Fine-needle aspiration may replace skin biopsy for the collection of material for experimental infection of mice with *Mycobacterium leprae* and *Lacazia loboi*

Patrícia Sammarco Rosa, Andréa de Faria Fernandes Belone, José Roberto Pereira Lauris, Cleverton Teixeira Soares

**Summary**

**Background:** Procedures involving the use of *Mycobacterium leprae* and *Lacazia loboi*, uncultivated organisms, depend on the collection of material from the lesions of patients or experimental animals. This study compared fine-needle aspiration (FNA) and skin biopsy methods for obtaining bacilli and fungal cells to experimentally infect animals.

**Methods:** Lepromas from one armadillo and one enlarged footpad of a mouse previously inoculated with *L. loboi* were submitted to FNA and biopsy. Materials collected were processed for inoculation in mice.

**Results:** Acid-fast bacilli (AFB) collected by two FNA procedures yielded 7.2 × 10^7 and 5.3 × 10^6 AFB/ml and biopsies yielded 1.58 × 10^8 and 3.5 × 10^8 AFB/ml from each leproma. Yeast-like cells of *L. loboi* collected by FNA yielded 1.0 × 10^6 fungal cells/ml and biopsy 1.0 × 10^7 fungal cells/ml. After 8 months, inoculated animals were sacrificed and the inoculated footpads submitted to histopathological examination and counting of AFB and fungal cells. The results obtained by the two methods were comparable for both microorganisms.

**Conclusions:** Biopsy may be replaced by FNA during harvesting of material for different purposes, especially for experimental inoculation of mice in leprosy and Jorge Lobo’s disease, with the advantage of FNA being a simpler, less invasive, and less costly method.

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1. Introduction

Experimental animal models for leprosy and Jorge Lobo’s disease are commonly used to elucidate the pathogenesis of both infectious diseases, in drug susceptibility testing, and maintenance of strains. *Mycobacterium leprae*, the etiologic agent of leprosy, is the only species of the genus that has not yet been cultivated in vitro. *L. loboi*, similarly to *M. leprae*, is highly adapted to their respective hosts, the experimental procedures used for inoculation require viable microorganisms; therefore, samples should be processed rapidly and animals inoculated soon after the material is collected from the host, whether human or animal.

Jorge Lobo’s disease, also known as lacaziosis, is a chronic cutaneous mycosis that occurs in the Amazon basin. Its etiologic agent, the fungus *Lacazia loboi*, is another species of the genus *Lacazia* that has not yet been cultivated in artificial culture media. The fungal cells used in routine research procedures need to be collected from lesions of patients or experimentally infected mice. The fungal cells are obtained from surgical specimens or punch biopsies for the follow-up of patients during treatment with antimicrobial drugs. Infected mice are used for maintenance and passage of strains that are further used in molecular and phylogenetic studies and antigen preparation.

Because both *M. leprae* and *L. loboi* are highly adapted to their respective hosts, the experimental procedures used for inoculation require viable microorganisms; therefore, samples should be processed rapidly and animals inoculated soon after the material is collected from the host, whether human or animal.

One simple, low cost, and minimally invasive technique widely used in the routine cytologic diagnosis of neoplasias and non-neoplastic diseases, including infectious diseases, is fine-needle aspiration (FNA). The material collected by FNA is in general highly comparable to the results of biopsy, and for the diagnosis involving the use of *Mycobacterium leprae* and *Lacazia loboi*, uncultivated microorganisms, depend on the collection of material from the lesions of patients or experimental animals. This study compared fine-needle aspiration (FNA) and skin biopsy methods for obtaining bacilli and fungal cells to experimentally infect animals.

**Methods:** Lepromas from one armadillo and one enlarged footpad of a mouse previously inoculated with *L. loboi* were submitted to FNA and biopsy. Materials collected were processed for inoculation in mice.

**Results:** Acid-fast bacilli (AFB) collected by two FNA procedures yielded 7.2 × 10^7 and 5.3 × 10^6 AFB/ml and biopsies yielded 1.58 × 10^8 and 3.5 × 10^8 AFB/ml from each leproma. Yeast-like cells of *L. loboi* collected by FNA yielded 1.0 × 10^6 fungal cells/ml and biopsy 1.0 × 10^7 fungal cells/ml. After 8 months, inoculated animals were sacrificed and the inoculated footpads submitted to histopathological examination and counting of AFB and fungal cells. The results obtained by the two methods were comparable for both microorganisms.

**Conclusions:** Biopsy may be replaced by FNA during harvesting of material for different purposes, especially for experimental inoculation of mice in leprosy and Jorge Lobo’s disease, with the advantage of FNA being a simpler, less invasive, and less costly method.

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of a mycobacterial disease other than leprosy, it has been shown to be as specific as PCR.\textsuperscript{15}

Despite its use in diagnostic procedures, there are no reports of the use of FNA to collect material for the experimental inoculation of animals. This study therefore aimed to compare the two methods for obtaining viable bacilli and fungi, for further use in experimental leprosy and Jorge Lobo’s disease.

2. Materials and methods

2.1. FNA technique

FNA was performed with a 0.60 \( \times \) 25 needle (23 G 1) coupled to a 10 ml syringe, which was attached to a hand-grip syringe holder. The procedure consisted of introduction of the needle into the tissue and suction while moving the needle back and forth repeatedly, as recommended previously.\textsuperscript{15,16} Enough material was considered to have been obtained when the hub of the needle was filled with aspirate, then pressure applied on the syringe was released and the needle removed from the lesion.

2.2. M. leprae collection from armadillo lepromas by FNA and biopsy

Acid-fast bacilli (AFB) were collected from two lepromas of one armadillo (Dasyopus novemcinctus; samples FNA 1 and FNA 2), which had previously been inoculated with \( \text{M. leprae} \). The animal received pre-anesthetic medication (atropine sulfate 0.04 ml/kg, \( \text{M. leprae} \) 2.2. \( \text{M. leprae} \) collection from armadillo lepromas by FNA and biopsy

The FNA procedure for the fungal lesion in the mouse footpad was the same as that used for the armadillo lepromas. The \( \text{L. loboi} \) fungal cells were collected from a previously inoculated mouse. The animal was anesthetized (xylazine 10 mg/kg and ketamine 115 mg/kg, administered intraperitoneally) and after cleaning with alcohol, the footpad was aspirated twice to obtain enough aspirates were taken. The two tissue fragments obtained were weighed (0.08 g and 0.12 g) and processed individually for counting and mouse inoculation.

2.3. L. loboi collection from the footpad of a mouse by FNA and biopsy

The FNA procedure for the fungal lesion in the mouse footpad was the same as that used for the armadillo lepromas. The \( \text{L. loboi} \) fungal cells were collected from a previously inoculated mouse. The animal received pre-anesthetic medication (atropine sulfate 0.04 ml/kg, \( \text{M. leprae} \) 2.2. \( \text{M. leprae} \) collection from armadillo lepromas by FNA and biopsy

2.4. AFB counting

The FNA materials were homogenized with the same syringes used in the aspiration prior to counting of the AFB. The biopsies, however, were macerated in a tissue grinder containing 2.0 ml of Hank’s salt solution; 10 \( \mu l \) of the suspension were placed on three 1.0 cm diameter circles on a glass slide for counting. After air drying, the slides were fixed and stained using the Ziehl–Neelsen technique. The numbers of AFB present in the suspensions were estimated from 60 microscopic fields, calculated according to the protocol previously described.\textsuperscript{17}

2.5. Evaluation of viability and counting of fungal cells

The FNA materials were homogenized using the same syringes used in the aspiration prior to counting of the fungal cells. The footpads were macerated in 2.0 ml of saline solution and then the suspension was filtered in two-ply gauze to eliminate the cellular debris. The volume of the fungal suspension was adjusted to 2.0 ml for counting, viability determination, and inoculation. The viability of the fungi was determined by ethidium bromide–fluorescein diacetate (DF–BE) vital staining, according to the protocol described by Vilani-Moreno and Oprimolla.\textsuperscript{18}

2.6. Inoculation

Inoculation of both organisms obtained by FNA and biopsy was performed intradermally in both hind footpads of 8-week-old male BALB/c mice. Animals were maintained at 21 °C and received water and food ad libitum. For \( \text{M. leprae} \), 40 mice (80 footpads) were inoculated with \( 1 \times 10^6 \) AFB/footpad. Mice were divided into four groups of 10 animals: FNA 1 and FNA 2, groups that were inoculated with material from two different FNA procedures; BIOPSY 1 and BIOPSY 2, groups that were inoculated with material from two different biopsies. For \( \text{L. loboi} \), 10 mice (20 footpads) were inoculated with \( 1 \times 10^6 \) fungal cells/footpad. Mice were divided into two groups of five animals: FNA 3, group inoculated with FNA-collected fungi; BIOPSY 3, group inoculated with biopsy material.

2.7. Sacrifice of inoculated mice

All animals were sacrificed 8 months after inoculation by cervical dislocation after sedation. Both footpads were excised; the right was submitted to histopathological examination and the left was macerated for counting of fungal cells or AFB, as previously described for the preparation of the suspensions. This study was approved by the Animal Ethics Committee of the Universidade do Sagrado Coração, Bauru, São Paulo, Brazil.

2.8. Histopathological evaluation

The histopathological evaluation and bacterial index determination of the material obtained from the footpads of \( \text{M. leprae} \) inoculated mice were done in hematoxylin–eosin (HE) and Fite–Faraco stained sections. The bacterial index for histological sections was the same as that used for skin biopsies of leprosy patients, on a scale from 0 to 6+.\textsuperscript{19} The histopathological evaluation of the material obtained from footpads of \( \text{L. loboi} \) inoculated mice was done in HE and methenamine silver stained sections.

2.9. Statistical analysis

The non-parametric Mann–Whitney test was used for comparison of the mean and median of bacilli and fungi recovered from experimentally infected animals.

3. Results

The number of AFB and fungal cells obtained by the two different methods did not vary greatly for the lepromas of the armadillo or the \( \text{L. loboi} \) mouse footpad lesion (Table 1). The FNA 1 and FNA 2 procedures yielded \( 7.2 \times 10^6 \) and \( 5.3 \times 10^6 \) AFB/ml, respectively, while the biopsy procedures BIOPSY 1 and BIOPSY 2 yielded \( 1.58 \times 10^6 \) and \( 3.5 \times 10^6 \) AFB/ml, respectively. For \( \text{L. loboi} \), \( 1.0 \times 10^6 \) fungal cells/ml were collected by FNA and \( 1.0 \times 10^7 \) fungal cells/ml were obtained from the excised footpad (Table 1).

The mean number of bacilli recovered in suspensions from material collected from the mice footpads after 8 months of
inoculation was very similar between the FNA groups: 2.1 \( \times \) 10^5 AFB/ml for FNA 1 and 1.4 \( \times \) 10^5 AFB/ml for FNA 2. In the biopsy groups, the total numbers of bacilli recovered were 2.1 \( \times \) 10^5 AFB/ml in BIOPSY 1 and 1.9 \( \times \) 10^5 AFB/ml in BIOPSY 2 (Table 1). The median number of fungal cells recovered from the footpads of mice after inoculation was comparable in the two groups: the FNA 3 group had 1.44 \( \times \) 10^6 fungal cells/ml and the BIOPSY 3 group had 8.1 \( \times \) 10^5 fungal cells/ml, with viability indices of 4.5% and 7.0%, respectively (Table 1). Thus, comparison of the data obtained from the same site showed no statistical difference, which demonstrates that both methods yielded enough material for analysis and infection of mice.

The histological pattern in mice inoculated with *M. leprae* did not vary in any of the groups. A diffuse lymphohistiocytic granulomatous infiltrate penetrating the dermis, skeletal muscle layer, and nerves was observed in most examined slides. The bacterial index varied from 3 to 6+, with a predominance of granular bacilli. Foci of necrosis associated with neutrophilic infiltrate were observed in a few animals (Figs. 1 and 2).

The histological pattern in mice inoculated with *L. loboi* was identical in both the FNA and BIOPSY groups. A lymphohistiocytic granulomatous reaction involving the dermis, skeletal muscle layer, and nerve branches was observed. A large number of fungal cells were observed inside histiocytes and multinucleated giant cells. Lymphocytes and plasma cells were also observed permeating the granulomatous infiltrate (Figs. 3 and 4).

### 4. Discussion

*M. leprae* used in experimental laboratory procedures are commonly obtained from skin biopsies from leprosy patients. Because *M. leprae* has not yet been cultivated in vitro, animals are routinely inoculated to evaluate the drug sensitivity profile of *M.*

<table>
<thead>
<tr>
<th>Organisms collected for inoculation</th>
<th>Organisms recovered from infected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>No. of fungal cells or bacilli</td>
</tr>
<tr>
<td>FNA 1</td>
<td>7.2 ( \times ) 10^7 AFB/ml</td>
</tr>
<tr>
<td>FNA 2</td>
<td>5.3 ( \times ) 10^8 AFB/ml</td>
</tr>
<tr>
<td>FNA 3</td>
<td>1.0 ( \times ) 10^9 fungal cells/ml</td>
</tr>
<tr>
<td>BIOPSY 1</td>
<td>1.58 ( \times ) 10^3 AFB/ml</td>
</tr>
<tr>
<td>BIOPSY 2</td>
<td>3.5 ( \times ) 10^8 AFB/ml</td>
</tr>
<tr>
<td>BIOPSY 3</td>
<td>1.0 ( \times ) 10^7 fungal cells/ml</td>
</tr>
</tbody>
</table>

AFB, acid-fast bacilli; BI, bacterial index; FNA, fine-needle aspiration; NA, not applicable. Mann–Whitney non-parametric test, no statistical differences: *p=0.236; *b*p=0.606; *c*p=0.756; *d*p=0.968.
In both experimental models, for leprosy and Jorge Lobo’s disease, the etiologic agents are inoculated in mice footpads. For passage into other animals or for collection of material to be studied, it is necessary to sacrifice the animal host to obtain enough bacilli or fungal cells.

FNA eliminates the need for sacrificing animals to collect samples; in addition, it facilitates the preparation of cell suspensions. By using FNA, the infected mouse footpad showing large numbers of fungal cells inside the granulomas (methenamine silver stain, 400×).

Fig. 4. Histology image of the L. loboi inoculated mouse footpad showing large numbers of fungal cells inside the granulomas (methenamine silver stain, 400×).

References

1. Rosa PS, Belone AF, Silva EA, Pedrini SC, Pinca KE. Inoculação experimental de M. leprae bacilli: in order to detect viable bacilli in patients during and after the end of treatment and in patients undergoing successive reational episodes; and to maintain strains of M. leprae in passages into immunosuppressed mice. Another experimental model of leprosy is the nine-banded armadillo, which develops the disseminated form of leprosy after inoculation of massive doses of M. leprae derived from lesions of patients, inoculated mice, or even other armadillos. Doses of 10^8 to 10^9 M. leprae inoculated subcutaneously and intravenously lead to the formation of nodules at the site of inoculation (lepromas) within about 12 months and then dissemination of the infection. These animals, therefore, have largely been used for the production of bulk bacilli and in the maintenance of M. leprae strains in the laboratory.

Studies into Jorge Lobo’s disease progressed remarkably in the last decade after the successful experimental infection of BALB/c mice with L. loboi. Infected mice are used in the maintenance of L. loboi strains, for DNA extraction in phylogenetic studies, and also for the characterization of L. loboi antigens. The preparation of the fungal suspension to be inoculated in animals is labor-intensive, involving the collection and maceration of fragments of lesions in sterile conditions, washing to eliminate the host’s stroma. Examination of smears allows thorough evaluation of highly cellular material is obtained by FNA. Smears of the aspirated material, with results comparable to studies conducted on biopsy cytological characteristics and diagnosis in most cases. It is also used to remove stromal lesions in sterile conditions, washing to eliminate the host’s stroma. Examination of smears allows thorough evaluation of highly cellular material is obtained by FNA.

In the present study, the results from material inoculated with M. leprae were highly homogeneous between the FNA and biopsy maceration techniques (Table 1). In the animals inoculated with L. loboi, there was a small difference between the two groups – the FNA results appeared better than the results from material obtained by maceration of tissue, with respect to both the number of fungal cells and viability (Table 1); however, the difference was not statistically significant (p = 0.756). This may have resulted from the fact that FNA allows for immediate inoculation, and consequently preservation of the characteristics of the fungal cells and bacilli, while the material submitted to processing of the biopsy takes additional steps, as previously discussed in the present study, which may lead to microenvironmental changes that can ultimately hamper the growth of these microorganisms after inoculation.

FNA has been shown to be adequate for the preparation of material for animal inoculation, mainly because of the quantities of organisms collected, with a good viability index. In the material obtained by biopsy there were larger numbers of fungal cells and bacilli than in the material obtained by FNA; however, this was expected because the quantities of cells present in a biopsy, even a small one, is very high (Table 1). Nevertheless, the results indicate that the number of fungal cells and bacilli recovered from inoculated footpads is similar between the FNA and BIOPSY groups.

There are several advantages to using FNA compared to biopsy: lower cost of FNA, it is a less traumatic approach for the individual submitted to collection, the procedure can be done faster, and the collected material is well preserved; there is also no need to sacrifice the animal host and sampling can be repeated at time intervals. In addition, FNA can easily be used to collect samples in field conditions.

In summary, the results show that FNA may replace biopsy as a method to harvest material for inoculation of experimental animals and for determination of viability of L. loboi and M. leprae. Thus, we recommend the use of the FNA procedure instead of biopsy for experimental inoculation in both leprosy and Jorge Lobo’s disease.

Conflict of interest: The authors have no conflict of interests to declare. This work was supported by the Instituto Lauro de Souza Lima and the institution had no role in the development of the study or its publication. This study was approved by the Animal Ethics Committee of the Universidade do Sagrado Coração, Bauru, São Paulo, Brazil.


