MOLECULAR FINDINGS OF DISSEMINATED HISTOPLASMOSIS IN TWO CAPTIVE SNOW LEOPARDS (*UNCIA UNCIA*)

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Abstract: This paper reports two cases of disseminated histoplasmosis in captive snow leopards (*Uncia uncia*). Histoplasmosis was diagnosed based on histopathology, immunohistochemistry, transmission electron microscopy, and molecular findings.

BRIEF COMMUNICATION

Two young adults snow leopards died 2 days apart in a zoological park in Querétaro, Mexico. The animals had been kept for 2 yr in an open, 80-m² enclosure, provided with a 20-m² shelter with a concrete floor. The first animal died suddenly without clinical signs and was reported by the keeper as being apparently healthy the previous night. The second animal was found mildly depressed and with mild dyspnea, and it was being treated parenterally with broad-spectrum antibiotics and non-steroidal anti-inflammatory drugs. At necropsy, the attending veterinarian reported generalized lymphadenopathy and multiple coalescing white foci and nodules throughout the lung, liver, spleen, and thoracic and abdominal lymph nodes. Samples of those organs were placed in 10% buffered formalin and were sent to the Department of Pathology of the Guadalajara Zoo (Guadalajara, Mexico), where they were processed routinely to obtain 5-μm-thick paraffin-embedded tissue sections that were stained with hematoxylin and eosin. Some sections of spleen were cut, mounted on poly-L-lysine–coated slides and stained with a modified Gomori's methenamine silver nitrate method for fungi.

On light microscopy, the architecture of the liver, spleen, lung, and lymph nodes was replaced by eosinophilic cellular and karyorhectic debris, extravascular erythrocytes, fibrin, and multiple nodular aggregates of inflammatory cells characterized by numerous macrophages; few plasma cells, neutrophils, and lymphocytes; and occasional multinucleated giant cells. There was a moderate to severe proliferation of fibrous connective tissue. In all four organs examined, numerous macrophages containing few to many spherical to oval yeast measuring 2–4 μm in diameter, with slightly basophilic, centrally located 1–2-μm nucleus characteristic of *H. capsulatum* were noted. Free yeast were also observed. Gomori's methenamine silver stain revealed further morphologic details. Yeast had single buds attached to parental cells by a narrow base, or they were attached in short chains. (Fig. 1).[5] Additional microscopic findings included lymphoid depletion and lymphoid necrosis of the lymph nodes and spleen, and chronic lymphohistiocytic interstitial pneumonia and pulmonary edema.

For transmission electron microscopy (TEM), small fragments (1 mm³) of paraffin-embedded tissue sections were deparaffinized by immersion in xylene during a 24-hr period and rehydrated in a graded ethanol series (100% to 25%), processed routinely for TEM, and analyzed with a Zeiss TEM 900 (at 60 Kv).[2] Electron microscopy revealed a yeast-like morphology, with a multilayered cell wall, and a slightly electron-opaque granular cytoplasm, with a single nucleus and lack of recognizable organelles.

Immunohistochemistry to demonstrate *H. capsulatum* in tissue sections was performed after antigen retrieval treatment, as described by Shi et al.[15] Shortly after paraffin removal and rehydration, tissue sections of spleen mounted on positive-charged slides (Superfrost Plus®, Thermo Fisher Scientific, Pittsburg, Pennsylvania 19102, USA) were transferred to a plastic Coplin jar containing 0.1 M citrate buffer, pH 6.0. The slides were heated in a pressure cooker for 20 min at 200°C, and then they were placed over a hot-plate for 10 min. Slides were then placed in a Coplin jar for 15 min at room temperature (RT) and were transferred to phosphate-buffered saline (PBS), pH 7.2, for the immunostaining procedure. After the antigen retrieval treatment, endogenous peroxidase was inhibited by incubation in 3% hydrogen peroxide diluted in dis-

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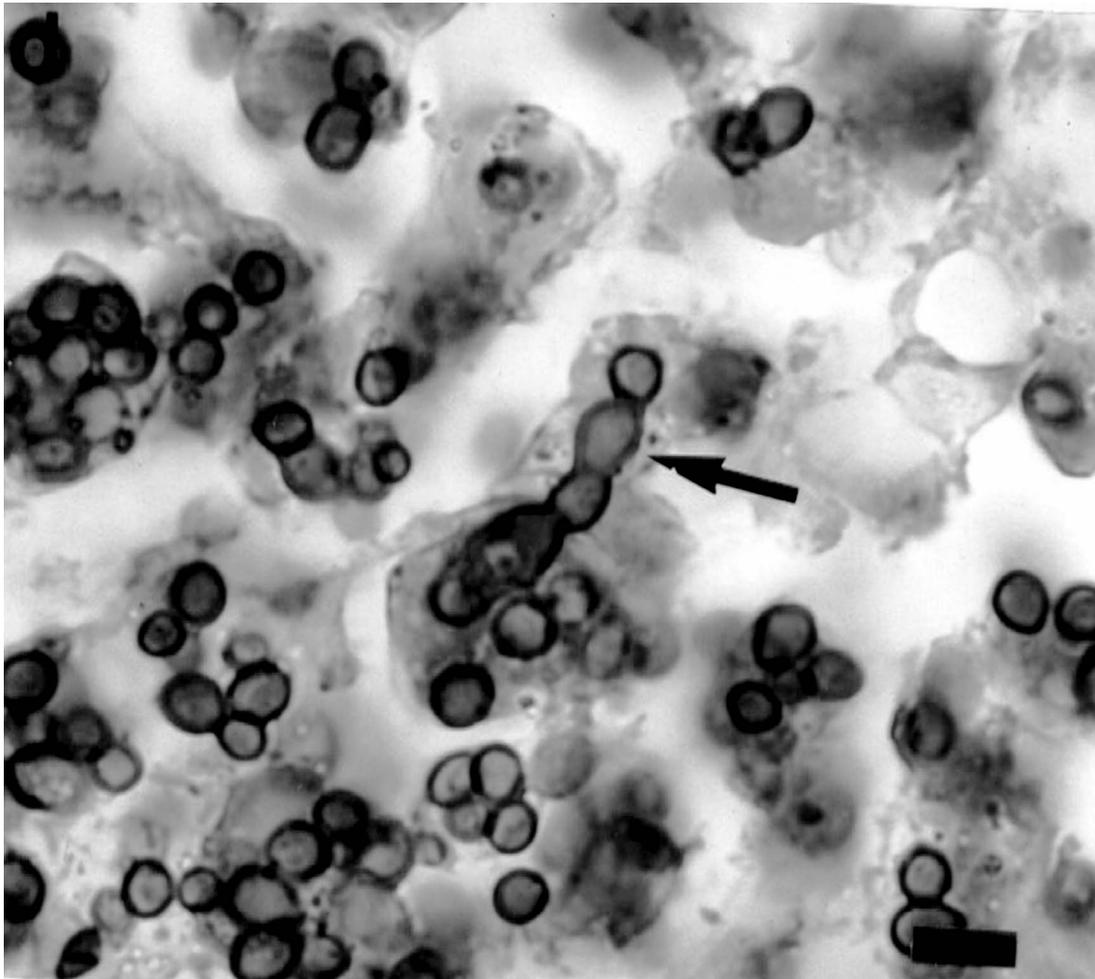
Figure 1. Spleen sections of a snow leopard with disseminated histoplasmosis. Arrows indicate *H. capsulatum* yeast. Gomori's methenamine silver stain was processed. Bar = 5 μm.

tilled water. The slides were then incubated for 1 hr at RT in a 2% PBS-0.01% normal mouse serum-Triton X-100 solution to reduce nonspecific background staining, followed by *H. capsulatum* rabbit hyperimmune serum diluted 1:50 in PBS for overnight incubation at 4°C. After three washes in PBS, slides were incubated in biotinylated mouse anti-rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, California 94080, USA) for 1 hr at RT, and then they were placed in the streptavidin-peroxidase complex and developed with diaminobenzidine as the chromogen, according to the manufacturer's recommendations (Dako North America, Inc., Carpinteria, California 93013, USA). Control slides were incubated in a normal rabbit serum. Positive staining was interpreted when intracellular and extracellular fungi showed dark

brown staining, confirming the etiologic diagnosis achieved by light microscopy.

For molecular studies, after paraffin removal, the spleen was crushed in liquid nitrogen with a mortar and pestle, and whole DNA was extracted with a commercial kit (DNeasy® Tissue, QIAGEN GmbH, Hilden 40724, Germany) based on DNA binding to silica gel membrane. The procedure was performed according to manufacturer's recommendations. Nested polymerase chain reaction (PCR) was developed as described by Bialek et al.[1] with minor modifications as described by Taylor et al.[19] Two sets of primers were used according to Bialek et al.[1] corresponding to the gene encoding for a 100-kDa protein (Hcp100) unique to *H. capsulatum*.[1,12] The outer primer set, Hc I (5′-GCGTTCCGAGCC TTCCACCTCAAC-3′) and Hc II (5′-ATGTCC
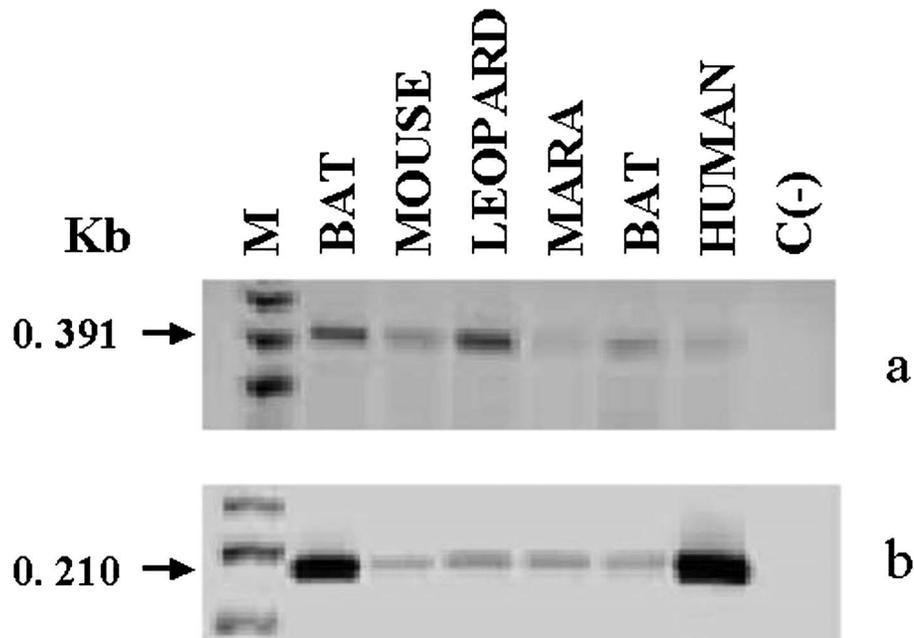
Figure 2. Nested PCR products of *H. capsulatum* in leopard tissue. The assay was performed with two sets of fungal-specific primers of the 100-kDa protein gene of *H. capsulatum*. PCR products were analyzed by electrophoresis through 1.5% agarose gels containing ethidium bromide. First (**a**) and nested (**b**) PCR reactions. M, 123-bp DNA ladder marker; bat (naturally infected with *H. capsulatum*, captured in Morelos, Mexico); mouse (experimentally infected); leopard (naturally infected in Querétaro, Mexico); mara (naturally infected in Puebla, Mexico); bat (naturally infected in Puebla, Mexico); human (clinical isolate from Guerrero, Mexico); C (–), negative control.

CATCGGGCGCCGTGTAGT-3′) delimit a 391-nucleotide sequence of the gene. The inner primers, Hc III (5′-GAGATCTAGTCGCGGCCAGGTTCA-3′) and Hc IV (5′-AGGAGAGAACTGTATCGG TGGCTTG-3′), delimit a specific 210 nucleotide sequence. The primers were supplied by Operon Technologies Inc. (Alameda, California 94501, USA). DNA amplification was performed on a PerkinElmer-Cetus DNA thermal cycler (Perkin-Elmer, Emeryville, California 94608, USA). Amplification products were electrophoresed through a 1.5% agarose in Tris borate-EDTA $0.5\times$ buffer. Electrophoresis was conducted at 90 V for 60 min. The 123-base pair (bp) DNA ladder (Invitrogen, Carlsbad, California 92008, USA) was used as a molecular marker. The bands were visualized with a UV transilluminator after ethidium bromide (0.5 μg/ml) staining. They were captured with a documentation system (GeneCam, Syngene, Cambridge, Massachusetts 02139, USA) and printed with a thermal printer (Sony 650, Sony, Tokyo 106-8620, Japan). The spleen DNA sample obtained from these snow leopards shared the same bands (0.391 and 0.210 kilobases in the first and nested amplifications, respectively) as isolates obtained from

five other *H. capsulatum* cases, including a mouse (*Mus musculus*), a mara (*Dolichotis patagonum*), and two bats (*Artibeus hirsutus* and *Leptonycteris nivalis*), and a human, that were used as positive controls (Fig. 2a, b).[14,16,19]

Microscopic findings in the present study were consistent with spontaneous disseminated histoplasmosis as described in other mammals.[3,7,8,9,11,13,14,22] The etiologic confirmation was achieved by immunohistochemistry and molecular studies.

Histoplasma capsulatum var. *capsulatum* is the causative agent of "histoplasmosis capsulati," a deep mycosis widely distributed in North and South America, Africa, eastern Asia, Australia, and occasionally in Europe.[3,4] In Mexico, the disease has been frequently associated with enclosed spaces where bats are present, and there are areas considered to be hyperendemic.[3,16,17,18] In general, the infection is benign with no overt clinical signs.[10] In symptomatic cases, four clinical forms can be observed: the mild to moderate flu-like form, the acute disseminated form affecting several organs, the chronic disseminated form that also affects several organs, and the chronic pulmonary form. The type of form an individual contracts depends on

that animal's immune function.[9,10,20] The organism is normally eliminated by a cell-mediated response and is not contagious.[10]

In animals, histoplasmosis caused by *H. capsulatum* is most commonly seen in dogs, cats, and horses involving the respiratory, gastrointestinal, or lymphoid systems.[9,10] It has also been reported in a variety of nondomestic animals, including a mara (*Dolichotis patagonum*), a sea otter (*Enhydra lutris*), badgers (*Meles meles*), a bottlenose dolphin (*Tursiops truncatus*), a chinchilla (*Chinchilla laniger*), a skunk (*Mephitis mephiti*), a two-toed sloth (*Choloepus didactylus*), a fennec fox (*Fennecus zerda*), a harp seal (*Phoca groenlandica*), and an owl monkey (*Aotus nancymai*).[3,7,8,9,11,13,14,21,22]

The animals in this report may have been exposed to a high concentration of *H. capsulatum* yeast, or they may have had an underlying dysfunction of the immune system because of stress or any other reason. A strong cell-mediated immunity is required to clear the infection in healthy animals.[10] A study of 571 cases of deep mycotic infections conducted by Davies et al.[6] showed an increased incidence of feline leukemia virus in cases of histoplasmosis. Limited availability of tissues prevented further characterization of the immune status in the animals of this report.

Although the specific immunoperoxidase reaction did not reveal the massive fungal distribution in tissues as observed with Gomori's stain, this could be explained by previous tissue processing with paraffin embedding, which hinders antigen exposure. To foster the immune reaction, a special tissue treatment was used to promote antigen retrieval and facilitate its recognition by the specific serum.[15]

Finally, molecular results support the pathologic findings. The use of a sensitive method for fungal identification, the nested PCR with the Hcp100 protein gene, confirms that the reaction was highly specific for *H. capsulatum* in tissue samples where fungi are scarcely found.

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