

NOTES

Use of Sera from Humans and Dolphins with Lacaziosis and Sera from Experimentally Infected Mice for Western Blot Analyses of *Lacazia loboi* Antigens[∇]

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Antibodies in the sera of patients with lacaziosis recognized an ~193-kDa antigen and other *Lacazia loboi* antigens. *Paracoccidioides brasiliensis* gp43 antigen was detected by all evaluated sera, but they failed to detect a protein with the same molecular mass in *L. loboi* extracts. This study is the first to examine the humoral response to *L. loboi* antigens by using multiple host sera.

Lacazia loboi is an uncultivated fungal pathogen predominantly restricted to Latin America. Lacaziosis has been recorded among humans dwelling in this geographical area, tourists visiting areas of endemicity (4, 10), and workers handling infected dolphins (19, 24). Lacaziosis has also been diagnosed in dolphins inhabiting the coastal areas of the United States (9, 12, 19), Brazil (16), and France (25). Using molecular methods, the phylogenetic features of *L. loboi* were only recently deciphered (13, 18, 31). These studies placed *L. loboi* as a sister group to *Paracoccidioides brasiliensis* and, in turn, linked to the other dimorphic members of the Onygenales in the family Ajellomycetaceae.

The clinical features of *Lacazia loboi* and *P. brasiliensis* infections are different, but both fungi develop yeast cells, which are difficult to differentiate microscopically, in the host's infected tissues (7). This resemblance was used by some investigators to classify this pathogen in the genus *Paracoccidioides* (1, 6, 8). Thus, due to *L. loboi*'s inability to grow in culture, immunological studies have traditionally been carried out with antigens extracted from mycelial cultures of *P. brasiliensis* (15, 17, 21, 23–27, 30). The objective of our study was to characterize the immunogenic proteins extracted from *L. loboi* yeast-

like cells in Western blotting analyses, using sera from humans and dolphins with lacaziosis and sera from experimentally infected mice.

Sera from four human patients with lacaziosis from Acre State, Brazil, and from one Brazilian patient with proven paracoccidioidomycosis and four sera from experimentally *L. loboi*-infected mice were collected and stored at –80°C until use (2). Sera from three bottlenose dolphins (*Tursiops truncatus*) infected with *L. loboi* were evaluated: one wild dolphin infected with *L. loboi* was captured in the Indian River Lagoon, FL, one was a stranded Indian River Lagoon bottlenose dolphin, and one was a captive bottlenose dolphin from SeaWorld in San Diego, CA. In addition, two sera from apparently healthy humans, two sera from healthy dolphins, and two sera from healthy laboratory mice were used as negative controls.

Lacazia loboi yeast-like cell extracts were obtained from the footpads of a previously infected BALB/c mouse 6 months after inoculation, as described by Belone et al. (2). Briefly, *L. loboi* yeast-like cells were then centrifuged for 10 min at 3,000 rpm, and the supernatant was discarded. Cells were washed twice with saline, and the pellet was suspended in 1 ml of saline. Fungal cells were macerated to a powder with liquid nitrogen inside a mortar and used in the experiments. The gp43 protein of *P. brasiliensis* was extracted and purified per the method of Tabora et al. (28). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were done per the methods of Laemmli (14) and Towbin et al. (29).

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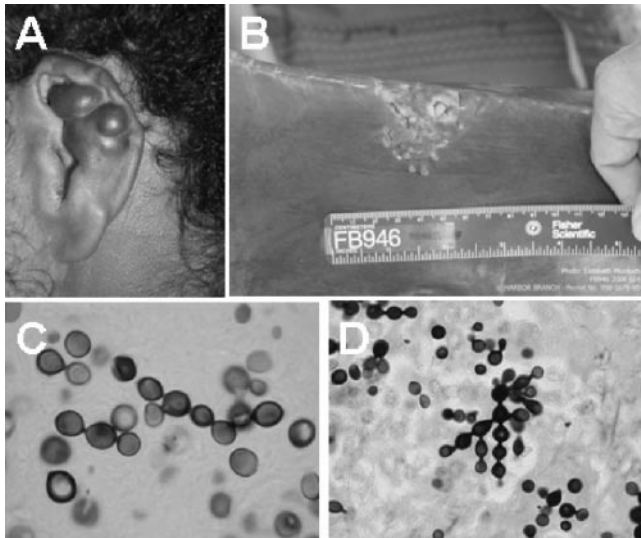


FIG. 1. Human parakeloidal (A) and dolphin verrucous (B) lesions from two of the hosts with lacaziosis used in this study. The histopathology of these lesions is shown in panels C (human) (silver stain; magnification, $\times 100$) and D (dolphin) (silver stain; magnification, $\times 40$).

The diagnosis of lacaziosis in the investigated humans and dolphins was confirmed by clinical and histopathological methods (Fig. 1). The stained gels of *L. loboi* protein extracts showed at least 10 bands, ranging from ~ 193 kDa to 9.0 kDa (Fig. 2). The *L. loboi* protein extract lacked a visible protein

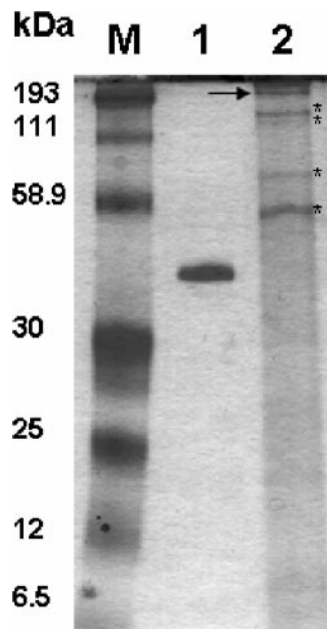


FIG. 2. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel showing purified *Paracoccidioides brasiliensis* gp43 (lane 1) and the profile of an *Lacazia loboi* protein extract (lane 2). The figure shows the ~ 193 -kDa immunodominant antigen (arrow) as well as other proteins in the *L. loboi* protein extract. The asterisks indicate common proteins detected by the sera tested in this study. Lane M, molecular mass marker.

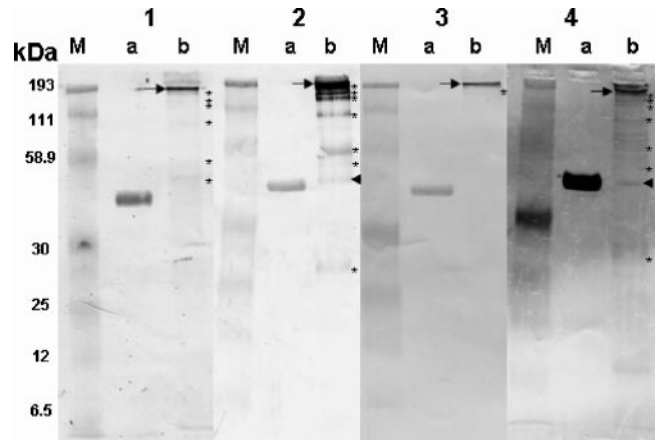


FIG. 3. Western blot analyses of transferred proteins on nylon membranes. The antibodies in the serum from a human with lacaziosis weakly detected *Paracoccidioides brasiliensis* gp43 (lane 1a). This serum strongly detected an ~ 193 -kDa immunodominant antigen (arrow) as well as six other weak bands (asterisks) (lane 1b). The IgG in the serum from the experimentally infected mouse weakly detected *P. brasiliensis* gp43 (lane 2a). The mouse antibodies strongly detected the ~ 193 -kDa immunodominant antigen (arrow) as well as seven other proteins (asterisks) and an ~ 48 -kDa protein not detected by the other sera (lane 2b, arrowhead). The antibodies in the dolphin with lacaziosis slightly detected *P. brasiliensis* gp43 (lane 3a). The antibodies strongly recognized the ~ 193 -kDa immunodominant antigen (arrow) and only one of the minor antigens previously detected in the *L. loboi* extract (lane 3b). The antibodies in the serum from the patient with paracoccidioidomycosis strongly detected gp43 (lane 4a). The antibodies of this patient also detected the immunodominant ~ 193 -kDa antigen (arrow) in the *L. loboi* extract as well as other antigens (asterisks), including a 43-kDa antigen (arrowhead) (lane 4b). The molecular mass marker (lanes M) appears in each of the panels.

band at 43 kDa. The purified gp43 protein of *P. brasiliensis* showed a strong band at the expected molecular mass and did not contain other proteins (Fig. 2). Normal mouse skin tissue samples processed as described above did not show visible bands (data not shown).

The immunoglobulin G (IgG) antibodies in the sera of all tested hosts with lacaziosis detected the gp43 protein of *P. brasiliensis* fairly well (Fig. 3). The tested dolphin, human, and experimentally infected mouse sera detected a major, immunodominant, ~ 193 -kDa antigen in the *L. loboi* protein extract (Fig. 3) and also reacted weakly with other immunogenic antigens (Fig. 3). The experimentally infected mouse antibodies mildly detected five other immunogens, including an ~ 48 -kDa protein not detected by the other sera (Fig. 3, panel 2, lane b, arrowhead). The IgG antibodies of the dolphins detected only two high-molecular-mass antigens in the *L. loboi* protein extract, including the immunodominant, ~ 193 -kDa antigen (Fig. 3, panel 3, lane b). The antibodies in the serum from a patient with paracoccidioidomycosis weakly detected at least 13 other antigenic components in the *L. loboi* protein extract, ranging from ~ 193 kDa to 9.0 kDa. Some of these antigens were not detected by the antibodies in the sera from humans and dolphins with lacaziosis or the antibodies in the sera from experimentally infected mice (Fig. 3, panel 4, lane b). Only the antibodies in the serum from the patient with paracoccidioidomycosis weakly detected a molecular antigen of 43 kDa in the *L. loboi* protein extract (Fig. 3, panel 4, lane b). The healthy

control human, dolphin, and mouse sera did not react with the antigens used in this study (data not shown). One of the two control dolphin sera possessed antibodies against the 43-kDa purified protein of *P. brasiliensis* and the major antigens of *L. loboi* normally detected by the dolphins with lacaziosis. Putative residual normal mouse skin cells were not detected by the tested sera (data not shown).

With the exception of three previous studies (1, 23, 30), in the past 75 years *L. loboi* immunological research has been carried out using antigens of *P. brasiliensis* (6, 8, 15, 17, 21, 24, 26, 27, 32). Our study showed that patients with lacaziosis possessed antibodies not only against the gp43 antigen of *P. brasiliensis* but mainly against an ~193-kDa immunodominant *L. loboi* antigen. The possibility that the ~193-kDa antigen comprises several compressed *L. loboi* immunogens is currently under investigation in our laboratory. The IgGs in the sera from patients with lacaziosis failed to detect the gp43-like antigen in the protein extract of *L. loboi*. However, the serum from the patient with paracoccidioidomycosis did detect an antigen with a similar molecular mass. The cross-reactive antigens reported in this study, including purified gp43 and those reported by other investigators (1, 6, 15, 21, 24, 26, 30), were not unexpected, since recent molecular studies have shown that both *L. loboi* and *P. brasiliensis* share the same ancestor (13, 18, 31).

The many reports of lacaziosis in dolphins have puzzled investigators for decades (3, 5, 9, 12, 19, 22, 25, 32). However, studies to elucidate its etiology, epidemiology, and immunology have been equally challenging (5, 22, 25). Our study showed that dolphins develop IgG antibodies against similar prominent antigens to those recognized by humans with lacaziosis and by experimentally infected mice. Interestingly, one of the negative control dolphins detected the gp43 antigen of *P. brasiliensis* and the other major antigens of *L. loboi* identified by dolphins with lacaziosis. This unexpected result suggests a previous exposure to *L. loboi* without disease, as it is frequent among the other dimorphic members of the Onygenales (15, 17, 20). Thus, Western blotting analyses could be a useful tool for epidemiological studies of *L. loboi*. Although some investigators have reported morphological differences between the yeast-like cells of *L. loboi* in both species (11), our study is the first to suggest that dolphins are infected with similar *L. loboi* strains to those infecting humans.

In contrast to previous serological reports (1, 23, 30), this study suggests that during infection, *L. loboi* presents immunogens to the immune system which are different from that in *P. brasiliensis* infection. The molecular characterization of these antigens, especially the ~193-kDa immunodominant antigen, could generate valuable information to better understand the immunology and serology of *L. loboi* infection and possibly aid in the development of new therapies for infections caused by this resilient fungal pathogen.

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