Survey of *Histoplasma capsulatum* in bat guano and status of histoplasmosis in Slovenia, Central Europe

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Abstract: There have been increasing reports on the presence of *Histoplasma capsulatum* in some European countries. The study investigated the presence of *Histoplasma* in bat guanos, speleologists with records of visiting *Histoplasma*-endemic regions and patients with histoplasmosis. A commercial ALPHA *Histoplasma* Antigen enzyme immunoassay was tested as an alternative methodology to detect *Histoplasma* in environment and compared with polymerase chain reaction (PCR) assays. The presence of *Histoplasma* antigen in bat guanos was not confirmed by PCR. Among 14 healthy speleologists, two were indicated as having the *Histoplasma* antigen in urine, but expressed negative PCR-specific results for the *Histoplasma* antigen. Five unequivocal cases of imported acute pulmonary histoplasmosis in Slovenia between years 2005 and 2016 were confirmed in patients returning from North and South America after visiting hazardous localities e.g., caves with guano, and places with dust. Currently there is no evidence of autochthonous histoplasmosis in Slovenia, or that bat guano is a source of *H. capsulatum*. Involvement of histoplasmosis in travellers' and cavers' morbidity might be underestimated in non-endemic areas. It is crucial to ensure the use of appropriate protective equipment in *Histoplasma* hazardous localities, to spread information about this hazardous microbe to vulnerable populations and to monitor the health of the environment. A differential diagnosis for a febrile respiratory disease outbreak in patients returning from endemic regions should trigger routine consideration of possible histoplasmosis.

Keywords: histoplasmosis, bat guano, caves, *Histoplasma* antigen EIA, speleologists

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INTRODUCTION

Histoplasmosis is an endemic mycosis caused by a dimorphic fungus with two distinct varieties that are pathogenic to humans, i.e., *Histoplasma capsulatum* var. *capsulatum* and *Histoplasma capsulatum* var. *duboisii*. The latter is known to be restricted to sub-Saharan Africa, whereas the former is distributed worldwide. *H. capsulatum* occurs in the soils of endemic areas, especially those contaminated by bird and bat droppings. Environmental disruption of *Histoplasma* habitats that introduces spores into the air is commonly a key factor associated with histoplasmosis outbreaks. Cave visitation, mining, building-site work, and agricultural activities are all associated with an increased risk of histoplasmosis (Kasuga et al., 2003; Kauffman; 2007 Antinori, 2014).

After inhalation of airborne *Histoplasma* microconidia, a majority of those exposed develop subclinical, self-limited, and commonly unrecognized disease symptoms. These are more likely to manifest after high-inoculum exposures, possibly with more intrinsically virulent strains, especially in immunocompromised patients and in very young and very old individuals. Those immunocompetent patients that do develop symptoms, most commonly present with acute pulmonary histoplasmosis, a »flu-like« illness, whereas severe or disseminated forms of histoplasmosis usually occur in immunocompromised individuals (Wheat et al., 2016).

Most reported cases of histoplasmosis in Europe are in immigrants or travellers returning from endemic areas, including the Americas, Africa, Southeast Asia, and East Australia (Bahr et al., 2015). According to an

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epidemiological survey of the European Confederation of Medical Mycology Working Group, some parts of Italy might be considered endemic for *H. capsulatum* (Ashbee et al., 2008). *H. capsulatum* var. *capsulatum* has been isolated from soil and animals in the Emilia Romagna region of Italy, and histoplasmin reactivity surveys in Lombardy, Tuscany and Apulia showed a low positivity in the general population (Sotgiu et al., 1966; Confalonieri et al., 1994; Mavropoulou et al., 2010; Reginato et al., 2014). In particular, the identification of some autochthonous cases in the Po Valley suggests the possible endemic presence of histoplasmosis in Italy (Manfredi et al., 1994; Farina et al., 2000, 2005; Calza et al., 2003; Ashbee et al., 2008). A few apparently autochthonous cases have been reported in other European countries, especially those in Southern Europe and those with badger populations (Ashbee et al., 2008; Craven 2013; Eisenberg et al., 2013), suggesting that extending the documentation of further case reports of histoplasmosis in Europe would give a completely different local picture of *Histoplasma* epidemiology.

There are several reports of point-source outbreaks of acute pulmonary histoplasmosis among speleologists, so called «cave disease», largely but not exclusively related to cave exploration in the tropics and subtropics. The documented areas of cave disease in Europe are confined to underground spaces in Cyprus and to Topolnița Cave in Romania (Craven, 2013). The diagnosis of histoplasmosis is indicated initially by the history of simultaneous exposure to bat guano and subsequent simultaneous onset of symptoms, and can then be confirmed in the individual by the presence of radiographic abnormalities and by the results of a suite of mycological and/or non-culture based tests. For such a potentially vulnerable population, it is of great importance to collect information about the prevalence of *Histoplasma* in any particular underground system. The isolation of *H. capsulatum* from environmental samples is difficult. The pathogen is typically retrieved as a cultivable strain after inoculation of laboratory animals (Richardson & Warnock, 2003). Direct identification of the fungus from complex environmental samples by use of molecular techniques is an alternative to the time-consuming culturing methods (Frias De Leon et al., 2012).

In this study we tested the usefulness of the commercial ALPHA *Histoplasma* Antigen EIA kit intended for human in vitro diagnostics of histoplasmosis (Cloud et al., 2007) as a rapid and cost-effective alternative to culturing methods of inoculated laboratory animals for environmental samples. Since there have been no reports from Slovenia, a small Central European country, on either the presence of *H. capsulatum* in caves or of bats infected with histoplasmosis (McMurray & Greer, 1979), bat guano samples from five Slovenian bat-inhabited karst caves were screened for *Histoplasma* antigen and the results compared with those of molecular analyses. Additionally, we analysed risk factors for acquiring histoplasmosis, both in a group of 14 speleologists and in five travellers with imported pulmonary

histoplasmosis who consulted the Infectious Disease Department at the Clinical Centre in Ljubljana between 2005 and 2016.

MATERIAL AND METHODS

Collection of bat guano and sample preparation

Five Slovenian bat-inhabited karst caves containing significant deposits of guano in a form of a heap, sampled in 2011-2012, were included in the study. Out of six samples, two samples of bat guano were obtained from Škocjan Caves (Škocjanske jame, E 13.994° N 45.665°) and one sample from each of the following caves: Huda Luknja Cave (Huda luknja, E 15.1743° N 46.4145°); Predjama Cave (Predjama, E 14.1265° N 45.8156°); Spodnja Klevevška Cave (Spodnja klevevška jama, E 15.2334° N 45.9067°); and Turjeva Cave (Turjeva jama, E 13.5046° N 46.2435°). These guano heaps had already been screened for the fungus *Pseudogymnoascus destructans* (formerly known as *Geomyces destructants*), a bat pathogen, and for free-living amoebae during previous studies (Mulec et al., 2013, 2016). The authors did not interact directly with any bats during sample collection. Briefly, in caves, the guano was sampled aseptically from the upper layer of the guano heaps, from zero to five cm in depth, and stored at -20°C until analysed in a laboratory. The age of samples was estimated at the sites of guano collection. “Fresh” guano samples were characterized by intact rod-shaped excrement, indicating the ongoing presence of roosting colonies of bats at the sites. A “recent” age attribution was given to those samples that included some relatively recent rod-shaped excrement over an older guano base. Samples were considered “old” when the guano showed no characteristics of fresh and/or recent rod-shaped excrement, and if no bat colony had been recorded above the guano heap in recent times, usually within a period of a few decades. Basic information on guano is summarized in Table 1. Sampling of guano was approved by the Slovenian Environment Agency, Ministry of the Environment and Spatial Planning, Republic of Slovenia (Nos. 35602-63/2009-3, 35602-136/2010-3).

Antigen detection in guano

In the laboratory, 1 g of each guano sample was suspended in 10 ml of sterile RPMI 1640 – Hepes cell culture medium (Sigma, USA) and vortexed vigorously. These biomass suspensions (1:11 [w/v]) were centrifuged for 5 minutes at 4°C and 9200g. The supernatants were collected undiluted and further diluted 10-fold with sterile RPMI 1640 – Hepes, and stored at -20°C pending further analysis. The ALPHA *Histoplasma* Antigen EIA kit (Immy, USA), a commercially available enzyme immunoassay (EIA) for *in vitro* diagnostic use in human serum and urine samples was used to detect *Histoplasma* antigen in the guano supernatants. Results were expressed as EIA Units of *Histoplasma* antigen. Two commercial *Histoplasma*-antigen preparations needed for the determination of anti-*Histoplasma*-antibodies using the complement fixation method, i.e., *Histoplasma*

Table 1. List of bat guano collection sites (Mulec et al., 2013, 2016).

Cave	Temperature (°C)	Bats number	Relative guano age / heap volume (l)
Huda Luknja Cave	9	2200	Recent (630)
Predjama Cave	12	1320	Recent (50)
Spodnja Klevevska Cave†	18	400	Fresh (30)
Škocjan Cave	12	5300	Fresh (400), Old (450)
Turjeva Cave	10	100	Old (800)

†Cave with a thermal spring

Mycelia CF Antigen and *Histoplasma* Yeast CF Antigen (Immy, USA), were included in the test as additional positive controls.

DNA isolation and molecular analyses of bat guano

DNA isolated, using the NucleoSpin® Soil kit with SL2 lysis buffer (optimized protocol for guano samples by Macherey-Nagel, Germany), in a previous study (Mulec et al., 2013), has been used in the present analysis. Using the whole-cell DNA, nucleotide sequences of a highly conserved region of the fungal rRNA gene were amplified with two primer sets, i.e., ITS1-ITS4 and ITS4-ITS5 in a control polymerase chain reaction – PCR (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>).

H. capsulatum-specific PCR assays using two sets of specific primers (H-anti3-H-anti4 and Msp2F-Msp2R), the regions of H-antigen precursor and M-antigen genes (Guedes et al., 2003; de Muniz et al., 2010) were performed. PCR was carried out with serially diluted positive-control DNA to determine the analytical sensitivity of the test. Amplification products were analysed on 1% agarose gels, stained with SYBR Safe DNA gel stain (Invitrogen, USA) and electrophoresed at 140 V for 20 minutes.

Extraction, amplification, and sequencing of DNA from clinical samples

The serum specimens from four histoplasmosis patients, who did not belong to the group of examined speleologists, were available from the archival serum collection of the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, and used to extract DNA. Additionally, total DNA was extracted from paired specimens (urine and serum) from three speleologist volunteers who exhibited positive or equivocal *Histoplasma* antigen assay in urine. DNA templates were obtained by using the automatic commercial isolation kit MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For the investigation of fungi-specific DNA in clinical samples, two sets of universal fungal primers were used as described above.

For sequencing, the amplified PCR products of the proper size (≈ 620 bp) were purified using Exonuclease I (Exo I) and FastAP™ Thermo sensitive Alkaline Phosphatase (Thermo Scientific, Waltham, USA) according to the recommendations of Werle et al. (1994). DNA sequencing reactions were purified using a BigDye X Terminator® Purification Kit (Applied Biosystems®, USA) and sequencing was done in a

3500 Genetic Analyser (Applied Biosystems®, USA). Finally, sequences were analysed using CLC Main Workbench 6.9.1 (CLC bio, Denmark). *H. capsulatum*-specific PCR was performed as previously described.

Speleologists and analysis of the risk factors for acquiring histoplasmosis

A questionnaire, which was addressed specifically at speleologists who work and explore caves and come into direct and/or indirect contact with aerosols derived from bat guano, was composed, and distributed to 14 speleologist volunteers. These speleologists professionally work in caves, most of them being active researchers. They were advised to test for histoplasmosis by their employer and physician as they are frequently exposed to work in potentially biohazardous environments. The questionnaire comprised three parts: travel history to known endemic areas and visits to caves in those regions, information about speleological activities, and data on any accidents in the caves that might have led to infection with *H. capsulatum* ([Appendix: Histoplasmosis Questionnaire](#)). Each speleologist was examined by the same physician to assess health status, and screened for the presence of anti-histoplasmin antibodies (in serum) by immunodiffusion (ID) assay (Meridian, USA), and *Histoplasma* antigen (in serum and urine) by ALPHA *Histoplasma* Antigen EIA (Immy, USA) according to the manufacturer's instructions. Urine samples were screened untreated and undiluted; however, serum samples were treated with pronase (Immy, USA) according to the manufacturer's instructions. The antigen EIA was considered to be negative when the antigen concentration was less than 2 EIA Units, to be equivocal when it was between 2 and 3 EIA Units, and to be positive when the concentration was above 3 EIA Units.

All samples from speleologists were obtained as a part of a preventive health examination recommended by the employer which initially included a complete physical examination and laboratory tests. Since laboratory testing did not include screening for the presence of anti-histoplasmin antibodies, *Histoplasma* antigen or *H. capsulatum*-specific DNA, further laboratory tests had been requested by the responsible clinician. Informed consent for additional laboratory testing and analysing the data was obtained in writing from all those who completed Histoplasmosis questionnaire. All speleologists and samples were anonymized, no additional sample was taken for the purpose of the study and only speleologist's age and gender were available to researchers.

Patients and criteria for classification of cases

Five cases of imported pulmonary histoplasmosis, diagnosed at the Infectious Disease Department in the Clinical Centre at Ljubljana between 2005 and 2016 were analysed retrospectively. A review of each patient's medical record was provided by the treating physician to categorize individuals with histoplasmosis. The cases were classified following the European Confederation of Medical Mycology Working Group definitions (Ashbee et al., 2008). Histoplasmosis was considered *probable* when the individual had a travel history to a known endemic area, presented positive results to serological tests and where imaging of the lung revealed lesions consistent with histoplasmosis. Cases were classified as *possible* when the individual had a history of travel to a known endemic area and either showed a positive result to serological tests or if imaging of the lung revealed lesions consistent with histoplasmosis. Since laboratory testing initially included screening for the presence of anti-histoplasmin antibodies, additional retrospective *Histoplasma* antigen EIA was performed.

With regard to disease presentation, patients presenting with an acute primary infection after recent exposure to *H. capsulatum* risk factors were classified according to Wheat et al. (2016) as acute pulmonary; patients presenting with milder symptoms than those with an acute infection (symptoms are the same as but milder than those of acute histoplasmosis, with chest imaging showing focal or patchy opacities instead of diffuse bilateral) was classified as subacute pulmonary histoplasmosis. A patient who was asymptomatic, developed subclinical, self-limited, disease.

The serum specimens for additional *Histoplasma* antigen EIA testing from four histoplasmosis patients were available from the archival serum collection of

the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. We followed the principles of the Helsinki Declaration, the Oviedo Convention on Human Rights and Biomedicine, and the Slovene Code of Medical Deontology. All samples were anonymized and only patient's age, gender, geographical destination of travelling, anti-histoplasmin antibodies result, and therapy were available to researchers.

RESULTS

Histoplasma in bat guano

Fungal broad range PCR on total DNA isolated from guano samples confirmed the presence of fungal DNA in all samples. However, *H. capsulatum*-specific PCR assays using H-antigen and M-antigen primers that displayed the analytical sensitivity of 26.6 pg/ μ l were negative (Fig. 1). In a direct approach to determine the specific fungus sequence, no sequence related to *H. capsulatum* was retrieved in any of the bat guano samples.

All six guano samples from Slovenian caves were positive for *Histoplasma* antigen in both undiluted and 10-fold diluted supernatants, determined by EIA (Fig. 1). The sample of old guano from Turjeva Cave had the highest antigen content. Because of the limited number of samples, the effect of guano age on *Histoplasma* antigen remains elusive. Because concentrations of *Histoplasma* antigen were substantially higher in all 10-fold diluted supernatants than in the undiluted ones, unspecific, false positive antigen results in guano samples were suspected. Both commercial antigen preparations (mycelial and yeast phase *Histoplasma* antigen), tested positive, showing 232.3 EIA Units and 145.4 EIA Units respectively.

Fig. 1. *Histoplasma* Antigen EIA content in bat guano samples was not supported by the molecular analyses. A) Two universal fungal primer pairs, ITS1-ITS4 and ITS4-ITS5, and two *H. capsulatum*-specific primer pairs, H-anti3-H-anti4 and Msp2F-Msp2R, were used for the analysis of genomic DNA; B) *Histoplasma* antigen was measured by the ALPHA *Histoplasma* Antigen EIA in undiluted (1:11 [w/v]) and in 10-fold diluted (1:110 [w/v]) guano supernatants.

Taken together, *Histoplasma* Antigen EIA results were not supported by the molecular analyses.

Epidemiological characteristics and diagnosis of histoplasmosis in speleologists

A total of 14 speleologists were studied (seven females, seven males). Their mean age was 39.7 years (the youngest was 27 and the oldest 58 years old). Two speleologists reported nonspecific health problems at the time of the visit at our clinic. Otherwise they were all healthy individuals, without any known immunosuppression and with unremarkable medical histories.

Most of the subjects had been active speleologists for a number of years, ranging from 4 to 43 years, with the mean of 17.5 years. Visits to caves once a week were reported in five cases (35.7%), once a month in seven cases (50%) and several times a year for one (7.1%) person. One person didn't specify the frequency. The speleologists visited caves in different regions of the world. North-American caves were visited by ten (71.4%) of the speleologists, Central-American by six (42.9%), South-American by four (28.6%), African by three (21.4%), Australian by five (35.7%), and Southeast-Asian and Indian caves by ten speleologists (71.4%). Five speleologists (35.7%) reported visiting only touristic caves, 8 (57.1%) also visited other wild caves, and one person (7.1%) didn't provide an answer. None of the persons complained about health problems when returning from caves.

Protective measures were employed by five persons (35.7%), all measures except for use of a protective face mask were reported by three speleologists (21.4%), whereas six people (42.9%) used no protection.

None of the 14 speleologists reported a bite by a bat. One had an accident in the cave, but with no serious consequence. Known contact with bats or guano were reported by nine (64.3%) of the subjects.

Results of the medical check-up were unremarkable in all 14 speleologists. In spite of having risk factors for acquiring infection with *H. capsulatum*, all speleologists tested negative by immunodiffusion and *Histoplasma* antigen EIA in serum (Table 2). For all but three, *Histoplasma* antigen EIA results in urine were negative. One person showed an equivocal antigen EIA test result in urine (2.0 EIA Units) and two results were low positive (3.9 and 3.2 EIA Units). PCR was performed on serum and urine samples from these three speleologists and results for *H. capsulatum* were negative in all cases (Table 2). The speleologist positive for the *Histoplasma* Ag (3.9 EIA Units) reported in his questionnaire that he visited some of the endemic areas (Northern America, Mexico, India and Indonesia) and he gets occasionally in contact with guano and bats. Broad range PCR revealed the presence of *Cladosporium* sp. in his urine. The second speleologist (3.2 EIA Units) reported visits in North America, India and Malesia, occasional contacts with bat guano, and she had *Malassezia restricta* in her urine and serum.

Table 2. Demographic characteristics of speleologists and results of diagnostic tests for histoplasmosis performed.

Speleologist	Sex	Sample	PCR result	ID result	<i>Histoplasma</i> Ag result (EIA Units)
1	Male	Serum Urine	ND ND	Negative	Negative Negative
2	Male	Serum Urine	Negative Negative	Negative	Negative Positive (3.89)
3	Female	Serum Urine	Negative Negative	Negative	Negative Positive (3.19)
4	Female	Serum Urine	ND ND	Negative	Negative Negative
5	Female	Serum Urine	ND ND	Negative	Negative Negative
6	Male	Serum Urine	Negative Negative	Negative	Negative Equivocal (2.02)
7	Female	Serum Urine	ND ND	Negative	Negative Negative
8	Male	Serum Urine	ND ND	Negative	Negative Negative
9	Male	Serum Urine	ND ND	Negative	Negative Negative
10	Male	Serum Urine	ND ND	Negative	Negative Negative
11	Female	Serum Urine	ND ND	Negative	Negative Negative
12	Male	Serum Urine	ND ND	Negative	Negative Negative
13	Female	Serum Urine	ND ND	Negative	Negative Negative
14	Female	Serum Urine	ND ND	Negative	Negative Negative

PCR, polymerase chain reaction; ID, immunodiffusion test; EIA, enzyme immunoassay; Ag, antigen; ND, not done

Epidemiological characteristics and diagnosis of histoplasmosis in travellers

During the last ten years five cases of histoplasmosis among travellers were diagnosed at the Infectious Disease Department of the Clinical Centre Ljubljana, Slovenia (Table 3). At the time of presentation, based on epidemiological data, clinical symptoms, and X-ray imaging, four patients had a highly-likely suspicion for pulmonary histoplasmosis (patients 1, 2, 4, and 5). The most common symptoms were cough, headache, myalgia, fever, and chest pain. One person was asymptomatic (patient 3) and was traveling with the patient who presented with acute pulmonary histoplasmosis (patient 2). They were all males, with mean age of 37.2 years without any underlying diseases, and with no known immune deficiency. The epidemiological background of patients included travel to *Histoplasma*-endemic regions. One patient had spent much time doing outdoor activities in National Parks throughout the Mississippi Valley, USA, two patients had collected bat guano for soil fertilizer in non-touristic caves in Jamaica, and two patients had visited rural areas, but not caves with bat guano in Venezuela and Ecuador. None of them was a speleologist. They were not aware of the potential presence of *H. capsulatum* in the region, and no one adopted any personal protection measures against the pathogen.

On medical examination, the lungs were normal on auscultation in four patients; one patient was displaying signs of pathological compromise of the lungs (patient 1). The rest of examination was not remarkable. Pathological chest radiographs for four patients were consistent with histoplasmosis (patients 1, 2, 4, and 5). Prompted by the pathology seen on the chest radiographs, computed tomography (CT) scans of the chest were performed. Pathology highly

indicative of pulmonary histoplasmosis was confirmed in all four patients.

Out of four patients with respiratory symptoms and indicative results of pathological chest imaging, three patients showed positive immunodiffusion results; one patient was seronegative. A patient who was asymptomatic tested positive by immunodiffusion. Out of four seropositive patients, two had the positive antigen EIA test in serum, one was equivocal, and one tested negative. Tests for *H. capsulatum*-specific PCR were performed on serum samples from four seropositive patients and results were negative in all cases.

In addition, three patients met the criteria for probable histoplasmosis and one patient for possible histoplasmosis. A patient who was asymptomatic and seropositive, had subclinical, self-limited histoplasmosis. In all, three patients presented with acute pulmonary histoplasmosis (patients 1, 2, and 5), of whom two patients were classified as probable and one patient as possible histoplasmosis; therapy with itraconazole was initiated. All three patients received an initial dose of 200 mg of itraconazole three times daily for three days. Thereafter, itraconazole was administered with the dose of 200 mg twice daily for a total of 12 weeks in patient 1, 6 weeks in patient 2 and 8 weeks in patient 5. All three patients responded well to therapy and the pathology seen on the chest radiographs was completely abrogated after several months. The patient with subacute pulmonary histoplasmosis and the asymptomatic one were not treated.

DISCUSSION

A small but significant number of autochthonous cases of histoplasmosis reported in European countries, especially in neighbouring Italy (Manfredi

Table 3. Epidemiological characteristics, results of diagnostic tests performed, classification of cases based on the European Confederation of Medical Mycology Working Group criteria, clinical category and therapy of Slovenian travellers with histoplasmosis.

Patient	Age	Sex	Travelled to	Sample	PCR result	ID result	<i>Histoplasma</i> Ag result (EIA Units)	Classification of histoplasmosis	Clinical category	Therapy
1	51	Male	USA (Mississippi Valley)	Serum	Negative	Positive (M band)	Negative	Probable	Severe acute pulmonary histoplasmosis	Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 12 weeks
2	32	Male	Jamaica (wild caves)	Serum	Negative	Positive (M band)	Positive (3.66)	Probable	Acute pulmonary histoplasmosis	Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 6 weeks
3	27	Male	Jamaica (wild caves)	Serum	Negative	Positive (M band)	Equivocal (2.51)	Not classified	Subclinical histoplasmosis	No
4	39	Male	Venezuela (outdoor activities)	Serum	Negative	Positive (M band)	Positive (3.91)	Probable	Subacute pulmonary histoplasmosis	No
5	35	Male	Ecuador (outdoor activities)	Serum	ND	Negative	ND	Possible	Acute pulmonary histoplasmosis	Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 8 weeks

PCR, polymerase chain reaction; ID, immunodiffusion test; EIA, enzyme immunoassay; Ag, antigen; ND, not done; TID, three times daily; BID, twice daily.

et al., 1994; Farina et al., 2000, 2005; Calza et al., 2003; Ashbee et al., 2008), leads us to consider whether *H. capsulatum* might not be confined to only a limited region but might actually be an autochthonous inhabitant of Central Europe, including Slovenia. In the study, the commercial ALPHA *Histoplasma* Antigen EIA kit designed for human in vitro diagnostics of histoplasmosis was applied for the first time to capture *H. capsulatum* antigen from bat guano heaps, not mixed with the surrounding cave material. The 10-fold diluted guano supernatants revealed a substantially higher antigen content than did undiluted supernatants (Fig. 1), implying that conditions in guano might have been the reason for discordant results and probably for non-specific reactions. For example, reactive antigen EIA might be linked to the cross-reactivity of rabbit anti-*Histoplasma* IgG antibodies used in the test with polysaccharide antigens of diverse and abundant guano-associated microorganisms rather than *H. capsulatum* (Chroňáková et al., 2009; Nieves-Rivera et al., 2009). Indeed, our previous study demonstrated that the sampled guanos are rich sources of cultivable fungi and bacteria (Mulec et al., 2013), but species identification was not carried out. Fungi associated with bat guano are diverse (Nováková, 2009). Besides being a source of nutrients and microbiota, guano as a complex environmental sample can also vary in pH, ranging from acidic to alkaline (Mulec et al., 2013) and is known to contain high levels of heavy metals (Walker et al., 2007; Krištůfek et al., 2010), which all together could facilitate the non-specific reactions in the *Histoplasma* antigen EIA.

The PCRs with the primers specific for *H. capsulatum* var. *capsulatum* (Guedes et al., 2003; de Muniz et al., 2010) were performed on total DNA isolated from the guano samples and nucleotide sequences of a highly conserved region of the fungal rRNA gene were analysed. Although the guano samples tested *Histoplasma*-antigen positive, no *H. capsulatum*-specific PCR products were detected and no sequences related to *H. capsulatum* were retrieved from any of the bat guano samples (Fig. 1). The results indicated that the commercial ALPHA *Histoplasma* Antigen EIA kit is probably not applicable for complex environmental samples such as bat excrement.

On the other hand, the negative results of the specific PCRs can point out that concentrations of *H. capsulatum* DNA in the samples were below the detection limit. It might also be that bat species identified in Slovenian caves are not the natural hosts of the fungus, and consequently the fungus is not yet present in guano. Several bat species have been identified in caves in Slovenia; however, the main origin of screened guano was from the bat *Miniopterus schreibersii* (Mulec et al., 2016), which is not yet reported to host *H. capsulatum* (Stančič-Pavlinčič and Grom 2005). Although the presence of reactive antigen EIA was not supported by the molecular analyses and therefore *H. capsulatum* was probably not present in bat guano, the possibility of transmission of the fungus by a different species of bat in Slovenia cannot be excluded. Further work is needed to screen guano

from other caves for the presence of *H. capsulatum* or the association of histoplasmosis in bats.

The increasing number of people travelling to *Histoplasma*-endemic areas is responsible of the growing number of reports of single or, more frequently, clusters of acute histoplasmosis cases. The continued reports of cave-associated outbreaks suggest that current caving practices continue to place cavers at risk from the infection, as a particularly vulnerable population (Senechal et al., 2012; Bahr et al., 2015; Benedict & Mody 2016). By analysing cases of imported pulmonary histoplasmosis in Slovenia retrospectively, our findings, reported here for the first time, suggest that visiting caves in *Histoplasma*-endemic areas and coming into contact with bat guano without the use of personal protective equipment were the major risk factors for acquiring the infection in the travellers group. Although several risk factors for acquiring histoplasmosis were indicated for the speleologists group, e.g., visiting confined underground spaces in endemic areas, contact with the bats or their guano and inadequate employment of protective measures, none of them showed clinical signs and/or symptoms of histoplasmosis at the time of medical examination. We highlight a general lack of awareness of this disease among professional speleologists and other cave explorers, who should use personal protective equipment to prevent infection. For example, two of the professional speleologists from the studied group (speleologists no. 4 and no. 6 in Table 2) did not acquire histoplasmosis during the international cave exploration in Viñales, (Cuba), whereas another speleologist from the international caving group who was not wearing a protection mask, and did not participate in the current study, got histoplasmosis. In the speleologists group, personal protective equipment and/or measures were used only by five persons (35.7%); nine persons (64.3%) did not use protective face masks and six persons (42.9%) did not use any protection. Travelers and not only speleologists who visit endemic areas should be better informed on risk factors for histoplasmosis and its prevention. The contribution of histoplasmosis to travellers' and cavers' morbidity is probably underestimated in non-endemic areas (Buitrago et al., 2011; Senechal et al., 2012). Even if it is usually a self-limited illness in immunocompetent individuals, European clinicians should consider it routinely when examining any person with febrile respiratory syndrome who has recently been involved in outdoor activities or visited caves, not only in endemic areas but also in Europe.

Serologic ID assay for the presence of M and H precipitin bands plays an important role in the diagnosis of acute pulmonary histoplasmosis (Kauffman, 2007); however, false-negative results may occur for patients with recent infection. Out of five Slovenian travellers with histoplasmosis, four had solely an M band and one patient was seronegative. It is noteworthy that the seropositive traveller with severe acute pulmonary histoplasmosis tested as *Histoplasma*-antigen negative in serum; however, urine was unfortunately not available for reaching a

diagnosis. Published reports note that antigenuria is far more common than antigenemia, not only in AIDS patients with disseminated histoplasmosis, but also in patients with acute pulmonary histoplasmosis (Wheat & Kauffman, 2003). The negative antigen result in the Alpha *Histoplasma* antigen EIA might be due to the lack of polyclonal antibodies to detect a specific antigenic epitope, but, alternatively, myriad antigenic epitopes on the target molecules that are present in biological samples may vary over the course of the disease (Cloud et al., 2010). PCR in serum was negative in all four travellers, probably due to the limited amount of DNA circulating in immunocompetent patients (Buitrago et al., 2011). In contrast with sera or blood samples, respiratory samples that would undoubtedly confirm the presence of pathogen (Kauffman, 2007; Wheat et al., 2016) have not been available and even contra-indicated for the patient with severe disease.

The final aspect deserving discussion is the inconsistency between *Histoplasma* antigen EIA in urine and other laboratory and clinical findings recorded in two speleologists (Table 2). In spite of having risk factors for acquiring infection with *H. capsulatum*, medical check-up results were unremarkable for all 14 speleologists. Additionally, none of them complained about health problems when returning from caves or developed histoplasmosis in the year following initial testing. Since all speleologists tested negative by immunodiffusion and *Histoplasma* antigen EIA in serum (Table 2), an additional medical check-up was not indicated and a phenomenon of seroconversion was hardly to expect. They were either out of *Histoplasma*-endemic regions or with no records of visiting caves at least 8 weeks before medical examination, therefore we did not repeat the serological tests. The above-mentioned two speleologists with discordant findings had weakly positive urine antigen test results (Table 2), no signs of either histoplasmosis or other endemic mycoses, which have been described as causing false-positive reactions for *Histoplasma* antigen in urine (Kauffman, 2007), and none of them developed histoplasmosis in the year following initial testing. However, DNA sequences related to *Cladosporium* sp. and *Malassezia restricta* were retrieved from urine samples, fungi that are not yet known to cause false-positive reactions. Since the specificity for healthy subjects and individuals without histoplasmosis was reported to be from 84-92% with the Immy EIA (LeMonte et al., 2007; Cloud, 2010) and because diagnosis should never be based on a urine *Histoplasma* antigen test alone (Kauffman 2007), the diagnosis of an active infection has not been established.

In conclusion, we have shown that the reactive Immy *Histoplasma* antigen EIA for complex environmental samples such as bat excrement was not supported by the results of molecular analyses. We highlight the widespread lack of awareness of histoplasmosis among persons exploring caves, who should use personal protective equipment to help prevent infection, particularly during exploration in endemic areas. Although five imported cases of pulmonary histoplasmosis were diagnosed in Slovenia during the

last ten years, we infer from our sequencing results from bat guano and the fact that, to date, there have been no reports of autochthonous histoplasmosis among tourists, speleologists or bat researchers visiting Slovenian caves, that *H. capsulatum* is probably not present in the caves of Slovenia. On the other hand, histoplasmosis should be considered in a differential diagnosis for a febrile respiratory disease outbreak in returning travellers with a history of geographical exposure.

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