ISSN 0325-7541
INFORME BREVE Revista Argentina de Microbiología (2009) 41: 102-104

Identification of the source of histoplasmosis infection in two captive maras (*Dolichotis patagonum*) from the same colony by using molecular and immunologic assays

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ABSTRACT

Histoplasma capsulatum was isolated from the spleen of a first infected mara (*Dolichotis patagonum*) and from a second mara's liver and adrenal gland, both in the same colony at the Africam Safari, Puebla, Mexico. Studies of *H. capsulatum* isolates, using nested-PCR of a 100-kDa protein coding gene (*Hcp100*) fragment and a two-primer RAPD-PCR method, suggest that these isolates were spreading in the environment of the maras' enclosure. By using a Dot-ELISA method, sera from mice inoculated with three homogenates of soil samples from the maras' enclosed space developed positive brown spot reactions to a purified *H. capsulatum* antigen, which identified the probable source of the maras' infection.

Key words: *Histoplasma capsulatum*, maras, PCR, RAPD-PCR, Dot-ELISA, infection source

RESUMEN

Identificación de la fuente de infección de histoplasmosis de dos maras (*Dolichotis patagonum*) cautivas procedentes de la misma colonia, utilizando ensayos moleculares e inmunológicos. *Histoplasma capsulatum* fue aislado del bazo de una primera mara (*Dolichotis patagonum*) infectada y del hígado y la glándula suprarrenal de un segundo ejemplar, ambos de la misma colonia en el Africam Safari, Puebla, México. Los estudios de los aislamientos de *H. capsulatum* mediante PCR anidada para un fragmento del gen *Hcp100* que codifica una proteína de 100 kDa y RAPD-PCR empleando doble iniciador sugieren que estos aislamientos estaban dispersos en el ambiente del refugio de las maras. Los sueros de ratones inoculados con tres homogenatos de muestras de suelo del refugio desarrollaron reacciones positivas a un antígeno purificado de *H. capsulatum* (manchas color castaño oscuro) por el método de Dot-ELISA; con lo cual se identificó la probable fuente de infección de las maras.

Palabras clave: *Histoplasma capsulatum*, maras, PCR, RAPD-PCR, Dot-ELISA, fuente de infección

The pathogenic fungus *Histoplasma capsulatum* var. *capsulatum*, the etiologic agent of the systemic mycosis histoplasmosis, usually occurs in geographic areas where tropical climates prevail. It is present either in confined spaces where bat guano is abundant or in open spaces where bird droppings are frequently found. Infection is acquired from the environment by inhalation of infective mycelial-phase propagules that are aerosolized (11).

Bats are probably the major fungal reservoir that contributes to maintain this parasite in natural foci. Histoplasmosis has been described in domestic and free-ranging wildlife animals. Occasionally, it has been documented in captive animals, including a sea otter (*Enhydra lutris*) (3) and an owl monkey (*Aotus nancymai*) (12). Disseminated histoplasmosis in captive maras (*Dolichotis patagonum*), also called Patagonian hares, at the Africam Safari, in the state of Puebla, Mexico, has been described elsewhere (8, 9). We herein report molecular and immunologic findings that support the identification of the source of infection in the maras' enclosure, which were probably associated with two histoplasmosis cases in captive maras from the same colony at the Africam Safari, Mexico.

Three isolates from maras were used: the EH-558B from the spleen belonging to case 1 and EH-574A and EH-574H from the adrenal gland and liver of case 2, respectively. Isolates were identified by morphological and antigenic procedures as described by Taylor *et al*. (10). One isolate (EH-406) from a naturally infected bat and another one from an Argentinean patient (01558) were processed as controls in different molecular assays. The mycelial phase of each *H. capsulatum* isolate was maintained at 26 ºC in brain-heart-infusion agar (BHI-agar) (Bioxón, México, DF).

DNA samples were extracted from the three maras' *H. capsulatum* isolates and from the two control isolates. Whole-cell DNA isolation was performed as described elsewhere by Reyes-Montes *et al*. (6).

Nested-PCR was performed as described by Bialek *et al*. (1) with minor modifications by Taylor *et al*. (10). DNA samples from the studied *H. capsulatum* isolates were processed together with a DNA sample of *Sporothrix schenckii*, used as negative control of heterologous fungal strain. Two sets of primers (Operon Technologies Inc., Alameda, CA) were used, corresponding to a fragment of the gene encoding for a 100-kDa protein (*Hcp100*) unique to *H. capsulatum* (10). The outer primer set, Hc I and Hc II, delimits a 391-nucleotide sequence of the gene (1). The inner primers, Hc III and Hc IV, delimit a specific 210-nucleotide sequence (1). DNA amplification and the electrophoresis of the PCR products were performed according to Taylor *et al*. (10).

The two-primer RAPD-PCR assay was performed as described by Hu *et al*. (2) with modifications by Taylor *et al*. (10). The amplification patterns of different isolates were analyzed to obtain an estimation of similarity for each pair of isolates. The similarity was calculated by using the Jaccard coefficient (5). A dendrogram was constructed based on the similarity matrix and through the unweighted pair-group method with arithmetic averages (UPGMA). Multivariate statistical methods were performed with the NTSYS-PC program (version 2.0; Exeter Software) (7).

To detect the maras' sources of infection, sera from mice inoculated with homogenates from some environmental samples in different places of the maras' enclosure at the Africam Safari (Table 1) were processed through Dot-ELISA (4, 10). Criteria to define positive reaction in sera from tested mice were standardized by taking into account that two out of three mice sera revealed brown spots in the assay.

H. capsulatum isolates from the maras' samples were previously identified by morphological and antigenic findings and are now preserved in the *H. capsulatum* Culture Collection of our laboratory.

Nested-PCR products targeting the *Hcp100* protein gene showed that all *H. capsulatum* isolates from the infected maras shared the same bands (391 and 210 bp in the first and nested amplifications, respectively) with two *H. capsulatum* isolates tested as controls. DNA of *S. schenckii* was not amplified (Figure 1 a and b).

The RAPD-PCR analysis distinguished the three maras' *H. capsulatum* isolates from the two different *H. capsulatum* isolates used as controls. The dendrogram in Figure 2 formed a highly related group of maras' *H. capsulatum* isolates, including the isolates from case 2 (EH-574A and 574H) with a 1.0 similarity and the isolate from case 1 (EH-558B) that showed a 0.84 similarity with those from case 2. In contrast, a less related group with a 0.36 similarity was formed by one isolate from a naturally infected bat captured in Puebla (EH-406), and another one from an Argentinean human clinical case (01558).

The presence of anti-*H. capsulatum* antibodies in sera of most mice at 10 and 17 days after inoculation with samples S-190, S-191, and S-197 suggests that the source of the maras' infection was associated with bat and bird guano collected from places related to the maras' enclosure, where both types of guano had accumulated near the maras' food container (Table 1).

Although *H. capsulatum* isolates from the mara samples were undoubtedly identified by morphological and antigenic findings, the molecular studies of these isolates were an important support to relate *H. capsulatum* isolates sampled from other sources and geographic origins. At the same time, the infection source at the maras' enclosure was immunologically monitored by detecting positive reaction to *H. capsulatum* antigen in sera of mice inoculated with different environmental samples, including from their burrows.

The nested-PCR assay established that the mara isolates shared the same bands in all *H. capsulatum* iso-

Table 1. Dot-ELISA from sera of mice inoculated with different environmental samples collected from the maras' enclosure at the Africam Safari, Puebla, Mexico

Soil samples	Type of samples	Place of collection	Dot-ELISA (10-days) N° positive/N° processed	Dot-ELISA (17-days)[1] N° positive/N° processed
S-190	Bat and bird guano	Mara shelter	2/3	1/3
S-191	Bat and bird guano	Mara shelter	3/3	3/3
S-195	Mara feces	Mara burrow	1/3	1/3
S-196	Mara feces	Mara burrow	1/3	0/3
S-197	Bat and bird guano	Mara shelter	2/3	3/3

[1]Sera from mice inoculated with homogenates of the environmental samples were harvested and tested at 10 and 17 days after mice inoculation. Dot-ELISA was performed by using a purified DPPC-Histo molecule as specific *H. capsulatum* antigen (10). Samples S-190, S-191, and S-197 were collected in distinct places of the maras' open shelter. Samples S-195 and S-196 were collected from different burrows in the middle of the maras' enclosure.

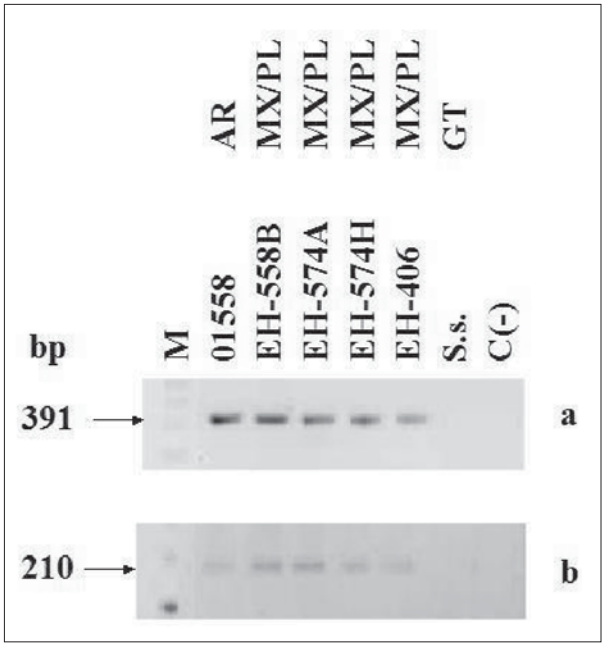

Figure 1. Nested-PCR of *H. capsulatum* DNA samples from infected maras. The assay was performed with two sets of fungal-specific primers of the 100-kDa protein gene of *H. capsulatum*. PCR products were resolved by electrophoresis in 1.5% agarose followed by ethidium bromide-staining. First (a) and nested (b) PCR reactions. Lanes refer to each processed *H. capsulatum* isolate. The *S. schenckii* (Ss) DNA was used as negative control of heterologous strain, and C (-) as negative control of the system. M- 123 bp DNA ladder molecular size marker. Abbreviations: AR- Argentina; MX/PL- Mexico/Puebla; GT- Guatemala.

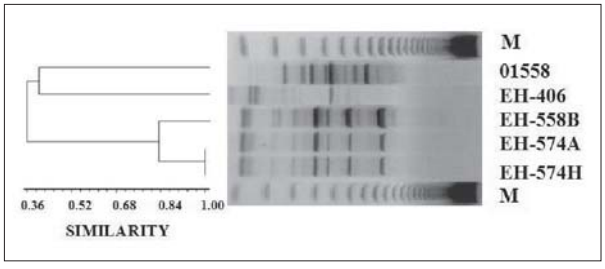

Figure 2. Polymorphic DNA profiles of the maras' *H. capsulatum* isolates. Three isolates from both maras (EH-558B, EH-574A, and EH-574H), one isolate from a naturally infected bat (EH-406), and another isolate from an Argentinean patient (01558) were processed, using 10 ng of DNA template in a two-primer RAPD-PCR assay. The PCR products were resolved by electrophoresis in 1.5% agarose followed by ethidium bromide-staining. The dendrogram was constructed based on the UPGMA algorithm and the similarity between each pair of isolates was calculated by the Jaccard coefficient. M- 123 bp DNA ladder molecular size marker.

lates studied, which supports their molecular recognition. Besides, the RAPD-PCR findings revealed, through the dendrogram based on the UPGMA analysis, that the mara isolates have high similarity in their polymorphic DNA bands, which differed from the DNA patterns of the two *H. capsulatum* control isolates processed. Thus, RAPD-PCR results suggest that the mara isolates shared the same source of infection.

The presence of *H. capsulatum* in the maras' enclosure was well-demonstrated by antibody production in mice inoculated with the environmental samples.

This work details infection data from two captive maras with disseminated histoplasmosis, regarding molecular findings of their *H. capsulatum* isolates and the immunological identification of their respective infection sources.

Acknowledgements: The authors thank Ingrid Mascher for editorial assistance.

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Recibido: 12/08/08 – Aceptado: 11/11/08