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A rapid DNA extraction protocol for a PCR-based detection of *Histoplasma capsulatum* in bat guano

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A 36-year-old male was hospitalised with histoplasmosis after he had visited a bat cave as part of a tourist tour to the Costa Rican rainforests. With the PCR-method described here, the bat guano in the cave was identified as infection source. Thereupon, the cave was closed to tourists to avoid future infections with *Histoplasma capsulatum,* an anamorphic ascomycete (teleomorph: *Ajellomyces capsulatus*). A PCR-based assay was developed, to permit a fast, animal-experiment independent identification of environmental infection sources. Different DNA-extraction protocols were tested with bat guano from the cave in Costa Rica. A DNA extraction protocol based on mechanical rupture of the samples, CTAB, skim milk, chloroform and a subsequent purification with PVPP-spin-columns in combination with nested PCR was successfully used to detect the pathogenic fungus in bat guano.

Keywords: DNA extraction, nested PCR, histoplasmosis, risk assessment, bat cave.

The dimorphic/anamorphic fungus *Histoplasma capsulatum* var. *capsulatum* Darling [teleomorph: *Ajellomyces capsulatus* (Kwon-Chung) McGinnis & Katz] is common in warm, humid regions in the south-eastern part of the United States and Latin America. The fungus grows as saprobe in soils enriched with organic compounds and a high proportion of nitrogen as well as in bird- or bat-excrements. Bat-infested caves have been repeatedly reported as source of histoplasmosis outbreaks (Lottenberg *et al.* 1979, Gordon *et al.* 1993, Lyon *et al.* 2004). A bat cave was supposed as infection source in the case inducing the presented study: A 36-year-old man, who returned

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from Costa Rica, was hospitalised with flue-like symptoms, cough, and chest pain. A serum sample analysed at the Robert Koch Institute, Berlin gave evidence of a recent infection with *H. capsulatum*. The patient had participated a guided tourist tour to a bat cave. To prevent potential infections with *H. capsulatum* in cave visitors, the infection source was identified and, consequently, the cave became closed to tourists.

The animal passage method as described by Emmons (1961) is still state of the art for the detection of *H. capsulatum* in soil samples or bat droppings. This method is very time consuming. The need of a quick and cost-effective assay for the detection of *H. capsulatum* in environmental samples has been postulated previously (Wheat *et al.* 2003, Lenhart *et al.* 2004). Aim of the presented study was to develop a quick and inexpensive PCR-based method for the detection of *H. capsulatum* in bat guano.

Materials and Methods

Sampling

About 500 g of bat guano were collected with a spade from different areas in a cave in the Golfo Dulce region of Costa Rica (province of Puntarenas). The guano was dried at 50 °C and subsequently stored at –20 °C. Additionally, 14 samples of rainforest top soil (ca. 5 g each) were randomly taken from the surrounding area of the cave within a distance of 1000 meters.

DNA extraction

Different protocols were evaluated in small scale experiments (n = 6): An apple seed-sized piece of guano or soil (approximately 50 mg) was suspended **(a)** in 400 µL CTAB-buffer (200 mM Tris HCl pH 7.5, 1.5 M NaCl, 20 mM Na-EDTA, 2 % CTAB w/v); **(b)** in 400 µL TES-buffer (100 mM Tris HCl pH 8.0, 10 mM Na-EDTA, 2 % SDS w/v); **(c)** in 250 µL skim milk (2 % aqueous suspension w/v, autoclaved) and 200 µL CTAB-buffer; **(d)** in 250 µL skim milk and 200 µL TES-buffer. Samples were vortexed and 400 µL of each mixture were transferred to 1.5-mL tubes with 0.2 g of glass-beads (2 mm and 425–600 µm; proportion 2:1). The suspensions were homogenized using a Retsch MM301 Ball Mill (Retsch Inc., Newtown, Pennsylvania) at maximum speed for 60 seconds. 10 µL Proteinase K (25 U mL^{-1}) were added and the samples were incubated at 65 °C for one hour. To TES-buffered samples (b, d) 70 µL of CTAB (10 % w/v) and 150 µL 5 M NaCl were added and incubated for 10 min at 65 °C. 400 µL chloroform/isoamylalcohol (24:1 v/v) were added to all samples (a, b, c

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and d), samples were centrifuged (5 min, 9500 × g), the aqueous upper phase was transferred to a fresh 1.5-mL tube. This step was repeated twice. 200 µL ammonium-acetate (5 M) were added, samples were incubated at 4 °C for 30 min and subsequently spun down (20 min, 4 °C, maximum speed). The supernatant was transferred to a fresh 1.5-mL tube an equal volume of isopropanol was added and samples were incubated at –20 °C overnight. DNA was pelleted (15 min, 4 °C, maximum speed), washed with ice-cold 70 % ethanol (10 min, 4 °C, maximum speed), air dried and re-dissolved (30 min, 60 °C) in 200 µL TE-buffer (10 mM Tris HCl pH 8, 1 mM Na-EDTA, 4.5 U RNase mL^{-1}).

DNA purification

(1) PVPP (Polyvinylpolypyrrolidone)-spin-columns were used to clean the DNA extracts (according to Damm & Fourie 2005): An opening (approximately 0.3 mm to 0.7 mm in diameter) was made in a 0.5-mL tube with a blood lancet. The tube was filled with PVPP/TE-buffer (0.4 g mL^{-1} PVPP in TE-buffer: 10 mM Tris HCl pH 8, 1 mM EDTA) and centrifuged for 1 min at 900 × g. The flow-through was discarded and the previous step repeated until the PVPP-spin-column was approximately 15 mm high. Subsequently the column was replaced in a 2-mL tube and spun dry for 3 min at 1400 × g. The completed column was placed into a fresh 1.5-mL tube, 50 µL of DNA extract were transferred to the column, incubated for 5 min at room temperature and spun down for 1.5 min at 1400 × g.

(2) Homogenised guano suspensions (prepared as described above in method "c") were purified without any further treatment with PVPP-spin-columns and with Sephadex G-50 columns (equilibrated for 60 min in TE-buffer, produced as described for the PVPP-spin-columns).

Polymerase chain reaction

A final volume of 20 µL PCR reaction mixture contained 1 × PCR-buffer (Promega), 2.5 mM $MgCl_2$, 200 µg mL^{-1} BSA (Bovine Serum Albumin, Sigma Aldrich), 0.2 mM dATP, dCTP, dGTP, dTTP (Promega, Mannheim, Germany), 1 µM of each primer ITS4, ITS5 (White *et al*. 1990; Tab. 1), and 0.5 U *Taq* polymerase (GoTaq® DNA polymerase, Promega). For the nested PCR, the *H.-capsulatum*-specific primer-pairs HistoAF/HistoAR, HistoCF/HistoCR, and HistoAF/HistoCR were used (Ueda *et al*. 2003; Tab. 1). The reaction mixture was the same as described above, except from the BSA concentration (100 µg mL^{-1}). 1 µL of the undiluted ITS4/ITS5 PCR

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product served as DNA template. Amplifications were carried out using a Primus 96 advanced-thermocycler (peqLab Biotechnologie GmbH, Erlangen, Germany) in 200-µL reaction tubes. The following amplification conditions were applied: 120 s at 95 °C initial denaturation; followed by 30 amplification cycles: 20 s at 95 °C, 40 s at 52 °C (ITS4/ITS5) or 65 °C (all Histo Primers), 40 s at 72 °C, and a final extension for 10 min at 72 °C.

Tab. 1. – Primers used for the detection of *Histoplasma capsulatum* in bat guano.

Primer	Primer sequence	Reference
ITS5	5'-GGA AGT AAA AGT CGT AAC CAG G-3'	White *et al.* 1990
ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'	White *et al.* 1990
HistoAF	5'-CAC GCC GTG GGG GGC TGG GAG CCT-3'	Ueda *et al.* 2003
HistoAR	5'-CGG TGT CCC CGG CGG ACA CGG GCC C-3'	Ueda *et al.* 2003
HistoCF	5'-TGA TTG GCG TCT GAG CAT G-3'	Ueda *et al.* 2003
HistoCR	5'-ATG GTG GGC AGG AGC CGG CC-3'	Ueda *et al.* 2003

Cycle sequencing

Excess primers and dNTP's were removed with chromatography columns (Microspin S-300 HR; Pharmacia). For sequencing primers, HistoAF and HistoCR at a 1.6 µM concentration were used. Sequencing was carried out with the ABI Prism BigDye Terminator Cycle Sequencing Kit (PE Biosystems) according to the manufacturer's recommendations. The parameters for cycle sequencing in the GeneAmp 2400 thermocycler (PE Biosystems) were 18 s of delay at 96 °C, followed by 25 cycles of 18 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C.

Results

In the small scale experiments only samples treated with skim milk and CTAB-buffer (c) yielded in positive results with the ITS4/ITS5 primer-pair (two out of six approaches). A subsequent nested PCR with the primer-pairs HistoAF/HistoAR, HistoCF/HistoCR, and HistoAF/HistoCR lead to PCR-products of a length of 150 bp, 352 bp, and 516 bp (Fig. 1). Blasting the 456 bp sequence (GeneBank accession number: DQ980237) of the HistoAF/HistoCR PCR product at the NCBI-server revealed a 99 % to 100 % identity with *Ajellomyces capsulatus* (anamorph: *H. capsulatum*) isolates.

When bat guano was suspended in CTAB-buffer without skim milk (a) or when TES-buffer was used instead of CTAB-buffer (b, d) no PCR products were obtained. Direct amplification of milled guano suspension purified by PVPP or Sephadex G-50 columns without any further treatment was not successful, too.

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To validate DNA-extraction method (c), 2 g of dried guano were mixed with 20 mL 2 % skim milk and 16 mL CTAB-buffer and suspended on an overhead shaker for 30 min. 500 µL of this suspension were ground and treated as described above. Twenty-three out of 24 approaches (95.8 %) led to positive results in the first PCR. Most PCR products displayed double bands on a 1.8 % agarose gel (Fig. 1). After nested PCR, 18 out of the 24 approaches (75 %) were positive for *H. capsulatum*.

Accordingly, the protocol (c) is proposed for the detection of *H. capsulatum* in environmental samples. To facilitate the analysis of an appropriate sample size it is recommended to suspend bat guano in 2% autoclaved skim milk (w/v) and CTAB-buffer in the ratio 1:10:8 (w/v/v) and placing it on an overhead shaker for 30 min. Aliquots (500 µL) of this suspensions should be used in quadruplicates for DNA extraction.

As controls, fourteen soil samples from the Golfo Dulce region (Costa Rica) were also tested with the above described method. PCR with fungal universal primers was successful for 13 samples, whereas *H. capsulatum* could not be detected in any of the samples.

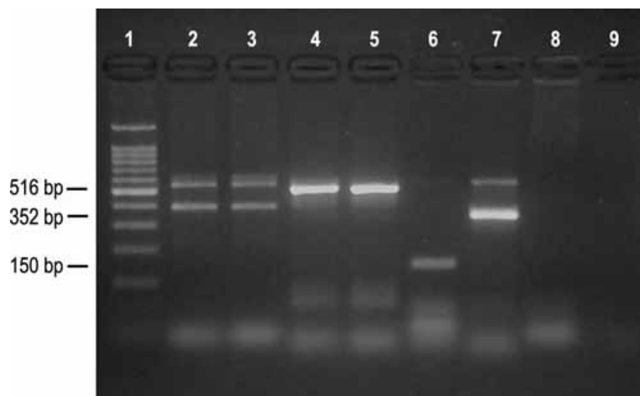

Fig. 1. – Agarose gel electrophoresis. Lane 1: 100 bp DNA-ladder. Lanes 2 and 3: PCR products obtained with universal fungal primer pair ITS4-ITS 5. Lanes 4 and 5: Products of the *Histoplasma capsulatum* specific primer pairs HistoAF-HistoCR (516 bp). Lane 6: Product of HistoAF-HistoAR (150 bp). Lane 7: Product of HistoCF-HistoCR (352 bp); Remnants of the ITS-primers product can be seen at 600 bp. Lane 8 and 9: Sephadex G-50 cleaned crude extracts processed with ITS4-ITS5 (lane 8) and nested PCR HistoAF-HistoCR (lane 9) led to no PCR products.

Discussion:

The development of a DNA-based diagnostic technology for bat guano faces some fundamental problems: bat guano contains 30 % to 65 % of organic matter and 15 % to 25 % humic substances. For the detection of *Histoplasma* in soil samples, a method based on mechan-

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ical rupture and purification with Sephadex G-50-spin-columns was described by Reid & Schafer (1999). In our study, this approach failed. The heat inactivated *Histoplasma*-spores added to soil-extracts in the study mentioned above (Reid & Schafer 1999) may not sufficiently mimic naturally infested bat guano, in which microorganisms live and survive in complex interactions with biotic and abiotic soil components. Sorption of DNA to humic substances and other high-molecular weight compounds has to be minimized (Tsai & Olson 1992, Wilson 1997). In our study, adding skim milk to the guano and the application of PVPP-spin-columns was essential to overcome these DNA-binding-effects. Using skim milk, similar results have been reported for soil DNA extracts (Volosiouk 1995, Takada Hoshino & Matsumoto 2005). The PVPP-spin-columns were highly effective to remove PCR-inhibiting substances: The DNA extracts were clear and colourless after purification. Nevertheless, direct PCR with *Histoplasma*-specific primers was insufficient for a reliable detection of *H. capsulatum*. Only very weak PCR products were obtained in two out of 24 preparations (data not shown). Applying a two step PCR, *H. capsulatum* was detectable in 75 % of our samples (n = 24). The introduced method allows a reliable detection of *H. capsulatum* in bat guano, and consequently, provides a quick and inexpensive risk assessment for bat caves frequented by tourists. It will also enable a risk assessment of bat guano used as manure: although the risk of acquiring histoplasmosis using guano for users is classified low (Kuepper 2003), at least one outbreak of histoplasmosis could be traced back to fertilizers of ornamental plants (Taylor *et al.* 2005)

It was proposed that soils and bat or bird droppings in regions where *H. capsulatum* is endemic should be considered as contaminated and appropriate precautions should be made including wearing respiratory masks and posting health risk warnings to prohibit people to enter the area (Lenhart *et al.* 2004). Tests with soil samples from Costa Rica indicate that the presented DNA extraction method is applicable for soil samples as well: 13 out of 14 samples treated with the described method yielded in positive PCR results with fungal universal primers.

The method presented here does not require animal testing and time-consuming culturing techniques. It is not necessary to establish pure cultures of *H. capsulatum* and, therefore, the infection risk for laboratory workers is kept to a minimum. Results are available within 48 h, in contrast to the animal passage method (Emmons 1961), which takes approximately four to five weeks to obtain results. The presented DNA extraction method allows large scale testing of environmental samples for the presence of *H. capsulatum* and provides the opportunity of an effective risk assessment in endemic areas.

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