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A Metrological Contribution to the Diagnosis of Bovine Tuberculosis

R. A. Valdés[1*], A. Piratelli-Filho[2], H. L. Costa[1], A. M. C. L. Ribeiro[3], M. D. Nascimento[1] and P. M. Soares[3]

[1]Faculty of Mechanical Engineering, Federal University of Uberlândia, (UFU), Av. João Naves de Ávila, 2121, Campus Santa Mônica, 38400-902, Uberlândia, MG, Brazil.
[2]Department of mechanical Engineering, Faculty of Technology, University of Brasilia, Campus Universitário Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil.
[3]Federal University of Uberlândia (UFU), Faculty of Veterinary Medicine, Av. Pará, 1720, Campus Umuarama, Bloco 2T, 38400-902, Uberlândia, MG, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. In this paper, author RAV identified the metrological criteria to be used, proposed the mathematical model to calculate measurement uncertainty, analysed the results and wrote the first draft of the paper. Author HCL helped analysing the data and writing the manuscript. Author APF helped designing the metrological criteria and the mathematical model and also helped analyzing the data. Author AMCLR supervised the Comparative Cervical Tuberculin (CCT) tests. Author PMS helped carrying out the CCT tests. Author MDN helped the metrological tests. All authors have approved the final article as it is now submitted.

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**Corresponding author: E-mail: arvaldes@mecanica.ufu.br;*

ABSTRACT

The present paper aims to evaluate the actual relevance of the application of metrological criteria for the diagnosis of bovine tuberculosis using Comparative Cervical Tuberculin (CCT) inoculation tests. The present work involves the following steps: identification of the instruments used to measure skin thickness in tuberculin inoculation tests; calibration of the measurement instruments (callipers) using gauge blocks; identification of the variables that can affect the calibration results and the measurement results from inoculation tests; development of a methodology to evaluate the uncertainties associated with both the calliper calibration and with the measurements carried out during diagnosis; mathematical modelling of calliper calibration process and measurement process with the calliper; CCT tests performed in a total of 40 cattle comprising Nellore breed and mixed-breed dairy animals. To determine the effects of uncertainty on the test diagnosis, callipers with resolutions of 0.1 mm and 0.01 mm were compared. The results obtained showed that measurement uncertainty influences the final diagnosis. Therefore, the application of metrological criteria can increase scientific rigor and quality of the results obtained with CCT tests, and consequently, the reliability of the final diagnosis.

Keywords: Bovine tuberculosis; tuberculin inoculation; calibration; measurement uncertainty.

ABBREVIATIONS

***CCT**: Comparative Cervical Tuberculin, **CF**: Caudal Foldal Test, **GUM**: Guide to the Expression of Uncertainty in Measurement, **MAPA**: Brazilian Ministry for Agriculture and Livestock, **PNCEBT**: Brazilian National Program for Control and Eradication of Animal Brucellosis and Tuberculosis, **PPD**: Purified Protein Derivative, **SCT**: Single Cervical Tuberculin, **TB**: Tuberculosis, **ΔB**: Increase in skin fold thickness, A_0: Thickness of the skin fold measured before inoculation with avian, A_{72}:Thickness of the skin fold measured after inoculation with avian, B_0: Thickness before injection with bovine PPD tuberculin, B_{72}:Skin thickness 72 hours after inoculation, c_i: Sensitivity coefficient of the input variable i, **k**: Coverage factor, **L**: Calliper indication, L_{0i} : Mean indicated value at the point i, **M**: Variation in the skin fold thickness between the two inoculation tests, **n**: Number of readings, **P**: Probability of the variable assuming a standard value higher than the calculated z-score , **R**: Calliper resolution, **RP**: Reproducibility of the calliper, **s(L)**: Variability of the value indicated by the calliper at each point, **u**:Standard uncertainty, u_c: Combined standard uncertainty, **U**: Expanded uncertainty, UC_{GB}: Uncertainty associated with the gauge block calibration, UC_C: Uncertainty associated with the calliper calibration, UC_{Ci}: Uncertainty of the value obtained with the calliper at the point i during calibration, **CV:** Conventional value, **X**: Upper legislation limit, **z**: Score, **s**: Standard deviation, X_i: Measur and, x_i: Estimation of measure and, δT : Temperature variation during calibration, $\Delta\alpha$: Differential expansion between the materials of the calliper and of the gauge blocks, **ΔA**: Thickness variation before and after inoculation with avian PPD tuberculin, **ΔB:** Thickness variation before and after inoculation with bovine PPD tuberculin, $\Delta s(L)_i$: Correction associated with the variability of the value indicated by the calliper at the point i, **ΔR**: Correction associated with the calliper resolution, ΔIC_{GBi}: Correction associated with the gauge block calibration, **ΔT**: Difference between the calibration temperature and the reference temperature of 20ºC, **ΔRp**: Correction associated with the calliper reproducibility, ΔUC_c: Correction due to the uncertainty associated with the calliper calibration, v_{ef} : Effective degree of freedom.*

1. INTRODUCTION

Bovine tuberculosis (TB) caused by Mycobacterium bovis was first described in 14 A.D, but only with the discovery of the tubercle bacillus in 1882 by Robert Koch it started to be properly researched [1].

Bovine tuberculosis still poses serious risks to human health, since cattle to man infection is possible via milk and unpasteurized dairy products and via the respiratory route [2].

Even though the impact on human health is a strong determinant for initiating programs for the control of bovine tuberculosis, economic losses have also been recognized [3]. Bovine tuberculosis has significant consequences for farming economies throughout the world [4].

The economic costs of this zoonosis associated to farming include direct losses due to death, reduction in weight gain, reduction in milk production, premature slaughtering for control of the disease, loss of cattle with high zootechnical value, condemnation of carcasses during slaughtering, etc. [5].

When suitable control measures are not taken, the effects on economy and health evolve slowly and steadily, and sometimes the consequences can be dramatic [6]. They can include direct life losses, mainly due to miscarriages, low reproduction levels, increase of the interval between births, death of calves, and interruption of genetic lineages. The commercial value of infected rural properties and of their animals decreases. The regions and properties where the disease is endemic are in disadvantage when disputing new markets. Indirect losses include human contamination. If it is not treated in due time, the chronic development of the disease in humans leads to economic losses resulting from diagnosis and treatment costs, besides the costs associated with the time away from work during treatment [5].

In industrialized countries, programs for control and eradication of bovine tuberculosis, together with pasteurizing techniques and vaccination [7], have drastically reduced the incidence of infection by *Mycobacterium bovis* both in cattle and in humans. North America, Europe [8,9], Australia and New Zealand [10] have been more successful in controlling and eradicating bovine tuberculosis than Latin American countries [3,11] and other developing countries [12,13]. However, bovine tuberculosis remains a problem for countries both with and without control programs [14-16].

The diagnosis of bovine tuberculosis can be carried out using both direct and indirect methods. The direct methods involve the detection and identification of the infecting agent in biological samples [17,18], whereas the indirect methods investigate immune responses of individuals to the infecting agent. An example of an indirect method is the tuberculin inoculation test, which involves a cellular immune response against *Mycobacterium bovis* manifested as a delayed hypersensitisation reaction [2].

Diagnosis using tuberculin inoculation is fast, safe and relatively cheap [5]. The tuberculin tests are the internationally accepted standard and the most robust tool currently available for the diagnosis of infection by *Mycobacterium bovis* [19].

The use of tuberculin inoculation tests has drastically reduced bovine tuberculosis [14]. However, the infection of feral animals in preservation areas around farms makes the eradication of this disease from cattle herds difficult even in countries with successful

tuberculosis control [20-22]. Cattle-to-cattle transmission has also lead to a slight increase of bovine tuberculosis in some developed countries [16, 23].

Diagnosis using results of inoculation tests involve measurements of skin thickness before and after tuberculin inoculation using callipers. However, the majority of the documents with norms and specifications for using tuberculin inoculation tests in eradication programs do not mention either the technical characteristics of the calliper or the qualification of the staff involved in the measurements. For example, the national program for bovine tuberculosis eradication in Spain [24] only states that the callipers must be in good condition, whereas the use of callipers which are specific for tuberculin inoculation tests are the sole recommendation by the Brazilian national program for bovine tuberculosis eradication [5].

In 2006, a report was a produced for the Defra (UK) and the Welsh Assembly Government reviewing risks involved in bovine TB tests [19]. The report emphasizes the need for a methodical and well-defined test procedure in order to guarantee a reliable result for each animal. In particular, this report revealed that equipment used during TB screening tests, including callipers for skin thickness measurements, can incur in deviations of the final results. This probably occurs because this equipment has not been improved for decades. They suggest that some fresh ideas and professional considerations should be given to help manufacturers improve the design of equipment used in TB tests, including skin measurement. Also, although manuals generally specify all the procedures to be followed during tuberculin inoculation tests, it is not uncommon that personnel involved in the tests do not follow strictly all the recommendations. This behaviour was associated to various reasons: the use of difficult language in manuals, many cross-references and a general failure to consider the level of knowledge of the users when designing and writing the procedures may jeopardize the understanding of the procedures; rules are broken, because they are felt to be irrelevant or because people no longer appreciate the dangers, creating a culture that tolerates violations; lack of local resources; and insufficient procedural guidance or inexperienced staff.

In order to obtain valid results from skin thickness measurements for tuberculosis diagnosis, the measurement instrument (calliper) must be adequate in terms of accuracy and precision and must be traceable in terms of the international length standard (metre). Traceability includes the declaration of the uncertainty at all levels of the traceability chain, including for the measurement results [25]. According to ISO TAG 4/WG 3 [26], popularly known as GUM (Guide to the Expression of Uncertainty in Measurement), any measurement result must declare the reliability associated with the measurement, denominated measurement uncertainty.

Therefore, improvements in the design of the equipment and conformity with procedure regulations would not suffice to reduce deviations that occur in the results from tuberculin inoculation tests. Manuals must be improved to include recommendations related to: the need for calibration of all the equipment involved, aiming the traceability of the results and the reduction of errors; the calculation of measurement uncertainty; the consideration of measurement uncertainty to interpret the results; and the technical specification of the metrological parameters of the equipment, such as accuracy, precision and resolution.

The present paper aims to evaluate the actual relevance of the application of metrological criteria for the diagnosis of bovine tuberculosis using Comparative Cervical Tuberculin (CCT) inoculation tests. The criteria investigated in this study are: calibration of the calliper using gauge blocks; development of a methodology to evaluate the uncertainties associated with

both the calliper calibration and with the measurements carried out during diagnosis; discussion of the effects of uncertainty on the test diagnosis; and comparison of results obtained using callipers with resolutions of 0.1 mm and 0.01 mm.

2. THEORETICAL BACKGROUND

A simple methodology to diagnose bovine tuberculosis involves the intradermal injection of tuberculin and assessment of the test site. In most cattle infected with *Mycobacterium bovis*, this will cause the immune system of the animal to react to the tuberculin and cause a localised allergic reaction (swelling) of the skin a few days after the injection. The presence of induration or swelling, or the measurement of these reactions in millimetres, is carried out at 72 (±6) hours following the injection. A variety of test methods have been used over the years, but they are classically described as a delayed-type hypersensitivity response, relying on the individual response in vivo of the animal to the injection. Estimates of the sensitivity of tuberculin tests range from 68% to 95% while specificity is estimated to be between 96% to 99% [27].

Although tuberculin was first produced by Robert Koch in 1890, Purified Protein Derivative (PPD) tuberculin was developed in 1934 by Seibert. PPD tuberculins, despite being commonly described as "pure", are complex mixtures of proteins, lipids, sugars and nucleic acids including a great variety of antigens, many of which are common to several mycobacterial species [27]. In Brazil, bovine PPD tuberculin is produced from *Mycobacterium bovis* AN5, containing 1 mg of protein per ml (32.500 IU) and avian PPD tuberculin is produced from *Mycobacterium avium* D4, containing 0.5 mg of protein per ml (25.000 IU) [5].

The Brazilian National Program for Control and Eradication of Animal Brucellosis and Tuberculosis (PNCEBT) presents three test methods that involve tuberculin inoculation: i) the caudal fold test; ii) the single cervical test, and iii) the comparative cervical test [5].

The Caudal Foldal (CF) Test is mainly used in North America, Australia and New Zealand [27]. In this test, a 0.1 ml dose of bovine tuberculin PPD is injected intradermally at the centre of the caudal fold approximately 6 cm to 10 cm distal to the base of the tail.

Reading of the test is by palpation of the injection site at 72 hours post injection. Cattle are classified as negative when there is no detectable response at the injection site. Any increase in the thickness of the caudal fold at the injection site result in an animal being classified as either "suspect" or "reactor".

The Single Cervical Tuberculin (SCT) test is carried out in the skin of the neck using bovine tuberculin. It is the main screening test used in most countries of the European Union [27] and is also largely used in Brazil [5].

During SCT tests, intradermal injection of 0.1 ml of approved bovine tuberculin is made at the junction of the anterior and middle thirds of the neck. The interpretation of reactions is based on clinical observations and records of the increase in skin fold thickness at the site of injection 72 hours later.

(Table 1) summarizes reference values used to interpret clinical observations and thickness measurements and therefore to diagnose the animal [5].

Table 1. Reference values for the interpretation of results obtained with SCT tests, *ΔB* is the increase in skin fold thickness at the injection site [5]

ΔB (mm)	Pain sensitivity	Consistency	Other interpretations	Diagnosis
0 to 1.9	–	–	–	negative
2.0 to 3.9	Some pain	endured	delimited	inconclusive
2.0 to 3.9	Intense pain	soft	exudation, necrosis	positive
≥4.0	–	–	–	positive

Therefore, the test involves two measurements of the skin fold thickness at the inoculation site. The thickness measured immediately before injection with bovine PPD tuberculin (B_0) and a second measurement of the skin fold thickness, carried out 72 hours after inoculation (B_{72}). The increase in skin fold thickness at the injection site (ΔB) is calculated using Eq. (1) as the difference in thickness due to PPD tuberculin inoculation.

$$\Delta B = B_{72} - B_0 \quad (1)$$

Cattle are sometimes infected with other types of mycobacteria which may cause the animal to react to the test. In order to distinguish between animals infected with *Mycobacterium bovis* and those infected by other mycobacteria, another test called Comparative Cervical Tuberculin (CCT) also involves the injection with tuberculin produced from *Mycobacterium avium*, an organism that can cause tuberculosis in birds. The size and nature of the reactions to both tuberculins (avian and bovine) is compared to determine whether the test result is considered positive, negative or inconclusive.

The CCT test is a confirmatory test to be used in animals that reacted in either CF tests or in SCT tests. The thickness of the skin fold is measured using callipers before (A_0 and B_0) and after inoculation with avian (A_{72}) and bovine PPD tuberculin (B_{72}). The increase in skin fold thickness due to avian (ΔA) tuberculin inoculation is then calculated as:

$$\Delta A = A_{72} - A_0 \quad (2)$$

A comparison of the values obtained with Eq. (1) and Eq. (2) is then carried out with reference values Table 2, in order to obtain a final diagnosis.

Table 2. Reference data for tuberculosis diagnosis using comparative cervical tests, *ΔB* is the increase in skin fold thickness due to bovine inoculation and *ΔA*is the increase in skin fold thickness due to avian inoculation [5]

	ΔB- ΔA (mm)	Diagnosis
$\Delta B<2.0$	–	Negative
$\Delta B<\Delta A$	<0	Negative
$\Delta B\geq\Delta A$	0.0 to 1.9	Negative
$\Delta B>\Delta A$	2.0 to 3.9	Inconclusive
$\Delta B>\Delta A$	≥4.0	Positive

The results of diagnosis carried out using SCT and CCT tests depend on the values obtained with the calliper. Therefore, the scientific rigor of the diagnosis depends on the quality of the measurements. Some uncertainty will always exist in relation to how correctly the measurement result represents the value being measured, *i.e.*, the measurement result

is only an approximation or estimative of measure and value. Many factors can influence the measurement quality, so that when measurement results are presented, some quantitative indication of the measurement quality must always be provided. This allows users of such results to evaluate their reliability. Measurement results cannot be compared without some indication of the measurement quality, either between themselves or with a reference value [26].

Measurement uncertainty is defined as a non-negative value that characterizes the dispersion of the values that can be attributed to the measure and, based on the used information. The methodology proposed by ISO TAG 4/ WG 3 [26] can be used to evaluate measurement uncertainty. However, this methodology does not substitute critical thinking, intellectual honesty and professional ability. The evaluation of measurement uncertainty is neither a routine task nor a purely mathematical task. It depends on a detailed knowledge about both the measure and nature and the measurement. The quality and usefulness of the uncertainty indicated for a result depend on knowledge, critical thinking and honesty of those involved in finding the uncertainty value.

The evaluation of the measurement uncertainty is particularly useful for decision making [28]. When maximum or minimum tolerance limits exist for the measure and, dictated, for example, by some legislation, uncertainty becomes essential for a correct interpretation of the measurement result. Weckenmann et al. [29] have graphically represented how measurement uncertainty can affect the established limits, reducing the conformance zone.

The authors show that all zones are affected by the expanded uncertainty value associated with the measurement. The expanded uncertainty is distributed around the limit values, generating ranges where no analysis can be obtained without risk.

The probability of the measure and value being above the maximum value allowed by specification (legislation) can be evaluated taking into account the uncertainty measurement. For that, first the variable is transformed into a *z*-score:

$$z = \frac{(X - x_i)}{u_c(y)} \tag{3}$$

Where X is the upper legislation limit, x_i is the measurement result and $u_c(y)$ is the value of the combined standard uncertainty, which is equivalent to a dispersion measurement of a standard deviation, obtained by U/k, where U is the expanded uncertainty and k is the coverage factor.

In sequence, the probability of the variable assuming a standard value higher than the calculated *z*-score is defined:

$$P(X > z) = 1 - P(X \leq z) \tag{4}$$

This type of information allows users to evaluate and define an acceptable risk during decision making. When a user of the measurement decides to approve a sample, he or she will know the risk of making the wrong decision, i.e., approving a sample that should be rejected. This concept of risk evaluation, which requires the knowledge of the measurement uncertainty, can be extended to various situations. Therefore, when uncertainty is not

evaluated and expressed properly, the interpretation of the results can be jeopardized, leading to errors.

3. MATERIALS AND METHODS

Comparative Cervical Tuberculin (CCT) tests were carried out in a total of 40 cattle comprising Nellore breed and mixed-breed dairy animals. The tested animals were from the Glory Experimental Farm of Federal University of Uberlândia, located in Uberlândia, MG. All animals, male and female sex, with age equal or superior to six week were tested. The tests were carried out in the morning, at environment temperature ranging from 22℃ a 28℃. In this farm, the animals are kept in pasture continuous stocking with approximately 1 hectare (ha) and the number of animals ranges from 30 to 40 animals, according to the accessibility of the trough and size of the animals. Therefore, it is estimated an area of 250 m^2 for each animal. The nutrition of the animals is performed by providing feed in the trough once a day approximately 1 kg for animal. During the dry season, silage is added in their food. The source of water comes from artesian post in shaded and cooler near the trough.

First, hair was shaved around the two injection sites located on the same side of the cervical area of each animal (Fig. 1a). A skin foldat both sites was measured with callipers (Fig. 1b). Readings using the analogic calliper combine a fixed scale and a moving scale. A trigger and combined with a spring system ensure the application of a constant measuring force. The spring system is responsible for returning the moving measuring surface, which makes manipulation by users easy and comfortable. A screw in the upper region of the instrument support allows to fix the moving measuring base in the correct position. A dial system facilitates readings during the tests.

Small amounts (0.1 ml) of bovine PPD tuberculin and of avian PPD tuberculin were injected at room temperature into the shaved skin using 22 G x 3 mm multi-dose syringes at two different sites separated by a distance between 15 mm and 20 mm.

Fig. 1. CCT test: (a) Inoculation of the bovine PPD tuberculin; (b) measurement of the skin fold with callipers, 72 hours after inoculation [5]

The PPD tuberculins were used according to the regulations by the Brazilian Ministry for Agriculture and Livestock (MAPA). They were stored at temperatures between 2℃ and 8℃, protected from direct sunlight and, after opening, bottles were completely used within 24 hours.

After 72 hours, the animal identity was checked, the skin folds at both sites were measured with the same calliper and the thickness of the skin fold was recorded.

For the measurements, an analog calliper (Fig. 2), manufacturer SUPRIVET, located in Divinopolis, MG, Brazil (http://www.suprivet.com.br/), with a resolution of 0.1 mm and a nominal range of 40 mm, was used.

3.1 Calibration of the Calliper

Initially, the calliper was calibrated using a box of steel gauge blocks (Fig. 2), model Starrett, with calibration certificate n.1505/11 issued in July 2011 by LAROY S. STARRETT Metrology Laboratory (LAROYLAB), located in Itú, SP, Brazil, (http://www.inmetro.gov.br/laboratorios/rbc/detalhe_laboratorio.asp?num_certificado=87&area=DIMENSIONAL).

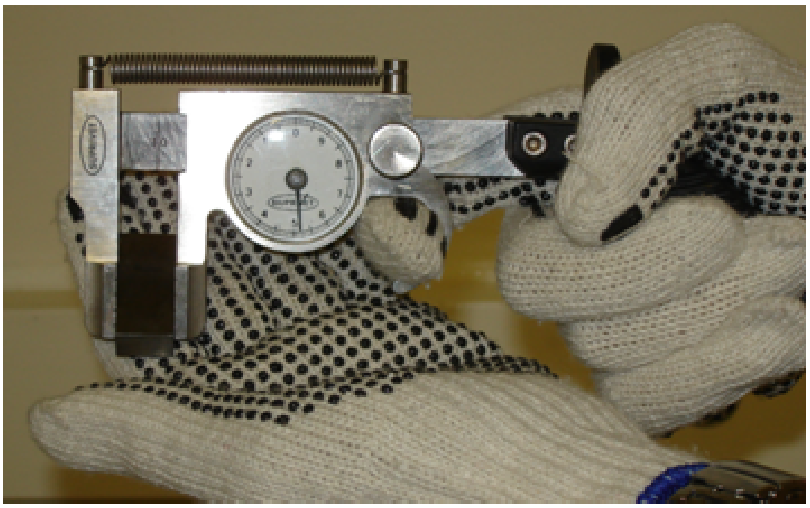

Fig. 2. Analog calliper during the calibration process

Calibration was carried out in a metrology laboratory at a controlled temperature of (20±1)℃, according to recommendations by NM-ISO 1 (Standard Reference Temperature for Industrial Length Measurements) [30]. During calibration, temperature was monitored using a digital thermo-hygrometer with a resolution of 0.1℃ and a nominal range of -20 to 60℃. Calibration in discrete points within the measurement range used gauge blocks with the following lengths: 5.1 mm, 15.0 mm, 22.8 mm, 25.0 mm, and 35.3 mm. The zero point was also calibrated. Five measurement cycles allowed the estimation of the arithmetic mean and of the standard deviation for each point, in order to obtain the error curve for the calliper.

3.2 Evaluation of the Uncertainty Associated with the Calibration of the Calliper using Gauge Blocks

The uncertainty was evaluated according to recommendations from ISO TAG 4 WG/3 (Guide to the Expression of Uncertainty in Measurement) [26]. Initially, the variables that could affect the calibration result were identified: *i)* variability of the value indicated by the calliper at each point $s(L)_i$; *ii)* calliper resolution (R); *iii)* uncertainty associated with the gauge block calibration (UC_{GB}); *iv)* difference between the measurement temperature and the reference temperature of 20℃ (ΔT); and *v)* temperature variation during the measurements (δT).

A mathematical model was proposed to assess the uncertainty associated with each evaluated point, which results from the algebraic sum of the corrections associated with the identified variables:

$$C_{C_i} = \Delta s(L)_i + \Delta R + \Delta UC_{GBi} + L_{0i}\Delta\alpha\Delta T + L_{0i}\Delta\alpha\delta T \quad (5)$$

Where:

C_{C_i} – Value obtained with the calliper at the point *i* during calibration;
$\Delta s(L)_i$ Correction associated with the variability of the value indicated by the calliper at the point *i*;
ΔR - Correction associated with the calliper resolution;
ΔUC_{GBi} - Correction associated with the gauge block calibration;
ΔT– Difference between the calibration temperature and the reference temperature of 20°C;
δT - Temperature variation during calibration;
L_{0i} - Mean indicated value at the point *i*;
$\Delta\alpha$ - Differential expansion between the materials of the calliper and of the gauge blocks.

3.3 Evaluation Associated With the Measurement of the Skin Fold

The mathematical model for the estimation of the uncertainty associated with the measurement of the skin fold is given by:

$$M = \Delta B - \Delta A \quad (6)$$

Where *M* represents the variation in the skin fold thickness between the two inoculation tests; ΔA is the thickness variation before and after inoculation with avian PPD tuberculin; and ΔB is the thickness variation before and after inoculation with bovine PPD tuberculin.

The variation of the measurement of the skin fold thickness due to bovine PPD inoculation is given by Eq. (1), as the difference between the measurement of the skin fold thickness 72 hours after inoculation (B_{72}) and the measurement of the skin fold thickness before inoculation (B_0). Similarly, the variation of the measurement of the skin fold thickness due to avian PPD inoculation is given by Eq. (2) as the difference between the measurement of the skin fold thickness 72 hours after inoculation (A_{72}) and the measurement of the skin fold thickness before inoculation (A_0).

In this case, the uncertainty associated with the variation of the skin fold thickness between the tests with bovine and avian inoculation depends on the uncertainties associated with the measurements of A_0, A_{72}, B_0 and B_{72}. Since those variables were obtained using the same measurement system, they can be considered correlated variables. Therefore, the mathematical model to evaluate uncertainty is given by Eq. (7).

$$M = \Delta B - \Delta A = (B_{72} - B_0) - (A_{72} - A_0) \quad (7)$$

The variables that can contribute to the uncertainty during the measurements of A_0, A_{72}, B_0 and B_{72} were identified as: *i)* reproducibility of the calliper (*Rp*), ii) resolution of the calliper (*R*), and (iii) uncertainty associated with the calliper calibration (UC_C). In this study reproducibility condition of measurement is a set of conditions that includes different locations, operators and replicate measurements on the same objects.

The variables that contribute to the uncertainty to determine A_0 are shown in Eq. (8), where ΔRp is the correction associated with the calliper reproducibility, ΔR represents the correction due to the calliper resolution, and ΔUC_C is the correction due to the uncertainty associated with the calliper calibration.

$$A_0 = \Delta Rp + \Delta R + \Delta UC_C \tag{8}$$

The mathematical model presented in Eq. (8) can also be used to evaluate the uncertainty associated with the measurement of A_{72}, B_0 and B_{72}. It must be pointed out that for the determination of the numerical value of measurement uncertainty, the factors that influence the measurements are the same.

Most measurement processes involve various readings of the same measure and under similar conditions in order to allow statistical treatment of the data, detection of possible gross errors, and evaluation of the uncertainty measurement. However, in the case of the tuberculin inoculation tests, repetition of the readings is almost impossible, since the inoculated site generally becomes sore.

In this case, to calculate the standard uncertainty associated with the variability of the readings, 30 measurements were carried out under reproducibility conditions. So, uncertainty can be evaluated with a Type A evaluation using a normal distribution and *n-1* degrees of freedom, as shown in Eq. (9).

$$u(\Delta Rp) = \frac{Rp}{\sqrt{n}} \tag{9}$$

Where *n* is the number of readings.

In relation to the calliper resolution, a Type B evaluation can be applied using a rectangular distribution and an infinite number of degrees of freedom, Eq. (10).

$$u(\Delta R) = \frac{Resolution}{\sqrt{3}} \tag{10}$$

The standard uncertainty associated with the calliper calibration ($u(\Delta IC)$) can be obtained by dividing the extended uncertainty (*U*) declared in the calibration certificate by the coverage factor (*k*), Eq. (11).

$$u(\Delta IC) = \frac{U(Calibration)}{k} \tag{11}$$

In this case, a Type B evaluation is applied using a normal probability distribution. The number of degrees of freedom can be determined using a *t*-student distribution table for the coverage factor *(k)* and the coverage probability, declared in the calibration certificate.

After the calculation of all standard uncertainties, the combined standard uncertainty (u_c) can be estimated. For that, the law of propagation of uncertainty is applied to the initial mathematical model, as shown in Eq. (12). In this equation, all the partial derivatives (sensitivity coefficients) assume unitary values.

$$u_c^2(A_0) = \left(\frac{\partial A_0}{\partial \Delta Rp}\right)^2 \cdot u^2(\Delta Rp) + \left(\frac{\partial A_0}{\partial \Delta R}\right)^2 \cdot u^2(\Delta R) + \left(\frac{\partial A_0}{\partial \Delta UC}\right)^2 \cdot u^2(\Delta UC) \quad (12)$$

Equation (12) also allows the evaluation of the combined standard uncertainty associated with the measurements of A_{72}, B_0 and B_{72}.

To calculate the expanded uncertainty *U*, the combined standard uncertainty was multiplied by a coverage factor *k,* obtained from the *t*-student table according to the measurement effective degree of freedom ν_{ef}, in order to increase the coverage probability to 95%, as shown in Eq. (13). The measurement effective degree of freedom ν_{ef} is obtained from the Welch-Satterwaite expression, Eq. (14), where c_i is the sensitivity coefficient of the input variable *i*.

$$U = k.u_c \quad (13)$$

$$\nu_{ef} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{u^4(y_i)}{\nu_i}} = \frac{u_c^4(y)}{\sum_{i=1}^{N} \frac{(u(x_i)\cdot c_i)^4}{\nu_i}} \quad (14)$$

4. RESULTS AND DISCUSSION

4.1 Calibration of the Calliper

(Table 3) shows the values obtained during calibration of the calliper, where *CV* represents the length of the gauge block; *L1* to *L5* represent the readings and *s* is the experimental standard deviation. The table also presents arithmetic mean and bias (error).

The bias values are positive within the whole calliper nominal range, reaching 0.2 mm for the points 5.1 mm and 15.0 mm. Therefore, the measurement instrument tends to provide values higher than the measure and.

The uncertainty associated with the calliper calibration was then evaluated. From the calibration certificate for the gauge blocks, the expanded uncertainty associated with their calibration is 0.09 µm for k = 2.00 and a coverage probability of 95%. The values of expanded uncertainty for each point evaluated during calibration are shown in (Table 4), which evidences identical values of 0.2 mm for k = 2.00 and a coverage probability of 95%, for all the points evaluated during calibration.

Table 3. Results of the calliper calibration (mm)

CV	*L 1*	*L 2*	*L 3*	*L 4*	*L 5*	Mean	*s*	Bias
0.000	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0
5.100	5.3	5.3	5.3	5.3	5.3	5.3	0.02	0.2
15.000	15.2	15.2	15.2	15.2	15.3	15.2	0.02	0.2
22.800	22.9	22.9	23.0	23.0	23.0	22.9	0.03	0.1
35.300	35.5	35.4	35.4	35.4	35.4	35.4	0.02	0.1

Table 4. Combined standard uncertainty (u_c) and expanded uncertainty (U) for the points evaluated during calibration

	0 mm	5.1 mm	15.0 mm	22.8 mm	25.0 mm	35.3 mm
u_c(mm)	0.1	0.1	0.1	0.1	0.1	0.1
ν_{ef}	125	124	125	125	125	124
k	2	2	2	2	2	2
U(mm)	0.2	0.2	0.2	0.2	0.2	0.2

4.2 Skin Fold Thickness Measurements

The measurement results of the skin fold thickness after inoculation tests are summarized in (Table 5), where: A_0 is the skin fold thickness before inoculation with avian PPD; A_{72} is the skin fold thickness 72 hours after inoculation with avian PPD; ΔA is the thickness difference before and after inoculation with avian PPD; B_0 is the skin fold thickness before inoculation with bovine PPD; B_{72} is the skin fold thickness 72 hours after inoculation with bovine PPD; and ΔB is the thickness difference before and after inoculation with bovine PPD. In the last column, the difference between the results with each inoculation is presented.

Comparing the values in (Table 5), which do not consider measurement uncertainty, with the reference values shown in (Table 2), the CCT tests carried out for the 40 cattle identified 39 animals with skin fold thickness variation (ΔB-ΔA) below 2 mm, indicating negative diagnosis. One animal (animal 33) showed positive diagnosis, which requires measurements to be taken according to regulations [5].

4.3 Measurement Uncertainty

(Table 6) exemplifies the calculation of measurement uncertainty (coverage probability = 95%) associated with A_0 for Animal 1. Similar procedures can be extended for the calculation of measurement uncertainties associated with A_{72}, B_0 and B_{72}.

(Table 6) shows that for this animal, the expanded uncertainty for k = 2 and coverage probability of 95% associated with A_0 was 0.2 mm. This uncertainty value can be extended to the values of A_0, A_{72}, B_0 and B_{72} for all the animals, since the variables that influence each value are the same and assume the same values. If a larger value of coverage probability is desired, for example, 99%, the coverage factor is 3.36 and therefore the extended uncertainty becomes 0.3 mm.

Table 5. Results of the measurements of the skin fold thickness (mm) for inoculation tests using bovine (*ΔB*) and avian PPD tuberculin (*ΔA*)

Animal	A_0	A_{72}	$A_{72}-A_0=\Delta A$	B_0	B_{72}	$B_{72}-B_0=\Delta B$	$\Delta B-\Delta A$
1	6.1	9.9	3.8	4.6	8.5	3.9	0.1
2	7.6	11.5	3.9	8.9	11.6	2.7	-1.2
3	6.4	7.0	0.6	8.2	8.6	0.4	-0.2
4	6.5	8.0	1.5	8.0	8.7	0.7	-0.8
5	5.3	6.6	1.3	5.3	5.9	0.6	-0.7
6	6.2	6.4	0.2	6.2	6.4	0.2	0.0
7	9.5	9.1	-0.4	9.9	10.0	0.1	0.5
8	6.4	6.5	0.1	6.5	6.8	0.3	0.2
9	5.0	5.5	0.5	5.2	5.7	0.5	0.0
10	7.6	7.6	0.0	8.7	9.8	1.1	1.1
11	6.7	7.2	0.5	7.7	9.7	2.0	1.5
12	7.4	7.5	0.1	8.3	8.3	0.0	-0.1
13	6.3	6.4	0.1	8.9	9.0	0.1	0.0
14	10.0	10.0	0.0	9.0	9.5	0.5	0.5
15	6.4	10.2	3.8	7.8	10.0	2.2	-1.6
16	7.6	9.0	1.4	6.8	7.8	1.0	-0.4
17	8.0	10.6	2.6	7.3	8.6	1.3	-1.3
18	8.5	10.1	1.6	8.7	9.5	0.8	-0.8
19	8.7	9.0	0.3	8.3	10.5	2.2	1.9
20	8.2	12.3	4.1	8.2	9.9	1.7	-2.4
21	7.3	8.5	1.2	7.1	8.0	0.9	-0.3
22	7.6	10.6	3.0	6.1	7.5	1.4	-1.6
23	8.1	9.5	1.4	6.4	7.5	1.1	-0.3
24	7.6	8.2	0.6	7.6	8.0	0.4	-0.2
25	7.4	7.5	0.1	7.0	7.4	0.4	0.3
26	8.6	9.5	0.9	7.3	9.4	2.1	1.2
27	7.6	7.6	0.0	7.3	7.7	0.4	0.4
28	7.6	7.8	0.2	7.1	8.2	1.1	0.9
29	6.0	8.5	2.5	6.5	8.5	2.0	-0.5
30	7.3	7.6	0.3	7.0	8.0	1.0	0.7
31	7.1	10.0	2.9	7.1	8.5	1.4	-1.5
32	7.0	7.5	0.5	7.9	8.7	0.8	0.3
33	7.1	7.9	0.8	6.7	15.5	8.8	8.0
34	8.5	9.6	1.1	7.8	10.6	2.8	1.7
35	9.7	9.4	-0.3	7.3	8.7	1.4	1.7
36	7.2	7.6	0.4	7.6	8.1	0.5	0.1
37	7.0	7.2	0.2	6.5	7.4	0.9	0.7
38	8.1	8.8	0.7	8.5	10.2	1.7	1.0
39	8.0	8.5	0.5	8.2	10.0	1.8	1.3
40	7.6	8.2	0.6	6.3	7.4	1.1	0.5

The uncertainty associated with the calliper calibration is the variable with the strongest influence on the combined standard uncertainty and therefore on the expanded uncertainty. (Tables 7 and 8) present as exemplified, for Animal 1, the uncertainties associated with the variation in the skin foldthickness due to both avian PPD inoculation *ΔA* and to bovine PPD inoculation *ΔB* were calculated.

Table 6. Parameters for the calculation of measurement uncertainty associated with the variation in the skin fold thickness due to avian PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (x_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
ΔRp	0.058 mm	Normal	1	29	0.0106 mm
ΔR	0.1 mm	Rectangular	1	∞	0.0577 mm
ΔIC	0.19 mm	Normal	1	100	0.0850 mm
Combined standard uncertainty (u_c), in mm					0.1033
Effective degree of freedom (ν_{ef})					218
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.2

Table 7. Uncertainty associated with the variation in the skin fold thickness due to avian PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (x_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
A_0	9.6 mm	Normal	1	218	0.1033 mm
A_{72}	12.2 mm	Normal	1	218	0.1033 mm
Combined standard uncertainty (u_c), in mm					0.1461
Effective degree of freedom (ν_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.3

Table 8. Uncertainty associated with the variation in the skin fold thickness due to bovine PPD inoculation for a coverage probability of 95%

Measur and (X_i)	Estimation (x_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
B_0	8.6 mm	Normal	1	218	0.1033 mm
B_{72}	10.1 mm	Normal	1	218	0.1033 mm
Combined standard uncertainty (u_c), in mm					0.1461
Effective degree of freedom (ν_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.3

Finally, the uncertainty associated with the difference in skin fold thickness variation between the two inoculations *(ΔB-ΔA*) was calculated for a coverage probability of 95%, which is exemplified in (Table 9) for animal 1.

For *ΔA* and *ΔB* the expanded uncertainty was 0.3 mm, whereas the difference (*ΔB-ΔA)* presented an uncertainty of 0.4 mm, for k = 2.00 and coverage probability of 95%. These values can be extended to all tested animals.

Table 9. Uncertainty associated with the difference (ΔB - ΔA)

Measur and (X_i)	Estimation (x_i)	Probability distribution	Sensitivity coefficient	Degrees of freedom	Standard uncertainty
ΔA	2.6 mm	Normal	1	436	0.1461 mm
ΔB	1.5 mm	Normal	1	436	0.1461 mm
Combined standard uncertainty (u_c), in mm					0.2066
Effective degree of freedom (ν_{ef})					436
Coverage factor (95%)					k = 2.00
Expanded uncertainty (U), in mm					0.4

The values from (Table 5) can be compared again with the reference values in (Table 2), but now taking into account measurement uncertainty. Animals 34 and 35 presented values of (ΔB - ΔA) = 1.7 mm and for animal 19 this value was 1.9 mm. Without taking measurement uncertainty into account, these animals had been diagnosed as negatives. Considering the expanded uncertainty of 0.4 for k = 2.00 and coverage probability of 95%, they fall into the uncertainty zone. Using Eq. (3), it is possible to calculate that deciding for a negative diagnosis for animals 34 and 35 implies in a risk of 7% of taking the wrong decision, when in fact the result is inconclusive. For animal 19, the chance of surpassing the maximum limit allowed for a negative diagnosis is significantly higher, around 31%.

A value of expanded uncertainty associated with (ΔB - ΔA) of 0.4 mm can be considered excessively high, since it reduces the maximum limit allowed for a negative diagnosis in around 20%. Therefore, it is recommended the use of a calliper with a better resolution in order to reduce the uncertainty associated with the measurements.

As a comparative example, uncertainty values were obtained for a digital calliper with a resolution of 0.01 mm and a nominal range of 30 mm, manufacturer Agrozootec (Brazil), with calibration certificate n.1300/11 issued in February 2011 by QUALIMETRO metrology laboratory. The manufacturer is located in Itú, SP, Brazil (http://www.agrozootec.com.br/contatti.asp). The uncertainty associated with the calibration of the digital calliper was evaluated using the mathematical model given by Eq. (5). The only difference is that for the evaluation of the uncertainty associated with calibration and/or measurement using digital instruments or measuring systems, the resolution must be divided by two, since this is the maximum error expected during readings.

(Table 10) compares the results obtained using the methodology proposed in Eqs. (8-14), where Calliper A is the analog calliper, model SUPRIVET, with a resolution of 0.1 mm and Calliper B is the digital calliper, model Agrozootec, with a resolution of 0.01 mm.

The use of a calliper with better resolution reduced the expanded uncertainty associated with the result from 0.41 mm (Calliper A) to 0.32 mm (Calliper B), which represents a reduction of 22%. The standard uncertainty associated with the resolution reduced from 0.0577 mm (Calliper A) to 0.0190 mm (Calliper B). Reproducibility varied from 0.0106 mm (Calliper A) to 0.0015 mm (Calliper B). (Fig. 3) summarizes the effect of expanded uncertainty on the values established for the final diagnosis for both callipers.

(Fig. 3) evidences that a calliper with better resolution (0.01 mm) must have a better precision and therefore the uncertainty zone for diagnosis is reduced. Despite the availability in the market of callipers of a variety of models and resolutions generally varying from 0.1

mm to 0.01 mm, this work recommends the use of calibrated and traceable callipers with a resolution of 0.01 mm for tuberculin inoculation tests.

Table 10. Comparison of the uncertainty for both callipers

	Calliper A (mm)	Calliper B (mm)
$u(\Delta Rp)$	0.0106	0.0015
$u(\Delta R)$	0.0577	0.0029
$u(\Delta IC)$	0.0850	0.0800
$u_c(A_0)$	0.2066	0.0801
$u_c(A_{72})$	0.2066	0.0801
$u_c(B_0)$	0.2066	0.0801
$u_c(B_{72})$	0.2066	0.0801
$u_c(\Delta A)$	0.2922	0.1133
$u_c(\Delta B)$	0.2922	0.1133
$U(\Delta B-\Delta A)$	0.4132	0.3205

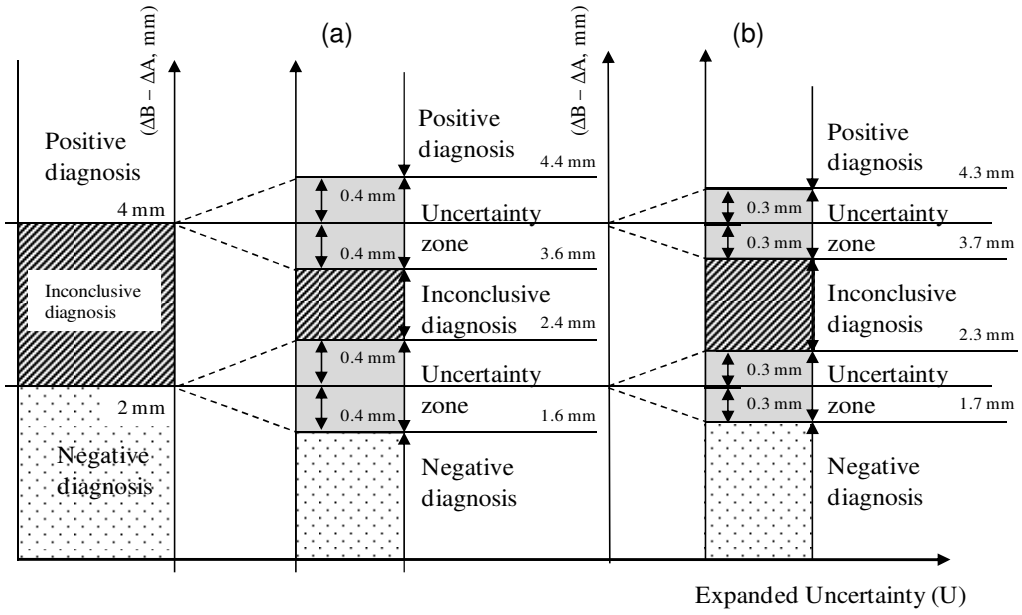

Fig. 3. Effect of the uncertainty on limit values for diagnosis; (a) Calliper A; (b) Calliper B

5. CONCLUSION

This work investigated metrological aspects associated with the diagnosis of bovine tuberculosis using tuberculin inoculation tests.

A methodology was applied to evaluate uncertainty of the measurements carried out during diagnosis in order to increase scientific rigor and reliability of the measurements, and therefore the quality of diagnosis obtained from tuberculin inoculation tests.

It was observed that when measurement uncertainty is used to interpret the results, the final diagnosis can change, so that animals that could be diagnosed as negatives should in fact have an inconclusive diagnosis.

The expanded uncertainty associated with the final result was 0.4 mm for an analog calliper with a resolution of 0.1 mm, but it was reduced to 0.32 mm (22%) when a digital calliper with a resolution of 0.01 mm was used.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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