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Major Article

New strategy to improve quality control of Montenegro skin test at the production level

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Abstract

Introduction: The production of the Montenegro antigen for skin test poses difficulties regarding quality control. Here, we propose that certain animal models reproducing a similar immune response to humans may be used in the quality control of Montenegro antigen production. **Methods:** Fifteen *Cavia porcellus* (guinea pigs) were immunized with *Leishmania amazonensis* or *Leishmania braziliensis*, and, after 30 days, they were skin tested with standard Montenegro antigen. To validate *C. porcellus* as an animal model for skin tests, eighteen *Mesocricetus auratus* (hamsters) were infected with *L. amazonensis* or *L. braziliensis*, and, after 45 days, they were skin tested with standard Montenegro antigen. **Results:** *Cavia porcellus* immunized with *L. amazonensis* or *L. braziliensis*, and hamsters infected with the same species presented induration reactions when skin tested with standard Montenegro antigen 48-72h after the test. **Conclusions:** The comparison between immunization methods and immune response from the two animal species validated *C. porcellus* as a good model for Montenegro skin test, and the model showed strong potential as an *in vivo* model in the quality control of the production of Montenegro antigen.

Keywords: Cutaneous leishmaniasis diagnosis. Montenegro skin test. *Cavia porcellus.*

INTRODUCTION

Cutaneous leishmaniasis (CL) is a serious social and public health problem, because it can result in sequels[1]. Early diagnosis and a suitable treatment are the best tools to control the disease[1]. Immunological methods have been largely used as a screening tool for diagnosis[1,2]. One method widely used in Brazil is the Montenegro skin test (MST) based on the delayed-type hypersensitivity reaction (DTH)[2]. The *in vivo* manifestation of cellular immune response is an induration that can be measured and semi-quantified by skin tests[3]. The method has been widely used as a complementary leishmaniasis diagnosis because of its high sensitivity and specificity. Furthermore, MST is an easy method to perform, low cost, does not require sophisticated equipment, and can be performed *in loco*[4].

The Montenegro antigen available in Brazil is provided by the *Centro de Produção e Pesquisa de Imunobiológicos* (CPPI) in Paraná State, Southern Brazil. The production is authorized and inspected by the *Agência Nacional de Vigilância Sanitária* (ANVISA) that establishes the standard evaluation of internal testing for antigen production (RDC 59/2000; RDC 167/2004)[5-7]. During the production process, methods for the qualitative and quantitative control of the produced antigens are necessary to evaluate the antigen efficiency and to validate the lots of antigen[5-7]. Currently, this analysis is performed in *in vivo* systems that are exposed to the antigen in order to evaluate the biological response to exposure[5-7]. This control is performed in CL human patients[5]. Such approach demands clinical cases of CL and poses ethical questions, making the quality control of antigen a complicated process. This study aimed to address these issues in order to establish an experimental model capable to replace the current *in vivo* model in the quality control process of Montenegro antigen production.

METHODS

This study was divided in two stages. To evaluate the immune response of *Cavia porcellus* to standard Montenegro antigen in order to establish this species as an experimental model capable of replacing the current *in vivo* process used in the quality control of Montenegro antigen production. Secondly, to validate *C. porcellus* as a suitable animal model, *Mesocricetus auratus*, which is considered a susceptible bio model for infection with *Leishmania* sp.[8,9], was chosen.

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1. Evaluation of *Cavia porcellus* immune response to skin test

Fifteen adult male guinea pigs, albino strain, weighing 250-350g, were maintained in the animal facility, housed in polypropylene cages, and fed a balanced diet with water and food *ad libitum*. The animals were maintained in groups of five animals. The animal number for this study was calculated as follow: $N = (Z_{\alpha/2}\ ?\ (\delta/E))^{2}$ where $Z_{\alpha/2}$: 1.96; E: standard error (5%); δ: standard deviation = amplitude/4. The standard deviation was 9.5. Our aim was to minimize the number of animal used in the experiment while maintaining statistically relevant data (according to the 3 Rs concept)[10].

The experiments were conducted in four steps (**Figure 1**). The first step consisted in the preparation of *Leishmania* antigen to immunize *C. porcellus*. Promastigotes forms of *L. amazonensis* (MHOM/BR/73/M2269) and *L. braziliensis* (MHOM/BR/94/M2903) from the Centre de Ressources Biologiques des *Leishmania*, Montpellier, France, were cultivated until a concentration of 10^7 parasites/mL was reached. After washing three times with saline solution at 0.9, 0.3, and 0.9%, the pellet was stored at -20°C until further use. The soluble *Leishmania* antigens were prepared as described by Szargiki et al.[11]. Briefly, the promastigotes were defrosted and centrifuged at 4°C for 15 min at 800 x *g*. The pellet was diluted in sterile distilled water in a volume equal to half of the pellet volume. The cell suspension was disrupted by heat shock, alternating between -80°C and 50°C for 5 min, and further sonicated six times at 30% of potency at intervals of 1 min in an ice bath. The extracted solution was centrifuged at 14,000 x *g* at 4°C for 60 min and lyophilized. The antigen emulsions (*L. amazonensis* and *L. braziliensis*) were prepared by diluting 20.5mg of each lyophilized antigen in 5.1mL of sterile water, and 3.3mL of this solution was added to 3.3mL of incomplete Freund's adjuvant (IFA). The final concentration of the antigen emulsion was 4mg/mL. The guinea pigs were divided into three groups with five animals in each. The groups were as follows: **G1:** immunized with *L. amazonensis* antigen emulsion; **G2:** immunized with *L. braziliensis* antigen emulsion; and **G3:** inoculated with IFA emulsion and saline solution (negative control group). The guinea pigs were intramuscularly inoculated with 1mL (4mg/mL) of antigen emulsion or IFA. The inoculum (0.5mL) was intramuscularly injected in two sites of guinea pig chest. After 30 days, *C. porcellus* was skin tested with standard Montenegro antigen (CPPI - lot 01/12- registration number: 80151040004). For the skin test process, the guinea pigs were depilated, and four inoculation sites were delimited. The inoculations with disposable syringes for each site were as follows: 0.1 or 0.2mL of standard Montenegro antigen, or 0.1 or 0.2mL of intradermally inoculated vehicle. The vehicle was represented by phenol saline solution in which the Montenegro antigen was diluted. The guinea pigs were skin tested with this solution to discriminate

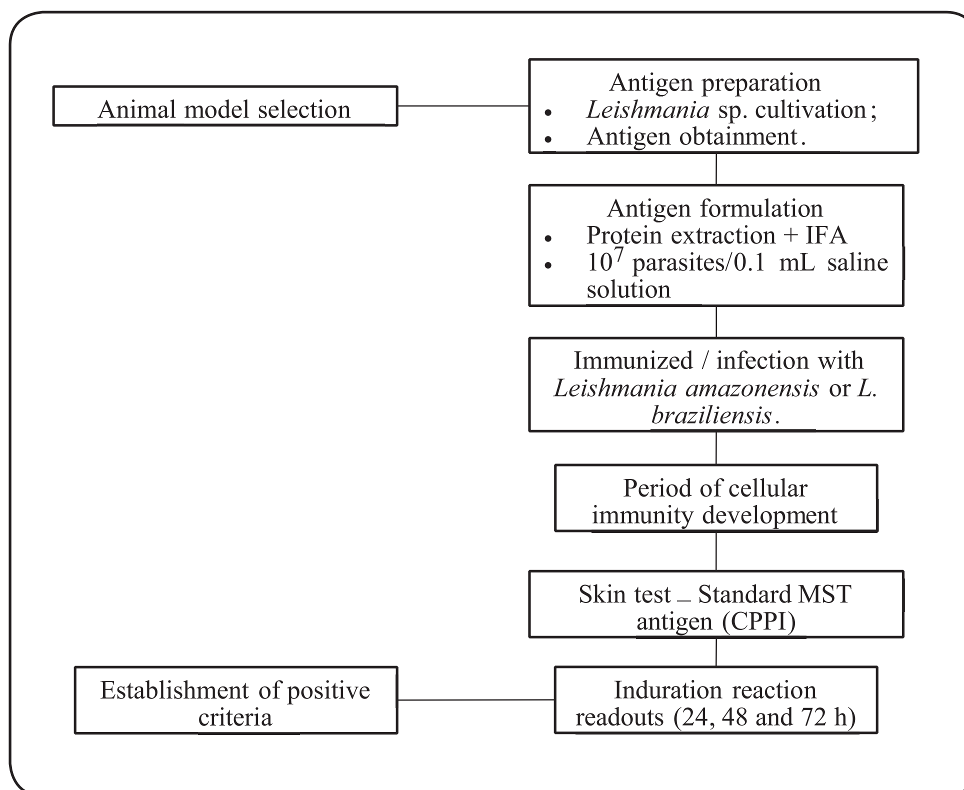

FIGURE 1 – Flowchart outlining the methodology steps. **IFA:** Incomplete Freund's Adjuvant. **MST:** Montenegro skin test.**CPPI:** *Centro de Produção e Pesquisa de Imnunobiológicos do Paraná*.

unspecific reaction or cross reactivity to it. The Montenegro antigen was inoculated at a concentration of 4µg/0.1mL. The readouts of the reaction were performed at 24, 48, and 72h after the intradermal inoculation by measuring the diameter of the indurations using a caliper.

2. Validation of *Cavia porcellus* as an animal model for skin test

To validate the proposed animal model, the same experimental design was applied to gold hamster (*M. auratus*), capable of developing the disease through infection with *L. amazonensis* or *L. braziliensis*. Eighteen male, weighing 140-160 g and 6-8 weeks old, were used. The animals were housed in polypropylene cages and fed with water and food *ad libitum*. The animals were maintained in groups of six. The animal number for this study was calculated as previously mentioned.

For experimental infection, promastigotes forms of *L. amazonensis* and *L. braziliensis* were separately cultivated in biphasic brain heart infusion culture medium and incubated at 24ºC. Promastigotes cultured until stationary phase (5 days) were used to infect the hamster groups. Briefly, the parasites were harvested, diluted in ultrapure water (5x), counted, and adjusted to a concentration of 1×10^7 parasites in 0.1mL of saline solution for inoculation. Three groups with six animals each were formed as follows: **GH1:** infected with 1×10^7 *L. amazonensis*; **GH2:** infected with 1×10^7 *L. braziliensis*; and **GH3:** saline solution (negative control group). The hamsters were intradermally inoculated in the right hind paw with 0.1mL of saline solution containing 1×10^7 parasites. After 45 days, when initial lesions were observed, hamsters were skin tested with standard Montenegro antigen. First, they were depilated, and the inoculation was performed as described above. The skin test solutions were intradermally inoculated using an antigen concentration of 4µg/0.1mL. The readouts of the reaction were performed at 24, 48, and 72h after measuring the diameter of the indurations using a caliper.

Statistical analysis

The homogeneity of variance analysis and the Kolmogorov-Smirnov test for the normal condition of variable evaluation were performed using the Statistica 7 software. A Kruskal-Wallis one-way analysis with a Dunn post-test was performed to analyze the significant difference among groups using GraphPad Prism 6 software, assuming a significance level of 5%.

Ethical considerations

This study was approved by the Research Ethics Committee of the Federal University of Paraná (Process n. 101328/2015-69).

RESULTS

The results showed that *Cavia porcellus* immunized with *Leishmania amazonensis* presented an induration reaction with 0.1 and 0.2mL of antigen (**Figure 2A**). With 0.1mL of Montenegro antigen, the average induration diameters ranged from 10.4 (24h) to 4.8mm (72h). After 48h, the induration diameter was 4.9mm. With 0.2mL of Montenegro antigen, the average induration diameters ranged from 9.4 (24h) to 4.4mm (72h). After 48h, the induration diameter was 5.1mm (**Table 1**).

In *C. porcellus* immunized with *L. braziliensis*, the results showed that both volumes (0.1 and 0.2mL) promoted induration reactions (**Figure 2B**). Inoculation with 0.1mL of Montenegro antigen resulted in indurations with average diameters ranging from 7.6 (24h) to 4.5mm (72h). After 48h, the induration diameter was 5.0mm. With 0.2mL of Montenegro antigen, the average induration diameters varied from 6.7 (24h) to 4.5mm (72h). After 48h, the induration diameter reached 4.8mm (**Table 1**).

In *C. porcellus* immunized with IFA and saline solution (negative control group), induration reactions were not observed. Statistical analysis showed that there was no significant difference between the induration reactions with inoculation of 0.1 and 0.2mL of Montenegro antigen in the guinea pigs immunized with *L. amazonensis* or *L. braziliensis* (p-value < 0.01) after 24, 48, and 72h.

The skin test in *M. auratus* showed that the six animals infected with *L. amazonensis* presented induration reaction with both 0.1 and 0.2mL of Montenegro antigen (**Figure 3A**). The average induration diameters ranged from 3.25 (24h) to 3.42mm (72h). After 48h, the induration diameter was 3.75mm. With 0.2mL of Montenegro antigen, the average induration diameters ranged from 5.6 (24h) to 5.4mm (72h). After 48h, the induration diameter was 6.2mm (**Table 2**). In *M. auratus* infected with *L. braziliensis*, all animals presented induration reactions in both inoculation sites with 0.1 and 0.2mL of Montenegro antigen (**Figure 3B**). The average induration diameters of inoculation site with 0.1mL Montenegro antigen ranged from 3.67 (24h) to 3.58mm (72h). After 48h, the induration diameter was 4.83mm. At inoculation site with 0.2mL of Montenegro antigen, the average induration diameters ranged from 5.25 (24h) to 3.92mm (72h). After 48h, the induration diameter was 5.58mm (**Table 2**).

In hamsters infected with saline solution (negative control group), induration reactions were not observed. Statistical analysis showed that, in the group infected with *L. amazonensis*, there was a considerable difference between induration reactions resulting from inoculation with 0.1 and 0.2mL of Montenegro antigen at 24 and 48h. After 72h, there was no significant difference between induration reactions with 0.1 and 0.2mL of Montenegro antigen (p-value = 0.01). For the group infected with *L. braziliensis*, there was no significant difference between induration reactions with 0.1 and 0.2mL of Montenegro antigen after 24, 48, and 72h (p-value = 0.04).

DISCUSSION

A volume of 0.1mL of Montenegro antigen is the standard amount used to inject patients when performing the skin test for cutaneous leishmaniasis[12]. In this study, the higher level of 0.2mL was tested to evaluate if such increase in volume would show a better induration reaction. The results showed that both injected volumes (0.1 and 0.2mL) promoted induration reactions, and the difference between these reactions was not considerable. However, the skin test reaction to the 0.2-mL Montenegro antigen inoculation was different in some animals

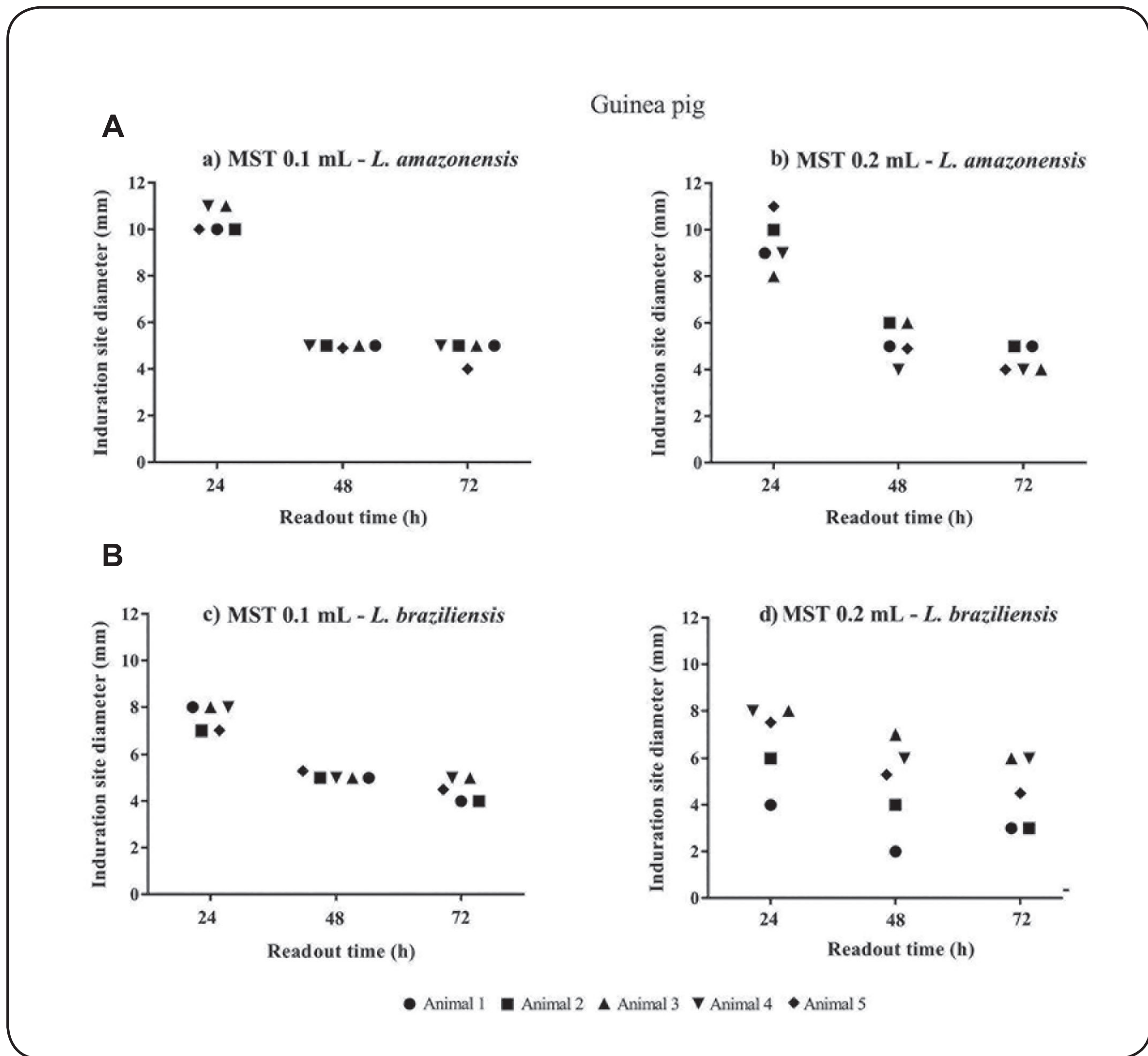

FIGURE 2 - A. Montenegro skin test in *Cavia porcellus* previously immunized with *Leishmania amazonensis*. **a:** Induration reactions against standard Montenegro antigen with an inoculation of 0.1mL. **b:** Induration reactions against Montenegro antigen with an inoculation of 0.2mL. **B.** Reaction to Montenegro antigen in *Cavia porcellus* previously immunized with *Leishmania braziliensis*. **c:** Induration reactions against standard Montenegro antigen with an inoculation of 0.1mL. **d:** Induration reactions against standard Montenegro antigen with an inoculation of 0.2mL. The diameter of indurations was measured 24, 48, and 72h after the antigen inoculation. Each value corresponds to individual induration reaction of the five animals in each group. **MST:** Montenegro skin test.

TABLE 1: Diameter in mm of induration after Montenegro skin in *Cavia porcellus* immunized with *Leishmania amazonensis* or *Leishmania braziliensis*.

		Leishmania amazonensis		***Leishmania braziliensis***	
Group	**Readout**	**Mean (SD) mm**	**95% CI**	**Mean (SD) mm**	**95% CI**
	24h	10.40 (0.54)	9.72-11.08	7.60 (0.54)	6.92-8.28
MST antigen	48h	4.98 (0.04)	4.92-5.03	5.06 (0.13)	4.89-5.22
0.1mL	72h	4.80 (0.44)	4.24-5.35	4.50 (0.50)	3.87-5.12
	24h	9.40 (1.14)	7.98-10.82	6.70 (1.71)	4.56-8.83
MST antigen	48h	5.18 (0.84)	4.13-6.22	4.86 (1.93)	2.45-7.26
0.2mL	72h	4.40 (0.54)	3.72-5.08	4.50 (1.50)	2.63-6.36

SD: standard deviation. 95% **CI:** 95% confidence interval; **MST:** Montenegro skin test.

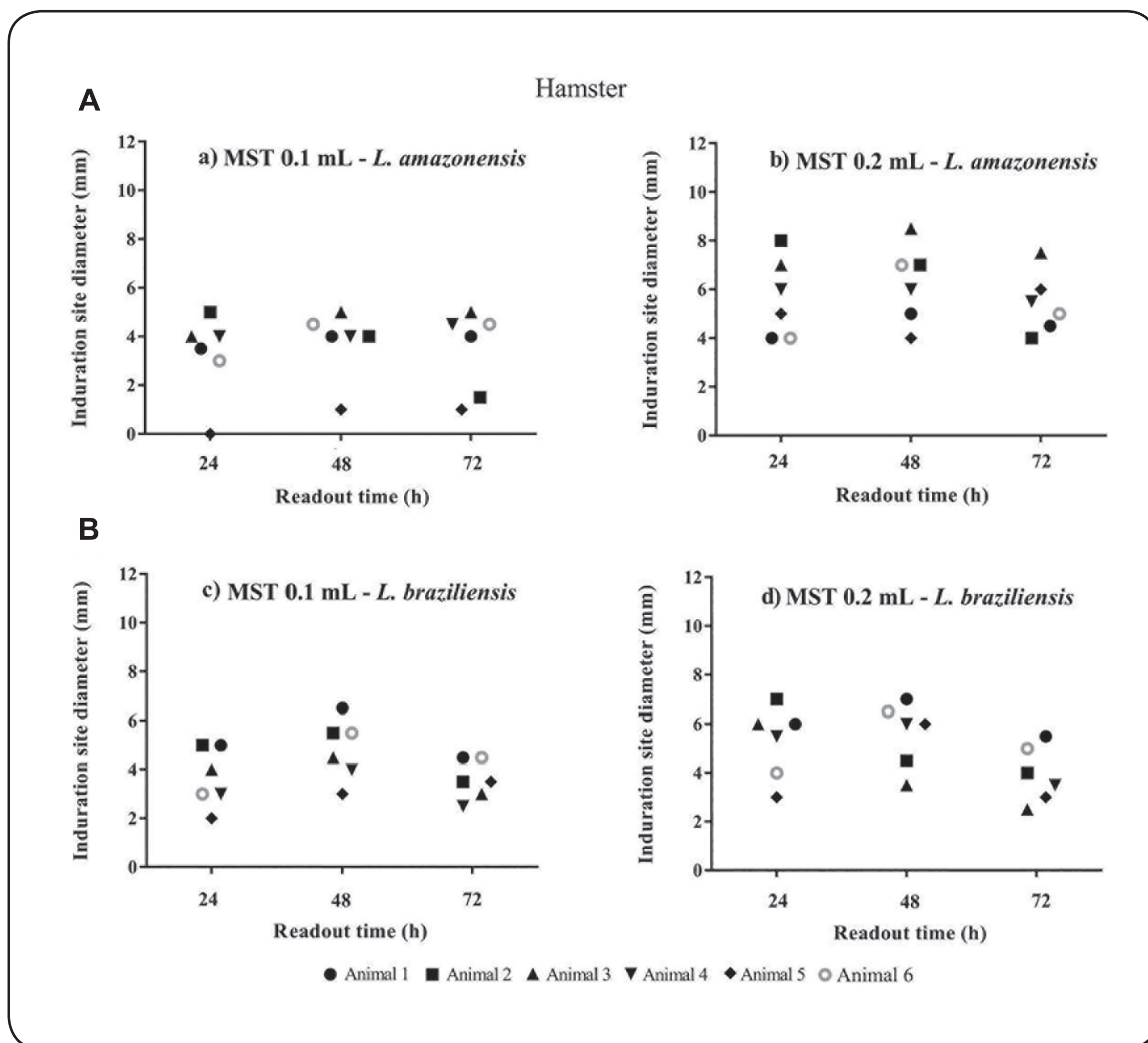

FIGURE 3 - A. Montenegro skin test in *Mesocricetus auratus* infected with *Leishmania amazonensis*. **a:** Induration reactions against standard Montenegro antigen with an inoculation of 0.1mL. **b:** Induration reactions against standard Montenegro antigen with an inoculation of 0.2mL. **B.** MST in *Mesocricetus auratus* infected with *Leishmania braziliensis*. **c:** Induration reactions against standard Montenegro antigen with an inoculation of 0.1mL. **d:** Induration reactions against standard Montenegro antigen with an inoculation of 0.2mL. The diameter of indurations was measured 24, 48, and 72h after skin test. Each value corresponds to individual induration reaction of the six animals in the group. **MST:** Montenegro skin test.

TABLE 2: Diameter of induration after reaction against Montenegro antigen in *Mesocricetus auratus* infected with *Leishmania amazonensis* or *Leishmania braziliensis*.

		Leishmania amazonensis		***Leishmania braziliensis***	
Group	**Readout**	**Mean (SD) mm**	**95% CI**	**Mean (SD) mm**	**95% CI**
MST antigen 0.1mL	24h	3.25 (1.72)	1.44-5.06	3.66 (1.21)	2.39-4.93
	48h	3.75 (1.40)	2.27-5.22	4.83 (1.25)	3.52-.14
	72h	3.41 (1.71)	1.61-5.21	3.58 (0.80)	2.74-4.42
MST antigen 0.2mL	24h	5.66 (1.63)	3.95-7.38	5.25 (1.47)	3.70-6.79
	48h	6.25 (1.60)	4.56-7.93	5.58 (1.31)	4.19-6.96
	72h	5.41 (1.24)	4.11-6.72	3.91 (1.15)	2.70-5.13

SD: standard deviation. 95% **CI:** 95% confidence interval; **MST:** Montenegro skin test.

compared to the group immunized with *L. braziliensis*. These results showed that 0.1-mL Montenegro antigen inoculation is the best volume of antigen for skin test in the *C. porcellus* model.

The positivity criteria established for skin test in guinea pigs in the present study were induration reactions ≥ 0.5cm and readouts at 48-72h after the intradermal test. Some studies are in accordance with the positivity criteria established in the present study[13-15]. The readouts after 24 h were not considered in the test evaluation, because 24h characterized the initial stage of the immunology response in DTH reactions, representing the cell migration to the injection site[3]. The influx of T cells reaches the maximum levels at 48-72h, indicating the time to determine the result of skin test[3].

The animal model chosen for this study was *C. porcellus*, because it is a well-established animal model to study DTH in different types of diseases and is also used in the investigation of several infectious diseases[16-20]. Furthermore, some studies on the genetics of guinea pig have shown immunological analogies between these species[21]. For instance, guinea pig genes that encode for major histocompatibility complex (MHC) proteins are homologous to human proteins, and genetic expression pattern of interferon-γ (IFN-γ) and interleukin 12 (IL-12) are similar in the two species[21].

The skin test in hamsters showed that they developed induration reaction for Montenegro antigen in both infected groups (*L. amazonensis* and *L. braziliensis*), irrespective of the inoculum volume. The skin test volume of 0.2mL promoted higher induration reaction in hamsters than volume 0.1mL in both the infected groups, but induration responses were not statistically different. The induration response difference was considerable just in hamsters in the *L. amazionensis* group after 48h. However, this result was in accordance with induration reaction developed in guinea pigs injected with the same Montenegro antigen volumes. As in guinea pigs, there was no considerable difference between induration reactions promoted by 0.1 or 0.2mL of Montenegro antigen. These results in hamsters validated the experimental model proposed in this work.

The guinea pigs proposed as a model for quality control have many advantages compared to hamsters. Firstly, in all infected groups (*L. amazonensis*, *L. braziliensis*, and negative control saline), hamsters presented lesions resulting from the phenol in the vehicle solution, which were not observed in guinea pigs. This could be a result of the more sensitive skin of the hamsters. Furthermore, a sensitive skin makes the intradermal injection difficult, because the needle can easily punch the skin or produce lesions, leading to difficult readouts. Secondly, a volume of 0.2mL of Montenegro antigen was required for the skin test in hamsters in order to observe induration reactions. Thirdly, hamster fur grows faster than that of guinea pigs, causing difficulties in performing the readouts after 48 and 72h. Fourthly, induration reaction response in hamsters was heterogeneous between the animals, while in guinea pigs this response was homogeneous, reducing the standard deviation and making the test more reliable. Fifthly, hamsters have strait dorsal compared to guinea pigs, making it difficult to organize the skin test inoculation sites. Furthermore, guinea pigs need be immunized with dead parasites (parasite proteic extract) and do not develop the disease, while hamsters need to be infected (live parasites) to respond to skin test[8,9,14]. Once immunized, the risk of contamination is reduced, and the time needed to develop an immune response is faster than that of hamsters (guinea pigs = 30 days; hamsters = minimum of 45 days). Thus, guinea pigs are better animal models to be used in an industrial qualitative control process. Moreover, they are already used as bio models for skin test antigen evaluation in tuberculosis[19], leprosy[22], and immunodeficiency virus type 1 infection[20]. Additionally, the process of experimental infection in hamsters is time consuming and requires well-trained professionals[8,9]. Observations made during experimental tests with hamsters also confirm that guinea pigs are a suitable animal model for Montenegro antigen quality control process.

We conclude that the experimental tests performed confirm that this experimental model may be used to replace the current human *in vivo* model in quality control process of Montenegro antigen production. The induration reaction was observed in all animals in both immunized groups. The establishment of this experimental model for skin tests represents a good alternative for qualitative and quantitative control process of antigen production.

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Conflict of interest

The authors declare that there is no conflict of interest.

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