Multilocus sequence typing of *Histoplasma capsulatum* in formalin-fixed paraffin-embedded tissues from cats living in non-endemic regions reveals a new phylogenetic clade

S. ARUNMOZHI BALAJEE*, STEVEN F. HURST†, LORETTA S. CHANG†‡, MACON MILES§, EMILY BEELER#, CHRISTA HALE‡¶, TAKAO KASUGA+, KAITLIN BENEDICT‡, TOM CHILLER† & MARK D. LINDSLEY†

*Center for Global Health, Centers for Disease Control and Prevention, Atlanta GA, †Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta GA, ‡Epidemic Intelligence Service, Centers for Disease Control and Prevention, Atlanta, GA, §Los Angeles County Department of Public Health, Los Angeles, CA, †Colorado Department of Public Health and Environment, Denver, CO, +USDA-ARS, Department of Plant Pathology, Hutchison Hall, University of California, Davis, CA, and §Animal Emergency and Referral Center, Torrance, CA, USA

Infections caused by *Histoplasma capsulatum* are found most often in endemic regions of North, Central, and South America. *H. capsulatum* has been divided into eight geographic clades by multi-locus sequence typing (MLST). Recently, one isolate and five formalin-fixed paraffin-embedded (FFPE) tissue samples were received from six of 15 suspected cases of histoplasmosis in cats residing in areas not known to be endemic for *H. capsulatum*. Polymerase chain reaction (PCR) amplification and sequence analysis of the rDNA ITS-2 region confirmed the diagnosis of *H. capsulatum*. Since these cases were not, as noted, from the accepted endemic areas, it was of interest to understand the molecular epidemiology of these isolates. Results of molecular analysis indicated that the *H. capsulatum* recovered from the cats were most closely related to the North American-1 clade, but clustered separately outside this clade, suggesting that the *H. capsulatum* infecting the animals may represent a separate clade or phylogenetic species. This study also demonstrated the utility of obtaining valuable molecular subtype data directly from archived FFPE tissue blocks, particularly when a fungus culture was not performed or is otherwise unavailable.

**Keywords** FFPE tissue, feline histoplasmosis, *H. capsulatum*

---

**Introduction**

*Histoplasma capsulatum* has been demonstrated to cause infections in both wild (mice, rats, foxes, raccoons, bats, opossum, and skunks) and domesticated (cattle, horses, dogs and cats) animals [1]. As the second most common fungal infection in cats [2], histoplasmosis usually presents with non-specific signs and symptoms including malaise, anorexia, weight loss, fever, dyspnea, and enlarged liver, spleen, and/or lymph nodes. Involvement of the gastrointestinal tract may occur as a consequence of disseminated infection, resulting in diarrhea and vomiting. Gastrointestinal infection may also occur through ingestion of conidia when no other signs of disseminated disease are present [3].

*H. capsulatum* is found worldwide but is most frequently observed in endemic regions of the Mississippi and Ohio River valleys in North America and in regions of Central and South America.

Multi-locus sequence typing (MLST) has divided the species into the following eight geographically separate clades: North American-1, North American-2, Latin American group A, Latin American group B, Australian, Netherlands (of Indonesian Origin), Eurasian, and African [4]. Thus far, all MLST characterizations of *H. capsulatum* have been performed using genomic DNA extracted from fungal isolates and not from formalin-fixed, paraffin-embedded (FFPE) tissues. Polymerase chain reaction (PCR) amplification using DNA from FFPE tissue can be
severely limited due to varying degrees of DNA fragmentation and cross-linking resulting from the type of formalin used and the length of time tissues are exposed to the fixative [5–8].

Recently, cases of suspected histoplasmosis were reported in cats residing in locations that are traditionally considered non-endemic for *H. capsulatum* (California [CA], Colorado [CO], New Mexico [NM], and Texas [TX]) from which FFPE tissues were available for further investigation. Since a MLST approach and a well-populated sequence database were already available for *H. capsulatum* [4], this study sought to use a slightly modified MLST strategy to better understand the molecular epidemiology of the cat isolates. Results of the study demonstrated the presence of a unique clade of *H. capsulatum* causing infections in these cats and the utility of the novel MLST approach directly using FFPE tissue.

**Materials and methods**

**Cat cases and specimen collection**

Between February 2008 and August 2011, 15 cases of suspected histoplasmosis were reported in cats residing in locations not known to be endemic for *H. capsulatum* (CA, CO, NM, TX). Specimens were received from six cats, i.e., three from Colorado, two from California and one from Texas (Table 1). Only one cat had lived in a state other than its current residence. While it is now living in California, it had previously resided in Washington State, another state not known to be endemic for *H. capsulatum*. For five cats, presumptive diagnosis of histoplasmosis was made through postmortem histopathologic examination of FFPE tissues and one was diagnosed by isolation of *H. capsulatum* in culture from a lymph node aspirate.

**Extraction of DNA**

**DNA extraction from culture isolates.** Extraction of DNA from the isolate retrieved from the cat and two control *H. capsulatum* isolates (CDC B0896 and CDC B0898) was performed using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) with the following modifications. The paraffin removal was performed through the DNeasy Mini spin column. The column was then washed and DNA eluted as described by the manufacturer.

**Extraction of DNA from FFPE tissue.** Tissues were sectioned on a Leica RM2145 microtome (Leica Microsystems Inc., Buffalo Grove, IL, USA). To reduce the risk of cross contamination several measures were employed, including cleaning all surfaces of the microtome, utensils used in handling the tissue sections, and laboratory bench with DNA Zap (Ambion, Applied Biosystems, Foster City, CA, USA) prior to sectioning and between each sample. In addition, sections were prepared using a fresh blade for each sample and the first five sections were discarded prior to collecting five sections of 5 μm thickness each. The sections were placed into a 2 ml microcentrifuge tube and genomic DNA was extracted from the sections using the Qiagen FFPE tissue extraction kit (Qiagen Inc. Valencia, CA, USA) according to the manufacturer’s instructions and as described by Munoz-Cadavid et al. [9], with the following modifications. The paraffin removal was performed using Pro Par Cleartant (Anatech, Battle Creek, MI, USA), were then placed into a 55°C water bath for 1 h, vortexing the tubes every 10–15 min. Once the tubes had cooled to room temperature, 88 U of ribonuclease (RNase, Sigma, St Louis, MO, USA) was added and incubated for 5 min at RT. After this step, 560 μl of buffer AL was added, the tube vortexed three times and then placed in a 70°C water bath for 10 min. The entire volume of the tube was transferred to a 2.0 ml plastic microcentrifuge tube and centrifuged at 10,620 g. The supernatant was transferred to a second tube and a volume of absolute ethanol equaling 50% of the transferred buffer was added to the tube and the tube vortexed three times. Five hundred microliters of the lysate was added to a DNeasy Mini spin column and centrifuged for 1 min at 6,000 g. The supernatant was transferred to a second tube and a volume of buffer AL equaling 2 μl was added. The tube was vortexed three times and then placed in a 55°C water bath for 10 min. The entire volume of the tube was transferred to a 2.0 ml plastic microcentrifuge tube and centrifuged for 10 min at 10,620 g. The supernatant was transferred to a third tube and 20 μl of DNA Zap was added. The tube was vortexed three times, centrifuged for 1 min at 6,000 g, vortexed again, and centrifuged for 1 min at 6,000 g. The newly removed DNA was then washed with 70% ethanol, air-dried, and resuspended in 10 μl of TE buffer.

### Table 1 Information regarding the six cats for which specimens containing *Histoplasma capsulatum* were available.

<table>
<thead>
<tr>
<th>State of residence and cat ID number</th>
<th>Age (yrs)</th>
<th>Specimen</th>
<th>Fatal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO-1</td>
<td>13</td>
<td>Mix</td>
<td>Y</td>
</tr>
<tr>
<td>CO-2</td>
<td>13</td>
<td>Mix</td>
<td>Y</td>
</tr>
<tr>
<td>CO-3</td>
<td>4</td>
<td>Spleen</td>
<td>Y</td>
</tr>
<tr>
<td>CA-1a &amp; b</td>
<td>9</td>
<td>Liver &amp; lung</td>
<td>Y</td>
</tr>
<tr>
<td>CA-2</td>
<td>6</td>
<td>Culture Isolate (from lymph node)</td>
<td>N</td>
</tr>
<tr>
<td>TX-1</td>
<td>1.5</td>
<td>Lung</td>
<td>Y</td>
</tr>
</tbody>
</table>

*CO, Colorado; CA, California; TX, Texas. FFPE tissue block contained a sample of each organ: heart, lung, small intestine, kidney, liver, and spleen. Cat CA-1 had separate blocks submitted for each tissue.

© 2013 ISHAM, *Medical Mycology*, 51, 345–351
proteinase K digestion was incubated for 2 h at 56°C, vortexing every 15–20 min, and an additional 45 min incubation at 37°C with 2U recombinant lyticase/100 μl (Sigma-Aldrich Corporation, St Louis, MO, USA) [9] was performed after the 90°C incubation step. The DNA extract was further treated with 32U/200 μl RNase (R4642, Sigma-Aldrich) for 2 min at 24°C, prior to binding onto Qiagen columns, which were then washed and the DNA eluted as described by the manufacturer.

**PCR amplification and DNA sequencing**

Amplification and sequencing of the ITS2 region (350 bp) of the rDNA gene loci was initially performed to confirm the identity of the *Histoplasma*-like yeast cells observed in the tissue. Five microliters of the DNA extract was placed into 45 μl of PCR buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 1.5 U of *Taq* polymerase (Roche Diagnostics, Indianapolis, IN, USA), and 0.2 μM of each of the forward and reverse primers, ITS-3 and ITS4 [10]. Thermocycling was performed using a Gene Amp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), including an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min and then a final step at 72°C for 10 min. The PCR products were visualized on a UV transilluminator after electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide.

Prior to sequencing, PCR products were purified with either ExoSap-It (USB Corporation, Cleveland, OH, USA) or Thermo Scientific Ultra PCR Cleanup Kit (Thermo Fisher Scientific, Waltham MA, USA) following the manufacturer’s instructions. DNA sequencing was performed using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems) and the resulting sequences were edited and aligned using Sequencher version 4.8 software (Gene Codes Corp., Ann Arbor, MI, USA).

**Generation of modified *H. capsulatum* MLST sequence primers for use with DNA from FFPE tissue**

For sub-typing directly from FFPE tissue, the MLST method proposed by Kasuga et al. [4] was modified so the primer sets would amplify smaller regions of the three *H. capsulatum* MLST loci [arf (ADP-ribosylation factor), *H*-anti (H-antigen precursor), and *tub1* (α-tubulin)], yet still be discriminatory. The redesigned primers were developed using Primer 3 software [11]. Table 2 shows the primer sequences generated for the amplification the arf, *H*-anti and *tub1* genes, respectively. The newly designed MLST primers were first analyzed for cross reactivity with human and cat DNA by comparing to human and cat DNA sequences deposited in GenBank (NCBI) using Basic Local Alignment Search Tool (BLAST). The primers were evaluated by PCR amplification and DNA sequencing using genomic DNA from two *H. capsulatum* isolates, CDC B0896 and CDC B0898. PCR amplification using these MLST modified primers was performed, as noted above using the following cycling conditions: an initial denaturation of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, and a final extension of 72°C for 5 min. The number of cycles was increased to 45 cycles for use with DNA extracted from the FFPE tissue.

**Analysis of MLST generated data**

MLST sequences from 82 *H. capsulatum* strains representing the eight *H. capsulatum* clades were downloaded from the TreeBase database (http://www.treebase.org). These sequences and the *H. capsulatum* sequences isolated from the cat tissue were trimmed to the sizes derived from the redesigned primers and concatenated. All the sequences were aligned and edited using BioEdit version 7.0.9.0 [12] and then analyzed by Maximum Likelihood and Neighbor-Joining (NJ) tree using Phylip (version 3.68). Sequences of each MLST loci from the six cat tissues were deposited in GenBank under the accession numbers JX033948 through JX033965.

**Results**

**Diagnosis of histoplasmosis**

Five of the six cats were diagnosed with and eventually died from progressive disseminated histoplasmosis. Cat CA-2, the only cat to have a more localized infection with tarsal swelling and lameness, was treated with

![Table 2: Primers used for PCR amplification and sequencing of the *Histoplasma capsulatum* MLST gene loci.](https://academic.oup.com/mmy/article-abstract/51/4/345/1026477/347)
itraconazole and recovered from the infection. The diagnosis of histoplasmosis in the five cats that died was made during histopathologic examination of postmortem tissues, showing small budding yeast-like cells morphologically consistent with *H. capsulatum*. Fungal cultures were never ordered in these cases. The mould isolate from the sixth cat was identified by the local laboratory as *H. capsulatum* through the conversion of the mold to the yeast form at 37°C. The diagnosis of histoplasmosis was confirmed in our laboratory by PCR amplification and DNA sequencing of the internal transcribed spacer region 2 (ITS-2) in five of the six cases. DNA extracted from one FFPE tissue case did not amplify using the ITS-2 region primers. The diagnosis of histoplasmosis was confirmed in this case by PCR amplification using the primer sets of each of the three *Histoplasma* MLST gene loci and identified as *H. capsulatum* through BLAST analysis in GenBank.

**Evaluation of the modified *H. capsulatum* MLST sequence primers**

The redesigned primers for *arf*, *H-anti* and *tub1* MLST targets demonstrated no significant cross reaction with human or cat genomic DNA sequences. When evaluated using DNA extracted from known *H. capsulatum* isolates CDC B0896 and CDC B0898, the redesigned MLST primers amplified the three loci to the appropriate size (data not shown), with both isolates, and generated sequences matching those of similar *H. capsulatum* isolates.

Genomic DNA was extracted from all five FFPE tissue samples and successfully amplified using the three MLST primer sets. MLST analysis showed that all six cat samples had very similar sequences at all three loci. Phylogenetic analysis of the *H. capsulatum* MLST gene sequences using the Neighbor Joining algorithm (Fig. 1) demonstrated that even though shorter sequences were used for analysis, their distribution into the appropriate geographic clades, as previously described [4], was not affected. Using Neighbor Joining analysis, all *H. capsulatum* MLST sequences obtained from the six cats clustered together (Fig. 1), demonstrating 97% similarity to the NAm-1 clade. MLST analysis using the Maximum Likelihood algorithm provided similar results (data not shown).

**Discussion**

FFPE tissue specimens are a valuable source of easily transportable archival material, often with detailed patient information. This study demonstrates the value of obtaining molecular subtype data directly from archived FFPE tissue blocks in cases when a fungus culture was not performed or is otherwise unavailable. Previously, PCR amplification of portions of a single protein-encoding gene or a multi-copy ribosomal gene have been employed for (i) direct detection of a dominantazole resistance mechanism in *A. fumigatus* [13], (ii) identification of diverse fungi from tissue [14], (iii) detection of *H. capsulatum* DNA in human and animal tissue [15,16], and (iv) genotyping of *Paracoccidioides brasiliensis* directly from human tissue [17]. Here, a three locus MLST strategy was employed to subtype *H. capsulatum* from tissue and demonstrate the presence of a phylogenetically distinct *H. capsulatum* causing cat infections. The ability to apply an MLST strategy directly with FFPE tissues expands the scope and reach of genetic testing to sub-typing, permitting the use of this method as a powerful diagnostic and epidemiological tool.

The ability to perform subtyping analysis directly from FFPE tissue provided important information about the geographic boundaries of *H. capsulatum* which demonstrated that the *H. capsulatum* infecting these cats represents a new or phylogenetically distinct lineage, related to but distinct from the NAm-1 clade. The *H. capsulatum* described in this study came from cats that resided outside the known endemic areas of the fungus. Only one other report described histoplasmosis in two cats residing in California [18] and there have been no reports of histoplasmosis in cats from Colorado. Cases of histoplasmosis in other non-human mammals, such as dogs and badgers, in areas not known to be endemic for *H. capsulatum*, have also been reported [19–21].

Comparison of the sequences from the NAm-1 isolates and the cat isolates revealed the presence of multiple alleles among the different MLST loci. Although the sample size is limited, no alleles were shared between NAm-1 and the cat populations. We therefore judged that these two populations are likely to be genetically isolated. NAm-1 isolates were originally isolated from humans and a skunk from Missouri and Georgia [4], whereas, in the current study, the cats were from Texas, Colorado and California, implicating that the observed genetic isolation is due to geographic distance. However, host-driven genetic differentiation cannot be excluded, at this time.

The current knowledge of the geographic range described for *H. capsulatum* was generated approximately 50 years ago using histoplasmin skin reactivity data collected from military recruits [22]. However, since then, the geographic range of *H. capsulatum* may have changed, expanding its boundaries as a result of changes in climatic and ecologic conditions. Additionally, it is possible that *H. capsulatum* is ubiquitously distributed in temperate regions of the world, but only specific environmental conditions and/or phylogenetic species, increase the risk of infection. This may explain the presence of smaller, microfocal endemic areas that have been described in regions not
Fig. 1  Neighbor Joining phylogenetic analysis of concatenated Histoplasma MLST sequences from three gene loci: arf (263bp), H-anti (291 bp) and α-tubulin (204 bp). Select Histoplasma sequences representing the different geographic clades, similarly trimmed and concatenated, are included for comparison. LAm-A, Latin America group A clade; LAm-B, Latin America group B clade; NAm-1, North America 1 clade; NAm-2, North America 2 clade. The numbers in the dendrogram represent the bootstrap value after 1000 replicates.
known for histoplasmosis including areas around rivers or lakes in more arid environments that may be frequented by birds and areas outside caves frequented by bats [21]. The MLST data from this study also suggest that *H. capsulatum* strains infecting cats and possibly other mammals may be genetically different from *H. capsulatum* strains capable of infecting humans, and may have different mechanisms of pathogenesis. Alternatively, this phylogenetic difference may reflect the region in which the isolates originated, as all the North American isolates originally used in determining these phylogenetic relationships [4] originated in states no further west than Missouri, Arkansas, and Louisiana. Detailed epidemiological and environmental investigation is necessary to further understand the *H. capsulatum* population distributed in the Western USA.

A limitation in the use of FFPE tissue for molecular studies is that the quality of DNA retrieved from the tissue is dependent on its processing. Fixative formulation and length of time that the tissue is left in the fixative can affect the amount of DNA fragmentation and cross-linking, thereby further reducing the size of amplicon which can be detected [5–8]. Kobayashi et al. [23] may have observed this effect when they were unable to amplify *Histoplasma* DNA in FFPE cat tissue using primers ITS5 and ITS4, which amplifies a ~650 bp region of the rDNA. This may further explain why the DNA extracted from tissues of one of our cats did not produce an amplification product using primers ITS3/ITS4 (~350 bp), but produced an amplification product using the MLST primers (~250 bp). Our study provides evidence that when designing primers for use with FFPE tissue, it is important to select primer(s) that produces the shortest, yet still discriminating, amplicon size. Judicious primer design can help overcome the deleterious effects of formalin fixation on tissue samples, and allow analysis of such materials.

In conclusion, a strategy designed to perform MLST using DNA extracted from FFPE tissue was described for *H. capsulatum*. Using uniquely designed PCR primers which produce the shortest possible amplicon size, a phylogenetically distinct strain of *H. capsulatum* was found to cause infection in cats. The ability to apply an MLST strategy to FFPE tissues enhances the ability to provide diagnostic and epidemiological information in cases where culture was unable to be performed.

**Disclaimer**

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Los Angeles County Department of Public Health.

**Acknowledgments**

We acknowledge Drs Ronald A. Oyster, Eric Kufuor-Mensah, Barbara E. Powers, Bruce H. Williams, and Clifton Drew for providing the FFPE tissue blocks from the cat cases. We also acknowledge the CDC Genomics Unit for their support in performing the sequencing analysis.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

**References**


© 2013 ISHAM, Medical Mycology, 51, 345–351


This paper was first published online on Early Online on 22 October 2012.


