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First report of ocular histoplasmosis in a horse from Iran: molecular, clinical and pathological findings

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Abstract

Histoplasma capsulatum is a dimorphic fungus that is traditionally classified in three varieties: *Hc* var. *capsulatum*, *Hc* var. *duboisii*, and *Hc* var. *farciminosum* (HCF). Cytology, hematology, pathology, polymerase chain reaction (PCR), sequencing, and phylogenetic analyses were applied on samples collected from the blood and the eye of a horse with pustular lesions and ocular discharge. Physical examination and cytopathological tests showed *H. capsulatum* infection. Additionally, the results of two PCR tests confirmed *H. capsulatum* infection. The phylogenetic tree of the internal transcribed spacer sequence of Iranian *H. capsulatum* showed homology with the HCF variety. For the first time, *H. capsulatum* infection in the eye of a horse from Iran was detected and phylogenetically analyzed. This study revealed that *H. capsulatum* could establish infection in Iranian animals in addition to people, and indicated the role of soil enriched with bird dropping in the preparation of a favorable environment for *H. capsulatum* propagation. Further investigations are required to clarify the natural history and risk factors associated with histoplasmosis in Iran.

Introduction

Histoplasma capsulatum is a thermally dimorphic fungus. At room temperature, it is usually present in the mycelial form, characterized by septate hyphae, in the soil enriched with excreta of bats or birds; also, it is usually found in the unicellular, budding yeast form in the respiratory or cutaneous lesions.[1] The disease in the equid hosts showed four forms of clinical presentation including asymptomatic, cutaneous, respiratory, and ocular.[2] Traditionally, three distinct varieties of *H. capsulatum* have been described including *Hc* var. *capsulatum* (HCC), *Hc* var. *duboisii* (HCD), and *Hc* var. *farciminosum* (HCF). The HCC causes histoplasmosis in different mammals and mainly pulmonary histoplasmosis in humans. Infection with HCD causes cutaneous, subcutaneous, and osseous lesions in humans. The HCF usually infects horses, mules, donkeys, camels, cattle, dogs, and wildlife mammals. Human infection with HCF has been only reported sporadically.[3]

Histoplasma capsulatum could be identified in animals by direct microscopic examination on smears of the exudate or in histological sections of the lesion material, polymerase chain reaction (PCR), culture of mycelial form, and antibody detection tests including fluorescent antibody, enzyme-linked immunosorbent assay, hemagglutination test, and a skin hypersensitivity test.[4] The PCR tests have been described for the detection of almost all pathogens. In this test, a specified region of the pathogen genome is targeted by a primer pair and amplified it enormously. The culture of *H. capsulatum* usually takes a long time between 2 - 8 weeks, and serological tests may show unacceptable false-positive results due to long lasting antibody titer after an infection, and cross reactivity. Therefore, PCR could be included as a reliable confirmatory test especially for the direct microscopic examination.[5]

Here, the *H. capsulatum* infection was detected in the ocular lesion of a horse and analyzed phylogenetically based on the internal transcribed spacer (ITS) sequence. To the best of our knowledge, this is the first report of *H. capsulatum* infection from an animal, and the first phylogenetic analysis of *H. capsulatum* detected in all mammalian hosts, including humans from Iran.

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Materials and Methods

Clinical examination and sampling. A 3-year-old native mare with a history of long-term (four weeks) ocular discharge and unilateral cloudiness of the right eye (Fig. 1) was referred to the veterinary clinic in Marvdasht, Fars province, Iran (52.87° E, 29.86° N). Physical examination revealed multiple nodules in the conjunctival tissue suggestive of severe focal or diffuse granulomatous conjunctivitis. The animal had normal appetite, heart and respiratory rates, and body temperature without any involvement of the skin and other mucus membranes. After complete sedation of the animal, swab, tissue, and blood samples were collected. The sampling was done according to the State Committee on Animal Ethics, Shiraz University, Iran (IACUC no: 4687/63).

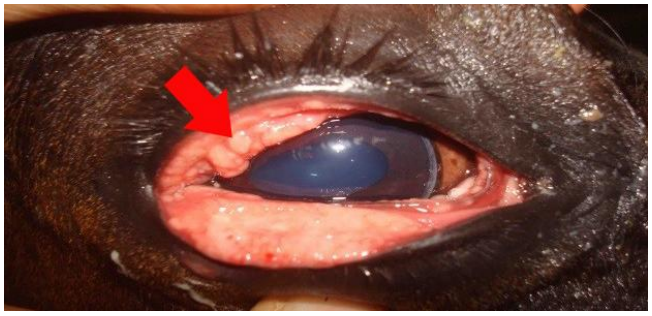

Fig. 1. Gross appearance of ocular histoplasmosis in a horse showing ocular discharge and cloudiness of the right eye with multiple nodular lesions in the conjunctival tissue (red arrow).

Hematology, cytology and histopathology. Complete blood count was determined using an automatic blood cell counter (Exigo, Stockholm, Sweden). Giemsa staining of fixed smears was used to obtain white blood cell (WBC) differential count in blood samples, and for the detection of *H. capsulatum* in swab samples of ocular discharge. Also, the tissue sections were prepared, and stained with Hematoxylin and Eosin (H&E) and periodic-acid Schiff (PAS) for histopathological studies. Also, seromocoid discharge smears were thoroughly searched for the eggs and/or larvae of the equine parasites of the eye (e. g., *Thelazia lacrymalis*, *Onchocerca cervicalis*, and *Habronema* spp.) using microscopic examination technique.

Polymerase chain reaction. The DNA was extracted from 100 µg of tissue samples using the PCR kit according to the manufacturer's instruction (MBST, Tehran, Iran). Two individual PCR reactions were conducted in an MJ mini thermal cycler (BioRad, Feldkirchen, Germany), (Table 1). In the first PCR run, the aim was the detection of *H. capsulatum*, and in the second PCR run, the ITS sequence of *H. capsulatum* was amplified and used for genome sequencing and phylogenetic analyses. After optimization, both PCR reactions were conducted in 20.00 µL total volume, containing 1.00 µL of each primer (10.00 µM), 10.00 µL RED Master Mix (Ampliqon, Odense, Denmark), 5.00 µL DNase free water, and 3.00 µL sample DNA. After PCR reactions, each PCR product was electrophoresed in 1.00% agarose gel to detect the presence or absence of expected band size.

Sequencing and phylogenetic analyses. For sequencing and phylogenetic analyses, a PCR product of the expected band size was bi-directionally sequenced. The ITS sequence was compared with other sequences using the BLASTN suite of NCBI and similar sequences were retrieved from the database. Collected sequences were aligned against the ITS sequence obtained in this study; then, the phylogenetic tree was constructed using the maximum likelihood method in MEGA Software (version 6.0; Biodesign Institute, Tempe, USA).

Results

Hematological and cytological findings. Eosinophilia was detected in the differential count of WBC; but other blood parameters were normal. In the Giemsa-stained smears of ocular discharge, *H. capsulatum* was clearly observed as free extra-cellular form and engulfed organisms in the macrophages and to some extent, in neutrophils. The tiny, yeast-like organisms were round to oval and 2.00 to 4.00 µm in diameter with a purple nucleus and lightly basophilic cytoplasm surrounded by a thin and transparent halo (Fig. 2A). In direct microscopic examination of smears, no eggs and/or larvae of the eye parasites were found.

Histopathological findings. The histopathological examination of H&E-stained tissue sections revealed a diffuse granulomatous reaction. The inflammatory nodular lesions of the conjunctiva mainly consisted of epithelioid macrophages and a few reactive lymphocytes and plasma cells. Also, numerous round-to-oval yeast-like structures (2.00 - 4.00 µm in diameter) were observed in the cytoplasm of the macrophages that were surrounded by a thin clear halo and an eccentric nucleus compatible with the yeast form of *H. capsulatum* (Fig. 2B). In the PAS-stained tissue specimens, the yeast form of the *H. capsulatum* was evident as reddish-purple fungal agents within the cytoplasm and free organisms outside of the inflammatory cells (Fig. 2C).

Table 1. Primer sequences, a common thermal program, and product lengths of each polymerase chain reaction test.[6,7]

Primer	5`-3` sequence	Thermal program	Product length
Hc-T-s	F: GAATCTCAGGTCAATCGGTG	95.00 ˚C, 5 min- 40X	373 bp
Hc-T-a	R: AGTTTCGCTGGAGTCAATTC	(95.00 ˚C, 30 sec- 60.00 ˚C, 30 sec- 72.00 ˚C, 30 sec)	
Modified P3 (MP3)	F: GCGGAAGGATCATTACCAC	72.00 ˚C, 10 min	586 bp
Modified 2R5 (M2R5)	R: GCGGGTATCCCTACCTGA		

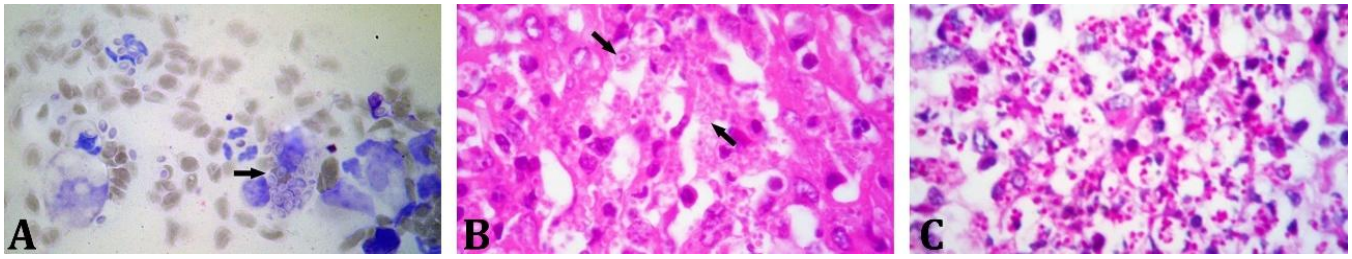

Fig. 2. Photomicrographs of cytological smear and histopathological features of ocular histoplasmosis in a horse. **A)** A macrophage is containing numerous round to oval yeast organisms of *Histoplasma capsulatum* (arrow), (Giemsa stain, oil immersion; 1,000×). **B)** Diffuse granulomatous conjunctivitis with numerous yeast forms of *H. capsulatum* inside the cytoplasm of macrophages surrounded by a thin halo (arrows), (H&E stain, oil immersion; 1,000×). **C)** The yeast form of *H. capsulatum* as reddish-purple organisms within the cytoplasm or free outside of the inflammatory cells (PAS stain, oil immersion; 1,000×).

Polymerase chain reaction results. The first PCR amplified a 373 bp fragment, and the second PCR product, used for sequencing, was a 586-bp fragment of ITS sequence of *H. capsulatum*. The phylogenetic analysis of the Iranian *H. capsulatum* sequence (MT125892) in line with other homologues sequences retrieved from the NCBI nucleotide BLAST based on the partial ITS sequences is shown in Figure 3. Phylogenetic tree obtained from ITS sequence showed that the present *H. capsulatum* strain was belonged to the *farciminosum* variety.

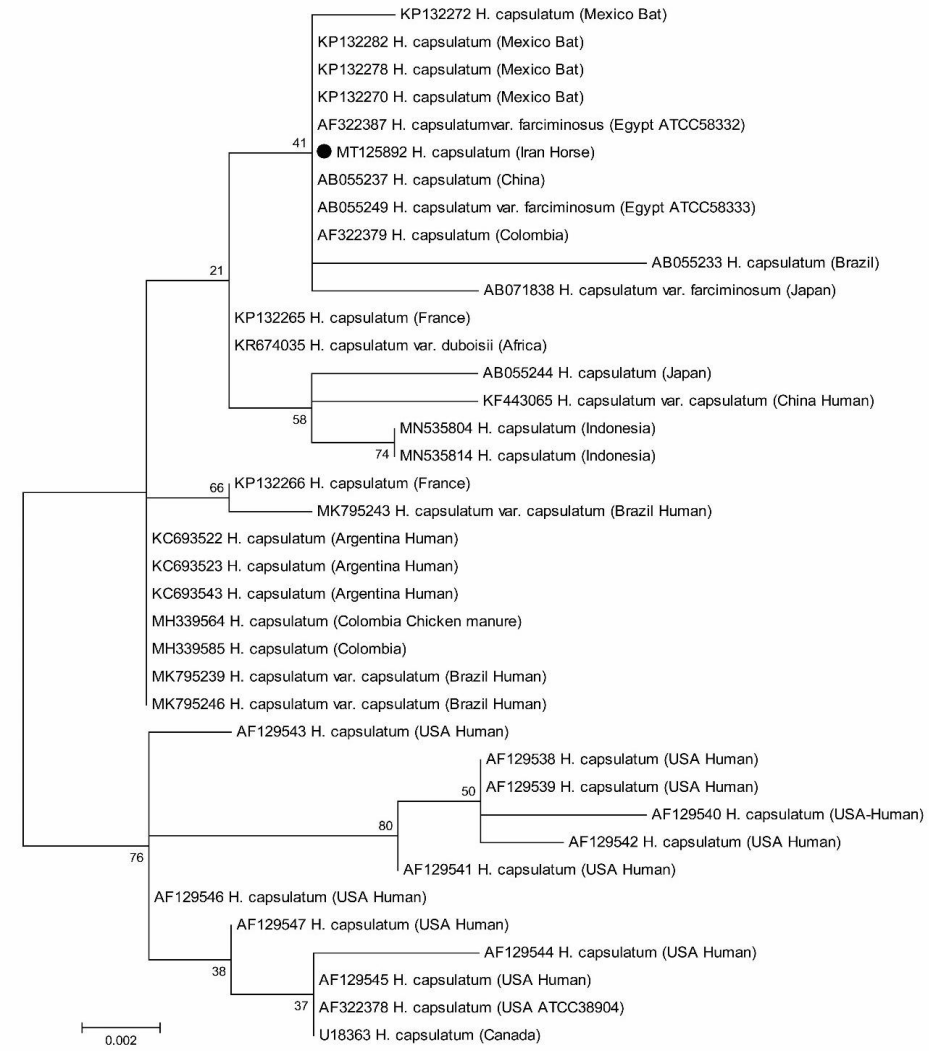

Fig. 3. Phylogenetic tree of the Iranian *Histoplasma capsulatum* (●) and other homologues sequences retrieved from the GenBank® based on the partial internal transcribed spacer sequences. Nucleotide substitutions are shown by scale bar and bootstrap values for 10,000 replications are indicated by the numbers at the nodes.

Discussion

In this study, the clinicopathological features and molecular confirmation of the *H. capsulatum* infection of an equine eye were reported and analyzed phylogenetically based on the ITS sequence for the first time in Iran. The phylogenetic tree of ITS sequence showed that *H. capsulatum* strain belonged to the *farciminosum* variety that is usually involved in the animal histoplasmosis. In the phylogenetic tree, the *H. capsulatum* strain matched with those from all over the world, i.e., from North America (Mexico), Latin America (Brazil and Colombia), North East Africa (Egypt), and the Far East (China and Japan). The homology of the strain and those other strains in the phylogenetic tree shows that all of them have deviated from a common ancestor during a long time.

Equine histoplasmosis is now only endemic in some countries of Sub-Saharan Africa, e. g., Ethiopia.[8] Although, the disease is eradicated from Europe and North America, there are sporadic case reports of equine histoplasmosis from Germany[9] and United States.[10] The phylogenetic tree, in line with the report from United States, showed that *farciminosum* variety is still circulating in the North America (Mexico). In the studies that the presence of *H. capsulatum* has been searched in human and animal samples from Iran, this pathogen was only isolated from a few human samples.[11] Only two researches aimed to detect *H. capsulatum* in cave-dwelling bats which were not successful and showed that cave-dwelling bats may not be involved in the transmission of infection in Iran.[12,13] This is known that the ideal environmental conditions of *H. capsulatum* to grow are acidic soils enriched with high concentrations of phosphorus and nitrogen, temperature of 22.00 and 29.00 ˚C, 1,000 to 1,200 mm annual raining, and 67.00% to 87.00% humidity.[14] It seems that the limiting factor of broader distribution of *H. capsulatum* in Iran is the climatic factors like the amount of annual rain and relative humidity. Iran's climate is assumed as dry and semi-dry. Based on official reports, the average annual rain in the last 51 years in Iran is about 250 mm which is significantly less than *Histoplasma* sp. ideal condition. Regarding the infected horse in this study, since the horse was kept in a semi-dark stable and in close contact with some pigeons, bird feces providing high nitrogen content alongside with warm temperature and high humidity of the stable provided a conducive environment for *H. capsulatum*.

This study showed the role of soil enriched with bird dropping in preparation of a favorable environment for *H. capsulatum* propagation. Further investigations are required to clarify the natural history of *H. capsulatum* in Iran.

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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