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An *Hcp100* gene fragment reveals *Histoplasma capsulatum* presence in lungs of *Tadarida brasiliensis* migratory bats

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SUMMARY

Histoplasma capsulatum was sampled in lungs from 87 migratory *Tadarida brasiliensis* bats captured in Mexico ($n=66$) and Argentina ($n=21$). The fungus was screened by nested-PCR using a sensitive and specific *Hcp100* gene fragment. This molecular marker was detected in 81·6% [95% confidence interval (CI) 73·4–89·7] of all bats, representing 71 amplified bat lung DNA samples. Data showed a *T. brasiliensis* infection rate of 78·8% (95% CI 68·9–88·7) in bats captured in Mexico and of 90·4% (95% CI 75·2–100) in those captured in Argentina. Similarity with the *H. capsulatum* sequence of a reference strain (G-217B) was observed in 71 *Hcp100* sequences, which supports the fungal findings. Based on the neighbour-joining and maximum parsimony *Hcp100* sequence analyses, a high level of similarity was found in most Mexican and all Argentinean bat lung samples. Despite the fact that 81·6% of the infections were molecularly evidenced, only three *H. capsulatum* isolates were cultured from all samples tested, suggesting a low fungal burden in lung tissues that did not favour fungal isolation. This study also highlighted the importance of using different tools for the understanding of histoplasmosis epidemiology, since it supports the presence of *H. capsulatum* in *T. brasiliensis* migratory bats from Mexico and Argentina, thus contributing new evidence to the knowledge of the environmental distribution of this fungus in the Americas.

Key words: Bats, *Hcp100* gene, *Histoplasma capsulatum*, *Tadarida brasiliensis*.

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INTRODUCTION

Histoplasma capsulatum var. *capsulatum* is a mammal fungal pathogen distributed worldwide. The infection associated with this pathogen is relevant in those geographical areas where histoplasmosis is endemic or epidemic, such as the Ohio and Mississippi valleys in the USA [1] and some Latin American regions with a high frequency of outbreaks [2]. In Mexico, histoplasmosis is extensively distributed throughout the country [2].

Infection is caused by the inhalation of aerosolized microconidia and hyphal fragments of the *H. capsulatum* saprobe mycelial phase, which develop in bird and bat droppings accumulated in specific areas, fostered by the biotic and abiotic factors of that particular environment. *H. capsulatum* causes a systemic mycosis with a primary respiratory infection and it is not transmissible from host to host through the air or through the host's faeces. The high risk of natural bat infection with this fungus in Mexican caves has been well documented by Taylor *et al*. [3].

In recent years, *H. capsulatum* bat isolates of different geographical origin have been grouped according to their DNA polymorphism, which has revealed an important genetic diversity [2, 4, 5]. Based on sequence analyses of four protein-coding gene fragments (*Arf*, ADP-ribosylation factor; *Ole1*, delta-9 fatty acid desaturase; *H-anti*, H antigen precursor; *Tub1*, alpha-tubulin) of 137 *H. capsulatum* isolates from 25 countries, Kasuga *et al*. [6] described eight clades, seven of which were considered as phylogenetic species. The Latin American A clade shows the greatest genetic diversity and includes most of the Mexican *H. capsulatum* isolates, either from human clinical cases or naturally infected bats, whereas, the Latin American B clade consists of a clonal Argentinean *H. capsulatum* clinical isolates' population [6].

H. capsulatum infection in humans was successfully diagnosed in tissue samples using a nested-PCR assay for a fungal-specific fragment of the *Hcp100* gene. This gene encodes an *H. capsulatum* 100-kDa protein, which might be essential for fungal survival in host cells [7]. This molecular marker was validated by Maubon *et al*. [8] and by Muñoz *et al*. [9], for the diagnosis of histoplasmosis in clinical human samples. Nested-PCR of the *Hcp100* gene fragment has also been applied to detect *H. capsulatum* infection in tissue samples of captive mammals, which has been well documented in the snow leopard (*Uncia uncia*) [10]. This molecular method was also used to detect the presence of *H. capsulatum* in contaminated compost, frequently believed to be a source of fungal infection [11], and to corroborate the identification of *H. capsulatum* isolated from captive infected maras (*Dolichotis patagonum*) [12].

In general, all bat species are considered as potential reservoirs and dispersers of this pathogen in nature [4, 5, 13]. Bats can become infected irrespective of their alimentary and migratory behaviours [3, 4, 14]. However, those colonial bat species with extensive migratory routes, such as *Tadarida brasiliensis*, are probably the best candidates to spread *H. capsulatum* in the environment throughout a wide geographical area. *T. brasiliensis* form colonies comprising of thousands to millions of individuals [15], these colonies produce a large amount of droppings, thereby enhancing the fungal infection risk in their different shelters, perhaps related to high exposure to *H. capsulatum* propagules.

In the current study, we used a very sensitive and specific molecular method, nested-PCR of the *Hcp100* gene fragment, to detect the presence of *H. capsulatum* in sampled *T. brasiliensis* lungs, the primary target organ of histoplasmosis. This is the first report using nested-PCR with the *Hcp100* marker to demonstrate the presence of *H. capsulatum* in bats. The study clearly highlights the close association between bats and *H. capsulatum*, contributing to the knowledge of histoplasmosis epidemiology by recording the distribution of *H. capsulatum* infection sources related to habitats of *T. brasiliensis* migratory bats that form high-density colonies.

METHODS

Bat samples

A total of 87 *T. brasiliensis* bats were captured with mist-nets in their roosts during the day. Female and reproductive adult male bats were released, in accordance with the international rules of capture and management of this bat species, as a consequence, only young male bats of non-reproductive stage were selected for this study. Sixty-six bats came from four Mexican states: Chiapas (La Trinitaria cave, samples: M-431P, M-433P–M-437P, M-439P–M-441P), Michoacán (Isla de Janitzio cave, samples: M-444P–M-450P, M-453P), Hidalgo (El Salitre cave, samples: M-454–M-463P, M-498P–M-508P), and Nuevo León (La Boca cave, samples: M-468P–M-471P, M-473P–M-487P, M-489P–M-497P). Twenty-one bats came

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from two Argentinean provinces: Tucumán (Dique Escaba grotto, samples: M-510P–M-525P) and Córdoba (Cemetery tunnel, samples: M-AR01–M-AR05). The bats from Mexico were delivered to the laboratory, alive if possible, where they were euthanized by cervical dislocation, according to the recommendations of the Animal Care and Use Committee of the Faculty of Medicine, National Autonomous University of Mexico (UNAM), and in accordance with the Mexican Official Guide (NOM 062-ZOO-1999). Each euthanized animal was placed on dry ice until necropsy. Animals captured in Argentina were euthanized in situ by cervical dislocation and their organs were preserved in 70% ethanol and sent to Mexico for DNA extraction. All captured animals were assigned a code number and they were prepared as described previously [3]. Data regarding sex, somatic measures, reproductive condition, weight, and age (determined by the nature of the hair and ossification of their phalanges) for all captured bats, from Mexico or Argentina, were recorded by bat researchers from the Instituto de Ecología, UNAM, Mexico, and from the Instituto Lillo, Tucumán, Argentina. All materials were identified and prepared as described by Anthony [16] and Handley [17]. Taxonomic determination was performed according to Hall [18] and Wilson & Reeder [19].

Bat lung samples were removed aseptically from all animals and frozen at −20 °C until DNA extraction. Systematically, each lung was processed for isolation of *H. capsulatum*, as described previously by Taylor *et al*. [3]. Briefly, the lung was homogenized in 0·1 mM phosphate-saline buffer (pH 7·2), supplemented with 50 mg/ml streptomycin and 100 U/ml penicillin, centrifuged at 300 ***g*** for 10 min, and 0·1 ml supernatant was placed on mycobiotic and brain heart infusion slants (Bioxón, México DF), containing 0·02% Bengal Rose to reduce non-pathogenic fungal contamination. Plates were incubated at 28 °C and checked daily during 6 weeks for fungal growth.

DNA samples

Each DNA sample was extracted from bat lungs using DNeasy Blood & Tissue kit (Qiagen Inc., USA) according to the manufacturer's instructions. Negative controls (Milli-Q water) were included in the extraction procedure to detect any contamination. Each extracted DNA sample was frozen at −20 °C and screened for *H. capsulatum*, using nested-PCR targeting a fragment of the *Hcp100* protein coding gene.

Nested-PCR assay of the *Hcp100* gene

This assay was performed as described by Bialek *et al*. [7] with minor modifications implemented by Taylor *et al*. [11], which did not change the specificity and sensitivity of the *Hcp100* marker. Two sets of primers, described by Bialek *et al*. [7], were used: the outer primer sets Hc I (5′-GCGTTCCGAGCCTTCCACC-TCAAC-3′) and Hc II (5′-ATGTCCCATCGGGC-GCCGTGTAGT-3′) delimit a 391-bp fragment of the gene; the inner primer sets Hc III (5′-GAGA-TCTAGTCGCGGCCAGGTTCA-3′) and Hc IV (5′-AGGAGAGAACTGTATCGGTGGCTTG-3′) delimit a 210-bp fragment unique to *H. capsulatum*. DNA amplification was conducted in a PerkinElmer Cetus DNA thermal cycler (USA) and the first PCR was set up in a 25-μl reaction mixture containing 200 μM of each dATP, dGTP, dCTP, and dTTP (Applied Biosystems Inc., USA), 2 mM $MgCl_2$, 100 pmol of each outer primer, 1 U *Taq* DNA polymerase (Applied Biosystems), and 4 μl of bat lung DNA template, which was tested at different concentrations to achieve the fungal DNA concentration required for the amplification. For the second PCR (nested reaction) the mixture consisted of 200 μM of each dNTP, 2 mM $MgCl_2$, 100 pmol of each inner primer, 1 U *Taq* DNA polymerase, and 2 μl of the first reaction product which was used as template. Cycling conditions for the first and nested reactions were performed as described previously [7]. Amplification products were electrophoresed. A 100-bp DNA ladder (Gibco Laboratories, USA) was used as molecular size marker. Heterologous DNA (20 ng) from other fungal species such as *Aspergillus fumigatus*, *Coccidioides immitis*, and *Sporothrix schenckii* (a kind gift from Dr Reyes-Montes, UNAM) together with rat and mouse lung DNA samples at different concentrations were processed as non-related DNA templates and Milli-Q water was used as a negative control. Positive amplification control was performed with DNA (20 ng) of the EH-53 *H. capsulatum* strain from a Mexican clinical case. The presence of PCR inhibitors was ruled out, since a positive control from a tissue sample of a naturally infected bat, confirmed by *H. capsulatum* culture isolation, always amplified the *Hcp100* marker generating the expected sequence.

After ethidium bromide (0·5 μg/ml) staining, the bands were visualized with a UV transilluminator and

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images were captured as TIFF files. Nested-PCR products were purified using QIAquick® PCR purification kit (Qiagen) and sent to the Molecular Biology Laboratory, Institute of Cellular Physiology (UNAM, Mexico) for sequencing in an ABI-automated DNA sequencer (Applied Biosystems). Sequencing of each amplified product was the main criterion to confirm fungal presence in bat lungs.

Sequences

Sequencing reactions were performed for forward and reverse DNA strands and a consensus sequence for each amplified bat lung sample product was generated. Sequences were trimmed to ensure that all sequences had the same start and end-point. Next, they were aligned and compared using MEGA software, version 4.0 [20]. Alignment consisted of the generated sequences from bat samples together with the *Hcp100* sequence of the G-217B strain from Louisiana, USA (GenBank accession no. AJ005963) being used as reference for base positions. Pairwise and multiple parameters were established for a gap opening penalty of 15 and a gap extension penalty of 6·66; transition weight was 0·5; the use of negative matrix was off, and the delay divergent cut-off was 30%.

Neighbour-joining (NJ) [21] and maximum parsimony (MP) [22] analyses for *Hcp100* gene fragments were performed, using MEGA-4 [20] and the G-217B sequence (GenBank) as reference. The NJ tree was drawn to scale, with branch lengths in the same units as those of the genetic distances used to infer the tree. Genetic distances were computed using the Kimura [23] two-parameter model. The MP method [22] was performed, using the close-neighbour-interchange algorithm [24]. NJ or MP bootstrap values [25] for internal branches were generated by 1000 replicates.

BLASTn and BLASTx algorithms were used to search the GenBank database for homologous nucleotide sequences and putative proteins, respectively, corresponding to the nested-PCR product of the *Hcp100* gene fragment from the sequences of bat lung samples analysed.

Statistical approach

The total infection rate was estimated taking into account all studied bats from those bats that had the fungus identified by sequencing of the *Hcp100* marker. A similar estimation was conducted for bat samples from either Mexico or Argentina. The corresponding 95% confidence interval (CI) was calculated by normal distribution.

Table 1. *Number and percentage of* H. capsulatum*-infected bats from different regions of Mexico and Argentina*

Mexico				Argentina	
CS	MN	HG	NL	Tucumán	Córdoba
Infected (captured) bats, *n*					
6 (9)	6 (8)	13 (21)	27 (28)	16 (16)	3 (5)
Infected bats (%)					
66·6	75	61·9	96·4	100	60

CS, Chiapas; MN, Michoacán; HG, Hidalgo; NL, Nuevo León.

RESULTS

Based on the molecular detection of a 210-bp fragment from the *H. capsulatum Hcp100* gene of 87 *T. brasiliensis* individuals studied, 71 bats were considered as probably infected; of these, 52 bats came from four different states of Mexico (six from Chiapas, six from Michoacán, 13 from Hidalgo, 27 from Nuevo León) and 19 bats came from Argentina (16 from Tucumán, three from Córdoba) (see Table 1). Sixteen samples did not amplify the *Hcp100* marker. Three *H. capsulatum* isolates were cultured from *T. brasiliensis* lung samples (M-431P, M-436P, M-485P) of bats captured in Mexico.

A total frequency of 81·6% (95% CI 73·4–89·7) of infection was registered taking into account all studied bats; discriminating for Mexico gave a 78·8% (95% CI 68·9–88·7) infection rate from 66 bats analysed, and for Argentina a 90·4% (95% CI 75·2–100) infection rate from 21 bats studied. The numbers of infected bats according to their capture region in relation to the total number of captured bats studied, as well as the percentages of infection in each bat capture region, are presented in Table 1.

Figure 1 illustrates amplified and non-amplified lung DNA samples for the 210-bp *Hcp100* nested reaction products. In all PCR assays, non-specific bands were never observed. The DNA from EH-53 strain (positive control) amplified the expected 210-bp product in all assays. The same was observed with the non-inhibition control (data not shown), whereas Milli-Q water and/or non-related DNA templates from heterologous fungi, as well as from rat and mouse lungs, did not amplify the *Hcp100* marker.

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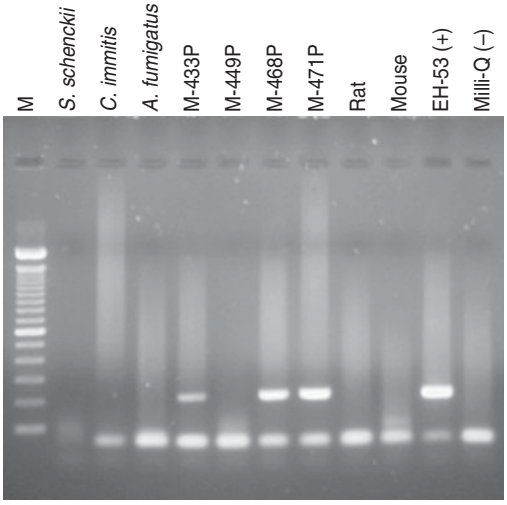

Fig. 1. Representative amplification of the *Hcp100* fragment by nested-PCR. A 100-bp DNA ladder was used as a molecular size marker (M). Heterologous DNA from other fungi and rat and mouse lung DNA samples were used as non-related DNA template. EH-53, Positive control; Milli-Q water, negative control.

Hcp100 specific DNA sequences were detected in the lungs of 71 bats sampled. These sequences were deposited in the GenBank database and bat samples with their respective accession numbers are given in Supplementary Table S1 (available online). Sequences were aligned from 2345 to 2500 (156 nt), using the *Hcp100* sequence of the G-217B strain as reference (Fig. 2), and they revealed four point mutations: thymine substituted by cytosine at position 2436 in all studied sequences; adenine substituted by guanine at position 2353 in sample M-445P (from the state of Michoacán, Mexico); cytosine substituted by thymine at position 2427 (sample M-AR03P from Argentina); and adenine substituted by guanine at position 2466 in samples from two Mexican states (M-490P–M-495P, and M-497P from Nuevo León, as well as M-503P from Hidalgo).

Based on NJ and MP analyses of 71 *Hcp100* sequences of *H. capsulatum* from bat lung samples, two clades are depicted in Figure 3. The optimal NJ tree was generated with the sum of branch length = 0·07. The MP data were: most parsimonious tree (MPT) = 170; tree length (L) = 4·0; consistency index (CI) = 1·0; retention index (RI) = 1·0, and rescaled consistency index (RC) = 1·0.

Clade I, in NJ or MP, was formed by 63 sequences of captured bats from Mexico and Argentina, which included 61 sequences with a common point mutation and two sequences, M-445P from Mexico and M-AR03P from Argentina, with one additional mutation site for each (Figs 2 and 3), achieving a 99% similarity in these sequences. Moreover, the sequence of G-217B strain, used as reference, was also included in this clade (Fig. 3). Clade II, supported by bootstraps of 64% and 99% for NJ and MP, respectively, was formed with eight sequences of captured bats from Mexico with a particular mutation (adenine substituted by guanine) (Figs 2 and 3). Seven of these sequences (M-490P–M-495P, M-497P) corresponded to La Boca cave in the state of Nuevo León and one (M-503P) from El Salitre cave in the state of Hidalgo (Fig. 3).

According to a search of the nucleotide database, using the BLASTn algorithm, all 71 sequences (156 nt each) of bat lung samples had 99% similarity with the corresponding sequence fragment of the *H. capsulatum* G-217B reference strain (GenBank), 83% similarity for the sequence of a transcription factor of *Ajellomyces dermatitidis* SLH14081 strain (GenBank accession no. XM_002628281), and 75% similarity for the fragment sequence of a hypothetic protein of *Paracoccidioides brasiliensis* Pb01 strain (GenBank accession no. XM_002793843).

A search of the protein database using the BLASTx algorithm identified a putative protein fragment of 51 amino acids (aa), corresponding to an open reading frame of 153 nt (2347–2499 nt), which is contained in the 156-nt *Hcp100* amplification product of the 71 *H. capsulatum* sequences tested. Most putative proteins resulting from the lungs of bats showed 100% identity with a fragment of 51 amino acids (731–781 aa) from a total 890 amino-acid sequence of the 100-kDa protein of the *H. capsulatum* G-217B strain (GenBank accession no. CAA06786), with only one exception (asparagine substituted by aspartic acid at position 733) for the putative protein of the M-445P sample (Michoacán, Mexico), which reached 99% identity (data not shown). The 51 amino-acid fragment had 91% identity with a transcription factor protein sequence of *A. dermatitidis* (GenBank accession no. XP_002628327) and 87% identity with a hypothetical protein of *P. brasiliensis* (GenBank accession no. EEH42596).

DISCUSSION

Molecular methods constitute a new tool to detect diverse microorganisms that infect bats and other mammals. The presence of *H. capsulatum* in bats could provide new epidemiological data concerning fungal genetic diversity in bat species and information

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          2345     2353
G-217B    TACGGAGAAACGACCGTGAAGCGAAAAAAGCCGACGTCGTTTACATCGACTACGGCAACTCCGAAACCGTTCCGTGGACC
M-445P    ........G.....................................................................
61-seq    ..............................................................................
M-490P    ..............................................................................
M-491P    ..............................................................................
M-492P    ..............................................................................
M-493P    ..............................................................................
M-494P    ..............................................................................
M-495P    ..............................................................................
M-497P    ..............................................................................
M-503P    ..............................................................................
M-AR03P   ..............................................................................

            2427      2436                                2466                          2500
G-217B    CGCCTCCGGCCTCTCACGCAGCCGCAGTTTTCCGTGCAGAAAATTCGACCCCAAGCCACCGATACAGTTCTCTCCT
M-445P    ...........C..................................................................
61-seq    ...........C..................................................................
M-490P    ...........C.............................G....................................
M-491P    ...........C.............................G....................................
M-492P    ...........C.............................G....................................
M-493P    ...........C.............................G....................................
M-494P    ...........C.............................G....................................
M-495P    ...........C.............................G....................................
M-497P    ...........C.............................G....................................
M-503P    ...........C.............................G....................................
M-AR03P   ..T........C..................................................................
```

Fig. 2. Sequence alignment of 156-nt *Hcp100* fragments amplified from *T. brasiliensis* lung samples. The *Hcp100* sequence from the G-217B strain (GenBank) was used as reference. Point mutations are indicated by nucleotide abbreviations. Sixty-one identical sequences are represented by one aligned sequence, whereas sequences with different mutations are shown.

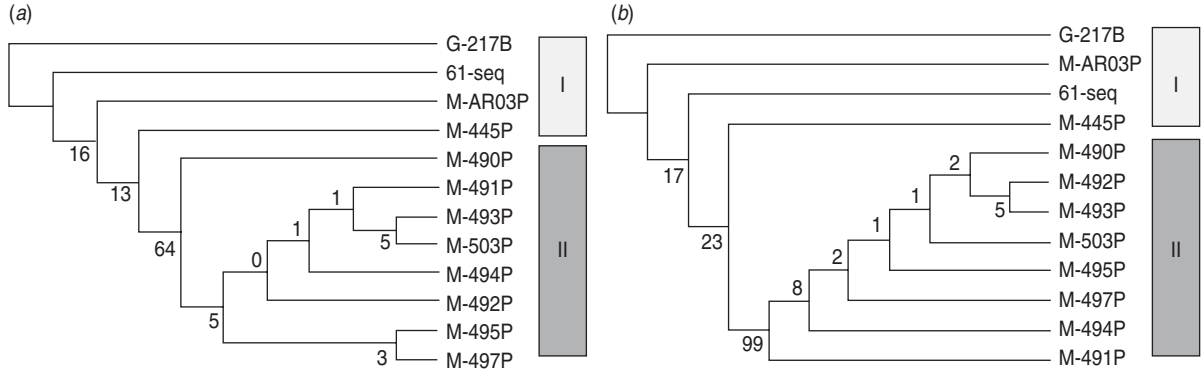

Fig. 3. Unrooted neighbour-joining and maximum parsimony trees of *Hcp100* sequences amplified from *T. brasiliensis* lung samples. (*a*) Neighbour joining; (*b*) maximum parsimony. The *Hcp100* sequence from the G-217B strain (GenBank) was used as reference. The bootstrap support values are represented below tree nodes.

regarding their behaviour related to their shelters and lifestyle, as has been suggested by Taylor *et al*. [3]. It is essential to recognize the importance of wild bats in spreading this fungus in nature and the respective repercussions for human disease. The association between bats and *H. capsulatum* had even been observed before the 1960s [26–28], and throughout the 1980s, other studies have also been published on this subject [14, 29–33]. However, this paper is the first to report on the use of molecular screening to detect the presence of *H. capsulatum* in a large sample (87 individuals) of the insectivorous migratory bat *T. brasiliensis* (Chiroptera: Molossidae), which is one of the most abundant bat species in the Western Hemisphere [15, 34–36], and therefore of importance for the study of bats from different geographical origins from Latin America. The findings described indicate the utility of the molecular marker studied, a unique *Hcp100* gene fragment that successfully identifies the presence of *H. capsulatum* in tissues of captured wild animals, supporting the role of this marker as an available epidemiological tool, as has been demonstrated in histoplasmosis diagnosis in human tissues [7–9].

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The present results emphasize an important percentage of *T. brasiliensis* infection with *H. capsulatum*, in either Mexican or Argentinean bats, representing a substantial rate of $\geqslant 60\%$ infection (Table 1). Establishing the presence of *H. capsulatum* in a wide number of lung samples of *T. brasiliensis* was our main aim. Young male bats in non-reproductive stage were selected for this purpose, according to the criterion mentioned in the Materials and Methods section. Further, based on our experience with other animal models, young male mice are one of the most susceptible hosts for experimental histoplasmosis [37, 38], which allow us to infer that young male bats may also be susceptible hosts for *H. capsulatum* infection compared to adults. Although the young male bat cohort is not representative of bat populations, our findings highlight the high risk of infection of *T. brasiliensis* bats and emphasize the role of bats as reservoirs and dispersers of this fungus. In addition, the high percentages of infected bats could be associated with the high density of *T. brasiliensis* colonies [15], enhancing their infection risk. Moreover, physical conditions of bat shelters could influence the infection risk factor for humans and bats, as has been documented by Taylor *et al*. [3].

Despite the fact that fungal isolation is the gold standard for demonstrating a state of fungal infection, this method has a major limitation in its low sensitivity (a high fungal burden is needed in tissue samples to obtain a successful fungal isolation). Moreover, in environmental samples, contamination with other fast-growing microorganisms interferes with a favourable *H. capsulatum* isolation process. *H. capsulatum* isolation in nature is an unusual finding and, in general, the rate of fungal isolation from bat droppings and/or other contaminated soil, as well as from infected bats, is low [14, 30–33, 39, 40]. However, a high infection rate of 66% in bats captured in Mexico was reported by Taylor *et al*. [3], through *H. capsulatum* cultures from different bat organs.

Although fungal isolation in organs of captured bats is a fortuitous event, three *H. capsulatum* isolates were recovered from cultured bat lungs. Assays were also performed to isolate *H. capsulatum* from other bat organs such as spleen, liver, and intestine, of which we also obtained three isolates (two from spleen and one from liver) of all *T. brasiliensis* bats captured in Mexico (data not shown), confirming the low fungal burden in the tissue of the bats studied. Correlation between mycological and molecular findings was very low, since only a small number of positive fungal cultures were attained. Molecular methods have been implemented in recent years to detect the presence of fungal pathogens in tissue samples of infected hosts, especially for the molecular diagnosis of histoplasmosis. For this reason, for the detection of *H. capsulatum* in bat tissues we selected a unique molecular marker (*Hcp100* gene fragment) which has been shown to be an excellent tool for revealing the presence of *H. capsulatum* in infected clinical samples [8, 9]. The *Hcp100* marker has a higher sensitivity than fungal isolation in culture media, irrespective of fungal burden in the host, supporting the use of this molecular marker for detection of the pathogen's presence in bat tissues. The high specificity of the *Hcp100* marker was corroborated by absence of non-specific bands, as well as by non-amplification of heterologous DNA from either other fungi or mammalian hosts and negative controls, as can be seen in Figure 1.

A 156-nt sequence included in the 210-bp fragment of the *Hcp100* marker was sufficient to reveal similarity in the sequences obtained from the lungs of bats. Hence, 61 bat lung samples presented identical sequences, supporting a high similarity in these samples. Moreover, 10 sequences, mostly from bats captured on the same date in the La Boca cave, and having an additional mutation site, also remained closely related to all sequences studied. Detected mutations are not due to sequencing errors, since they appeared in several *Hcp100* fragments and were confirmed in both DNA strands. The sequencing of 71 products of the 156 nt sequence revealed high similarity with the corresponding fragment of the *H. capsulatum Hcp100* gene from the G-217B strain (GenBank), confirming that all *Hcp100* sequences from the bat samples belong to *H. capsulatum*.

Despite the *Hcp100* marker showing low polymorphism, two clades associated with infected bats were identified based on the topologies of the NJ and MP trees. Analyses of NJ and MP topologies revealed differences in the studied sequences, irrespective of the geographical origin of *T. brasiliensis* bats sampled in Mexico. It is important to emphasize that some bat lung samples from Nuevo León (M-490P–M-495P, M-497P) and Hidalgo (M-503P), Mexico, diverge from other studied lung samples (clade II). These findings suggest that the clade II *Hcp100* genotype, from the Nuevo León and Hidalgo samples, is associated with distinct shelters for bat infection from these regions, and it may be related to *T. brasiliensis*

migration routes, which are shared throughout the Mexican territory [35, 36].

BLASTn analysis of the 156-nt sequences from 71 bat lung samples revealed the highest similarity for the *H. capsulatum* G-217B strain, followed by the *A. dermatitidis* SLH14081 strain and *P. brasiliensis* Pb01 strain (now classified as *P. lutzii* according to Teixeira *et al.* [41]), which supports the specificity of the 156-nt fragment to detect *H. capsulatum*.

Similarly, a BLASTx analysis also revealed identity in most protein fragments resulting from the sequences of the *Hcp100* marker amplified from the lungs of infected bats and the 100-kDa protein of the *H. capsulatum* G-217B strain, despite three silent mutations being found in most of the sequences analysed. A non-silent mutation (asparagine substituted by acid aspartic at position 2353) of sample M-445P (Michoacán, Mexico) has no known biological implication. BLASTx analysis also supports the specificity of the 156-nt fragment to detect *H. capsulatum*.

Wild bats have a high risk of infection with airborne propagules from this pathogenic fungus that are found in their shelters. Although bats are easily infected, they do not normally develop a severe course of the disease [3]. This study has a critical relevance that should be noted by specialized researchers who work with *H. capsulatum* and bats, which are considered potential reservoirs of this fungus and play an important role in the spread of *H. capsulatum* in the environment [3–5, 13].

NOTE

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/hyg).

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DECLARATION OF INTEREST

None.

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