Description of patients:	Cattle, 18 mnts, Female or Male
Diagnosis/purpose:	Bovine tuberculosis reactor animals
Authorisation number:	VCT/02/2019

Kindly note that this permit **allows you a once-off supply** of the imported product for use in the clinical trial. You are reminded to furnish this office with an interim report on the clinical trial.

Yours faithfully



For and on behalf of the CEO of SAHPRA