

A Metrological Contribution to the Diagnosis of Bovine Tuberculosis

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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. For that, the work involved the following steps: identification of the measurement 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 in a total of 40 cattle comprising Nellore breed and mixed-breed dairy animals. The discussion of the effects of uncertainty on the test diagnosis compared callipers with resolutions of 0.1 mm and 0.01 mm. 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

1. INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium bovis*, also called bovine tuberculosis, 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].

Nowadays, 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]. 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 difficult the eradication of this disease from cattle herds 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 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 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 to be investigated 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 control and eradicate 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 % [30].

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 [30]. 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 [30]. 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 first is the thickness immediately before injection with bovine PPD tuberculin (B_0). The second measurement of the skin fold thickness is 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) and bovine (ΔB) tuberculin inoculation is then calculated as:

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

$$\Delta B = B_{72} - B_0 \quad (3)$$

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.

185 **Table 2. Reference data for tuberculosis diagnosis using Comparative Cervical Tests,**
186 **ΔB is the increase in skin fold thickness due to bovine inoculation and ΔA is the**
187 **increase in skin fold thickness due to avian inoculation [5].**
188

	$\Delta B - \Delta A$ (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

189
190 The results of diagnosis carried out using SCT and CCT tests depend on the values
191 obtained with the calliper. Therefore, the scientific rigor of the diagnosis depends on the
192 quality of the measurements. Some uncertainty will always exist in relation to how correctly
193 the measurement result represents the value being measured, *i.e.*, the measurement result
194 is only an approximation or estimative of measurand value. Many factors can influence the
195 measurement quality, so that when measurement results are presented, some quantitative
196 indication of the measurement quality must always be provided. This allows users of such
197 results to evaluate their reliability. Measurement results cannot be compared without some
198 indication of the measurement quality, either between themselves or with a reference value
199 [26].

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

209 The evaluation of the measurement uncertainty is particularly useful for decision making
210 [28]. When maximum or minimum tolerance limits exist for the measurand, dictated, for
211 example, by some legislation, uncertainty becomes essential for a correct interpretation of
212 the measurement result. Weckenmann *et. al.* [29] have graphically represented how
213 measurement uncertainty can affect the established limits, reducing the conformance zone.

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

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

$$220 \quad Z = \frac{(X - x_i)}{u_c(y)} \quad (4)$$

222
223 Where X is the upper legislation limit, x_i is the measurement result and $u_c(y)$ is the value of
224 the combined standard uncertainty, which is equivalent to a dispersion measurement of a
225 standard deviation, obtained by U/k , where U is the expanded uncertainty and k is the
226 coverage factor.

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

229

$$P(X > z) = 1 - P(X \leq z) \quad (5)$$

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.

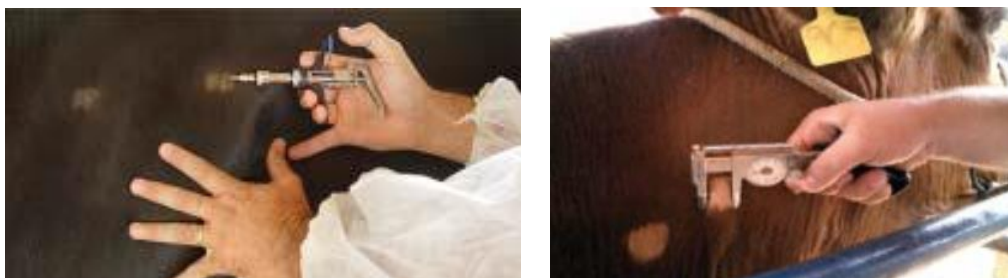
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241 3. MATERIAL AND METHODS

242

243 Comparative Cervical Tuberculin (CCT) tests were carried out in a total of 40 cattle
244 comprising Nellore breed and mixed-breed dairy animals. First, hair was shaved around the
245 two injection sites located on the same side of the cervical area of each animal (Figure 1a).
246 A skin fold at both sites was measured with callipers (Figure 1b). Small amounts (0.1 ml) of
247 bovine PPD tuberculin and of avian PPD tuberculin were injected at room temperature into
248 the shaved skin using 22 G x 3 mm multi-dose syringes at two different sites separated by a
249 distance between 15 mm and 20 mm.

250



251
252

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

255

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

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

262 For the measurements, an analog calliper model SUPRIVET (Figure 2) with a resolution of
263 0.1 mm and a nominal range of 40 mm was used.

264

265 3.1 Calibration of the Calliper

266

267 Initially, the calliper was calibrated using a box of steel gauge blocks (Figure 2), model
268 Starrett, with calibration certificate n.1505/11 issued in July 2011 by LAROY S. STARRETT
269 Metrology Laboratory (LAROYLAB).

270



Fig. 2. Analog calliper during the calibration process.

Calibration was carried out in a metrology laboratory at a controlled temperature of $(20 \pm 1) ^\circ\text{C}$, according to recommendations by NM-ISO 1 [30]. During calibration, temperature was monitored using a digital thermo-hygrometer with a resolution of $0.1 ^\circ\text{C}$ and a nominal range of -20 to $60 ^\circ\text{C}$.

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 Calliper Calibration

Uncertainty was evaluated according to recommendations from ISO TAG 4 WG/3 [26]. Initially, the variables that could affect the calibration result were identified: *i*) variability of the value indicated by the calliper at each point $s(R)$; *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 $20 ^\circ\text{C}$ (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:

$$C_{C_i} = \Delta s(L)_i + \Delta R + \Delta UC_{GBi} + L_{0i} \Delta \alpha \Delta T + L_{0i} \Delta \alpha \delta T \quad (6)$$

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 $20 ^\circ\text{C}$;

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:

$$M = \Delta B - \Delta A \quad (7)$$

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. (3), 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. (8).

$$M = \Delta B - \Delta A = (B_{72} - B_0) - (A_{72} - A_0) \quad (8)$$

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 caliper (Rp), ii) resolution of the calliper (R), and (iii) uncertainty associated with the calliper calibration (UC_C). The variables that contribute to the uncertainty to determine A_0 are shown in Eq. (9), 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.

$$U(A_0) = \Delta Rp + \Delta R + \Delta UC_C \quad (9)$$

Most measurement processes involve various readings of the same measurand 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. (10).

$$u(\Delta Rp) = \frac{Rp}{\sqrt{n}} \quad (10)$$

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. (11).

358

$$359 \quad u(\Delta R) = \frac{\text{Resolution}}{\sqrt{3}} \quad (11)$$

360

361 The standard uncertainty associated with the calliper calibration can be obtained by dividing
362 the extended uncertainty (U) declared in the calibration certificate by the coverage factor (k),
363 Eq. (12).
364

$$365 \quad u(\Delta C) = \frac{U(\text{Calibration})}{k} \quad (12)$$

366

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

370 After the calculation of all standard uncertainties, the combined standard uncertainty (u_c) can
371 be estimated. For that, the law of propagation of uncertainty is applied to the initial
372 mathematical model, as shown in Eq. (13). In this equation, all the partial derivatives
373 (sensitivity coefficients) assume unitary values.
374

$$375 \quad u_c^2(A_0) = \left(\frac{\partial A_0}{\partial \Delta R_p} \right)^2 u^2(\Delta R_p) + \left(\frac{\partial A_0}{\partial \Delta R} \right)^2 u^2(\Delta R) + \left(\frac{\partial A_0}{\partial \Delta UC} \right)^2 u^2(\Delta UC) \quad (13)$$

376

377 To calculate the expanded uncertainty U , the combined standard uncertainty was multiplied
378 by a coverage factor k , obtained from the t -student table according to the measurement
379 effective degree of freedom ν_{ef} , in order to increase the coverage probability to 95 %, as
380 shown in Eq. (14). The measurement effective degree of freedom ν_{ef} is obtained from the
381 Welch-Satterwaite expression, Eq. (15), where c_i is the sensitivity coefficient of the input
382 variable i .
383

$$384 \quad U = k \cdot u_c \quad (14)$$

385

$$386 \quad \nu_{ef} = \frac{u_c^4(y)}{\sum_{i=1}^N \frac{u^4(y_i)}{\nu_i}} = \frac{u_c^4(y)}{\sum_{i=1}^N \frac{(u(x_i) \cdot c_i)^4}{\nu_i}} \quad (15)$$

387

388 4. RESULTS AND DISCUSSION

389

390 4.1 Calibration of the Calliper

391

392 Table 3 shows the values obtained during calibration of the calliper, where CV represents
393 the length of the gauge block; $L1$ to $L5$ represent the readings and s is the experimental
394 standard deviation. The table also presents arithmetic mean and tendency (error).
395

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400

401 **Table 3. Results of the calliper calibration (mm)**

402

CV	L 1	L 2	L 3	L 4	L 5	Mean	s	Tendency
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0
5.1	5.3	5.3	5.3	5.3	5.3	5.3	0.02	0.2
15.0	15.2	15.2	15.2	15.2	15.3	15.2	0.02	0.2
22.8	22.9	22.9	23.0	23.0	23.0	22.9	0.03	0.1
35.3	35.5	35.4	35.4	35.4	35.4	35.4	0.02	0.1

403

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

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

413

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

416

	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

417

418 **4.2 Skin Fold Thickness Measurements**

419

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

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437 **Table 5. Results of the measurements of the skin fold thickness for inoculation tests**
438 **using bovine (ΔB) and avian PPD Tuberculin (ΔA).**
439

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

440
441 Comparing the values in Table 5, which do not consider measurement uncertainty, with the
442 reference values shown in Table 2, the CCT tests carried out for the 40 cattle identified 39
443 animals with skin fold thickness variation ($\Delta B-\Delta A$) below 2 mm, indicating negative

444 diagnosis. One animal (animal 33) showed positive diagnosis, which requires measurements
445 to be taken according to regulations [5].
446

447 **4.3 Measurement Uncertainty**
448

449 Table 6 exemplifies the calculation of measurement uncertainty (coverage probability =
450 95%) associated with A_0 for Animal 1. Similar procedures can be extended for the
451 calculation of measurement uncertainties associated with A_{72} , B_0 and B_{72} .
452

453 **Table 6. Parameters for the calculation of measurement uncertainty associated with**
454 **the variation in the skin fold thickness due to avian PPD inoculation for a coverage**
455 **probability of 95%.**
456

Measurand (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

457
458 Table 6 shows that for this animal, the expanded uncertainty for $k=2$ and coverage
459 probability of 95 % associated with A_0 was 0.2 mm. This uncertainty value can be extended
460 to the values of A_0 , A_{72} , B_0 and B_{72} for all the animals, since the variables that influence each
461 value are the same and assume the same values. If a larger value of coverage probability is
462 desired, for example, 99%, the coverage factor is 3.36 and therefore the extended
463 uncertainty becomes 0.3 mm.

464 The uncertainty associated with the calliper calibration is the variable with the strongest
465 influence on the combined standard uncertainty and therefore on the expanded uncertainty.
466 Table 7 and Table 8 present as exemplified, for Animal 1, the uncertainties associated with
467 the variation in the skin fold thickness due to both avian PPD inoculation ΔA and to bovine
468 PPD inoculation ΔB were calculated.
469

470 **Table 7. Uncertainty associated with the variation in the skin fold thickness due to**
471 **avian PPD inoculation for a coverage probability of 95 %.**
472

Measurand (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

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477 **Table 8. Uncertainty associated with the variation in the skin fold thickness due to**
 478 **bovine PPD inoculation for a coverage probability of 95 %.**
 479

Measurand (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

480
 481 Finally, the uncertainty associated with the difference in skin fold thickness variation between
 482 the two inoculations ($\Delta B - \Delta A$) was calculated for a coverage probability of 95 %, which is
 483 exemplified in Table 9 for animal 1.
 484 For ΔA and ΔB the expanded uncertainty was 0.3 mm, whereas the difference ($\Delta B - \Delta A$)
 485 presented an uncertainty of 0.4 mm, for $k = 2.00$ and coverage probability of 95 %. These
 486 values can be extended to all tested animals.

487
 488 **Table 9. Uncertainty associated with the difference ($\Delta B - \Delta A$).**
 489

Measurand (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

490
 491 The values from Table 5 can be compared again with the reference values in Table 2, but
 492 now taking into account measurement uncertainty. Animals 34 and 35 presented values of
 493 ($\Delta B - \Delta A$) = 1.7 mm and for animal 19 this value was 1.9 mm. Without taking measurement
 494 uncertainty into account, these animals had been diagnosed as negatives. Considering the
 495 expanded uncertainty of 0.4 for $k = 2.00$ and coverage probability of 95 %, they fall into the
 496 uncertainty zone. Using Eq. (4), it is possible to calculate that deciding for a negative
 497 diagnosis for animals 34 and 45 implies in a risk of 7% of taking the wrong decision, when in
 498 fact the result is inconclusive. For animal 19, the chance of surpassing the maximum limit
 499 allowed for a negative diagnosis is significantly higher, around 31 %.
 500 A value of expanded uncertainty associated with ($\Delta B - \Delta A$) of 0.4 mm can be considered
 501 excessively high, since it reduces the maximum limit allowed for a negative diagnosis in
 502 around 20 %. Therefore, it is recommended the use of a calliper with a better resolution in
 503 order to reduce the uncertainty associated with the measurements.
 504 As a comparative example, uncertainty values were obtained for a digital calliper with a
 505 resolution of 0.01 mm and a nominal range of 30 mm, model Agrozootec, with calibration
 506 certificate n.1300/11 issued in February 2011 by QUALIMETRO metrology laboratory. The
 507 uncertainty associated with the calibration of the digital calliper was evaluated using the
 508 mathematical model given by Eq. (6). The only difference is that for the evaluation of the
 509 uncertainty associated with calibration and/or measurement using digital instruments or
 510 measuring systems, the resolution must be divided by two, since this is the maximum error
 511 expected during readings.

Table 10 compares the results obtained using the methodology proposed in Eqs. (9-15), 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.

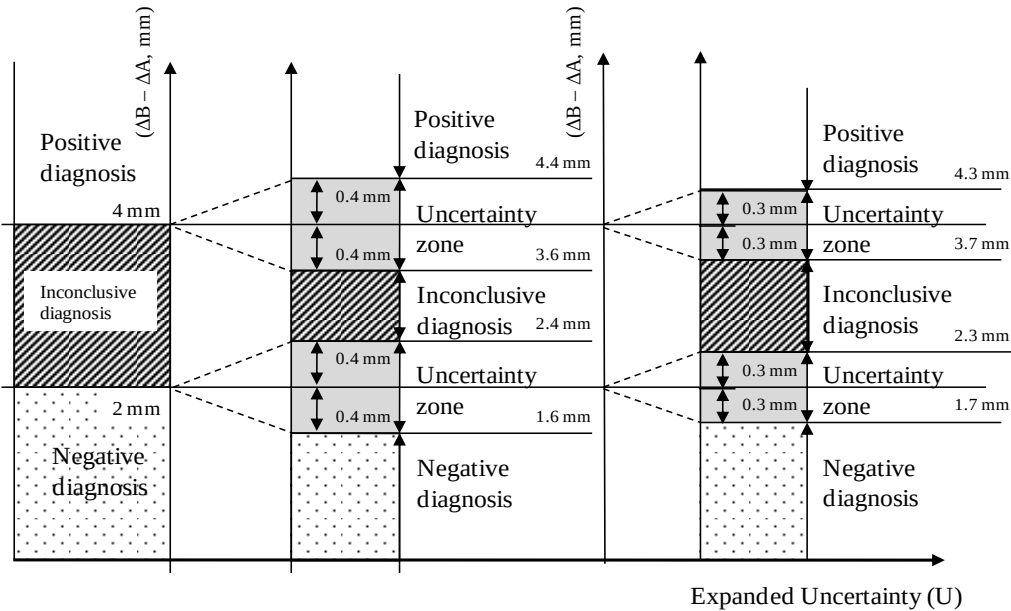
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Table 10. Comparison of the uncertainty for both callipers.

	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

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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 0.019 mm (Calliper B). Reproducibility varied from 0.0106 mm (Calliper A) to 0.0015 mm (Calliper B). Figure 3 summarizes the effect of expanded uncertainty on the values established for the final diagnosis for both callipers.



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

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Figure 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.

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.

AUTHORS' CONTRIBUTIONS

In this paper, Prof. Rosenda Valdes Arencibia, from the School of Mechanical Engineering, UFU, idealised the study, 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.

Prof. Henara Lillian Costa, from the School of Mechanical Engineering, UFU, helped analysing the data and writing the manuscript.

Prof. Antonio Piratelli-Filho, from the School of Mechanical Engineering, UNB, helped designing the metrological criteria and the mathematical model and also helped analysing the data.

Anna Monteiro Correia Lima Ribeiro Prof. from the School of Veterinary Medicine, UFU, supervised the Comparative Cervical Tuberculin (CCT) tests.

Pollyanna Mafra Soares, from the School of Veterinary Medicine, UFU, helped carrying out the CCT tests.

Murilo de Deus Nascimento, from the School of Mechanical Engineering, UFU, helped the metrological tests.

All authors have approved the final article as it is now submitted.

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DEFINITIONS, 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_{072} : 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

690 **R**: Calliper resolution
 691 **RP**: Reproducibility of the calliper
 692 **s(R)**: Variability of the value indicated by the calliper at each point
 693 **u**: Standard uncertainty
 694 **u_c**: Combined standard uncertainty
 695 **U**: Expanded uncertainty
 696 **UC_{GB}**: Uncertainty associated with the gauge block calibration
 697 **UC_c**: Uncertainty associated with the calliper calibration
 698 **UC_{ci}**: Uncertainty of the value obtained with the calliper at the point *i* during calibration
 699 **CV**: Conventional value
 700 **X**: Upper legislation limit
 701 **z**: Score
 702 **s**: Standard deviation
 703 **X_i**: Measurand
 704 **x_i**: Estimation of measurand
 705 **δT**: Temperature variation during calibration
 706 **Δα**: Differential expansion between the materials of the calliper and of the gauge blocks
 707 **ΔA**: Thickness variation before and after inoculation with avian PPD tuberculin
 708 **ΔB**: Thickness variation before and after inoculation with bovine PPD tuberculin
 709 **Δs(L)_i**: Correction associated with the variability of the value indicated by the calliper at the
 710 point *i*
 711 **ΔR**: Correction associated with the calliper resolution
 712 **ΔIC_{GBi}**: Correction associated with the gauge block calibration
 713 **ΔT**: Difference between the calibration temperature and the reference temperature of 20 °C
 714 **ΔRp**: Correction associated with the calliper reproducibility
 715 **ΔUC_c**: Correction due to the uncertainty associated with the calliper calibration
 716 **v_{ef}**: Effective degree of freedom