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Fungal species identification from avian lung specimens by single hypha laser microdissection and PCR product sequencing

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Accurate species diagnosis in cases of fungal pneumonia may be hampered by environmental contamination and colonization resulting in false-positive results. Our novel approach for fungal species diagnostics combines fluorescent staining of mounted cryosections with the optical brightener Blankophor, laser capture microdissection and PCR amplification with subsequent sequencing of the first internal transcribed spacer region (ITS-1). Using clinical specimens from infected birds, we show that the procedure is suitable for species identification from single hyphae of intralesional filamentous fungi. Our data also suggest that multiple *Aspergillus fumigatus* strain infections may occur frequently in pulmonary aspergillosis of birds.

Keywords LCM, microsatellite, FFPE, genotyping, *Ciconia ciconia*

Introduction

Invasive fungal infections (IFI) have emerged as a common cause of disease in immunocompromised patients. However, in birds respiratory IFI has been reported as a major cause of morbidity and mortality for decades [1,2]. Accurate species diagnosis of the etiologic agents of IFI is of major importance in choosing the appropriate therapy [3,4]. We have recently shown that certain wild avian species might be particularly susceptible to IFI during their first weeks of life [5]. *Aspergillus fumigatus* was isolated in 48.9% of histologically confirmed pulmonary IFI in white stork (*Ciconia ciconia*) chicks. Several birds had concurrent infections. However, results from conventional culturing methods may not always reflect invasive disease and could be influenced by false-positive results from colonization and environmental contamination [6]. Consequently, the European Organization for Research and Treatment of Cancer/Mycoses Study Group has recommended histopathology for confirmation of intralesional fungal growth [7].

Although several special histological stains exist to visualize fungal structures in tissue sections, accurate species determination based on morphology alone is often difficult [4,8]. Laser capture microdissection (LCM) has become a powerful tool to dissect single cells from histological sections and to study them separately from their heterogeneous environment and other contaminants [9,10]. Recently, this technique has also emerged in fungal research [11,12]. Our study aimed to develop a diagnostic approach for reliable and precise fungal species identification directly from tissue lesions based on PCR amplification and sequencing of DNA extracted from laser-dissected fluorescently labeled single fungal hyphae. Blankophor-stained hyphal strands were dissected under a light microscope with a pulsed UV-A laser from tissue sections mounted on glass slides, transferred by laser pressure catapulting into a sample tube and further processed for DNA extraction and PCR. LCM-based species identification clearly allowed the identification of *A. fumigatus* as the dominant species in IFI lesions in storks. We furthermore demonstrated that stork chicks can be infected by multiple *A. fumigatus* strains.

Materials and methods

Tissue samples

Specimens (S) from six animals with histologically confirmed IFI were selected. The lungs of five white stork

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chicks (S1 to S5) were obtained under sterile conditions. The axillar lymph node of a dog (S6) known to have fungal septicemia was used to test the applicability of the described method in specimens other than avian lungs. Each sample was divided into three representative parts. One was inoculated on solid malt-extract agar media (MEA) supplemented with streptomycin sulfate and chloramphenicol (Roth, Karlsruhe, Germany) and incubated at 37°C or 52°C for a maximum of 48 h. Primary fungal mycelia were subcultured to induce conidial formation and subsequent morphological identification to the family level as described previously [5,13]. For species identification, DNA was extracted from mycelium of pure subcultures for PCR amplification as described previously [5,13]. Sequencing of the ITS-1 region was performed as described below for laser microsdissected samples. Sequences were compared to those listed in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

One of the two other parts of the specimens was snap frozen at −80°C, while the second was fixed in 4% buffered formalin for 24 h and subsequently paraffin embedded. Consecutive 4–6 µm sections from frozen tissue samples were mounted on Starfrost adhesive microscope slides (Light Labs, Dallas USA) and stored at −80°C until use. Sections of 4–6 µm thickness from formalin-fixed and paraffin-embedded (FFPE) tissue were stained with haematoxylin and eosin, periodic acid-Schiff (PAS) reaction according to standard protocols or Fontana Masson (FM) with a prolonged incubation time of 90 min in silver solution [14].

Laser capture microdissection of fluorescent hyphal strands

Frozen tissue sections were stained with the optical brightener Blankophor (4,4′-bis[{4-anilino-subst.1,3,5-triazin-2-yl}amino]stilben-2,2′-disulfonic acid) to visualize

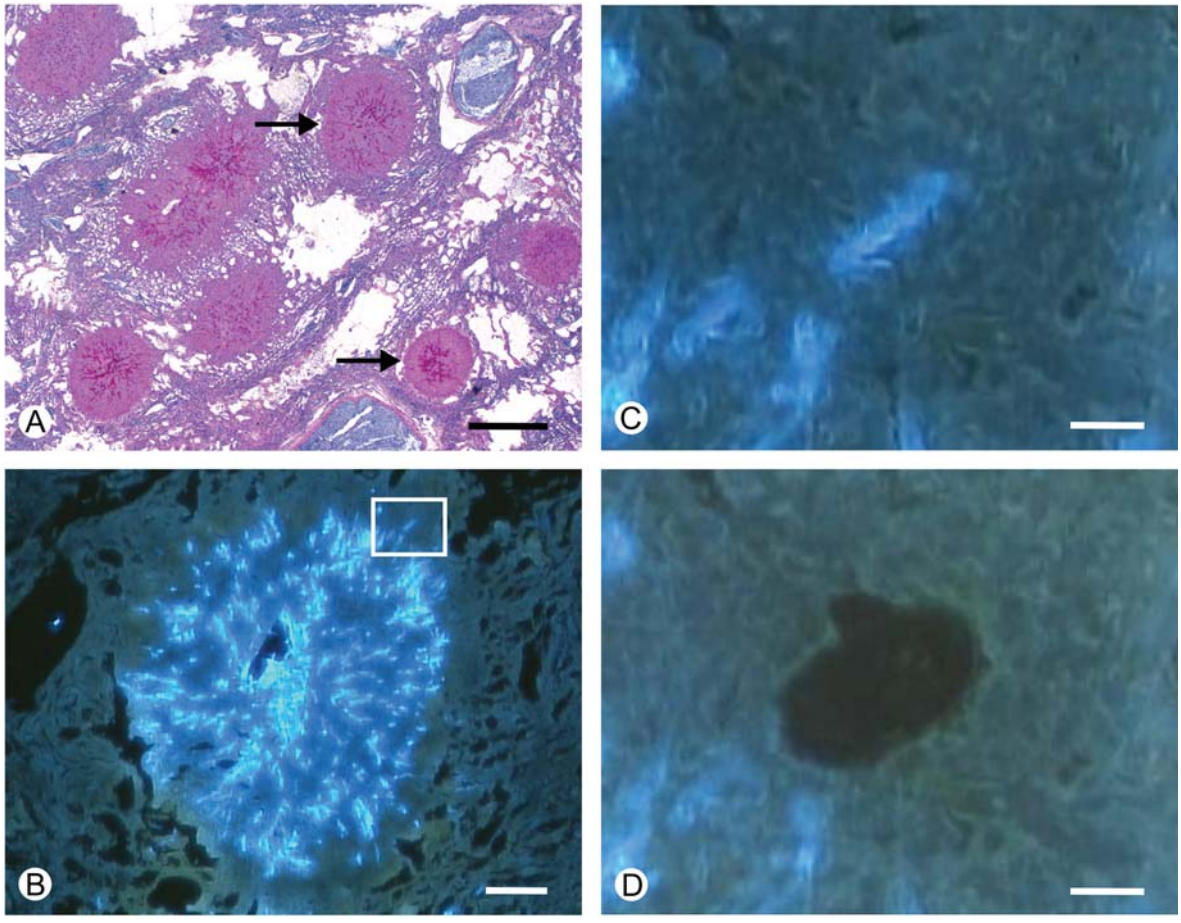

Fig. 1 (A) Consecutive tissue sections of the lung (specimen S1) from a white stork nestling with granulomatous pneumonia and intralesional filamentous fungal structures. Arrows: Granulomas chosen for laser microdissection. (A) Section 1: PAS staining of section (overview). (B) Section 2: Granuloma stained with Blankophor. (C, D) Inset of Fig. 1B with Blankophor-stained hyphae before (C) and after (D) laser capture microdissection of a single hypha. Bars = 500 µm (A); 100 µm (B); 10 µm (C, D).

fungal structures under a fluorescent microscope [15]. No counterstain was used. To prevent tissue maceration and PCR inhibition, a 20% [wt/vol] aqueous stock solution of P-Blankophor (Kemira, Leverkusen, Germany) was diluted 4×10^6-fold in sterile PBS. Directly before microdissection, tissue sections that had been stored at −80°C for 1 and 3 years were thawed at room temperature, stained for 15 sec, dehydrated in ascending graded ethanol and air dried at room temperature. Laser microdissection and laser pressure catapulting were performed using the PALM MicroBeam system (Carl Zeiss MicroImaging GmbH, Jena, Germany) under sterile conditions in a laminar flow biosafety cabinet. The system uses a pulsed UV-A laser of 337 nm wavelength to dissect cells or tissue areas of defined size from a glass slide [9]. The dissected cell or tissue area of interest is then transferred by laser pressure catapulting by a focused laser beam into the cap of a sample tube placed above the dissected area [10]. Single fluorescent hyphal strands were excised out of 10 independent lesions of each specimen and laser pressure catapulted into the lids of 0.5-ml reaction tubes containing 35 µl of lysis buffer (NucleoSpin Tissue XS kit; Macherey & Nagel, Düren, Germany) under sterile conditions (Fig. 1). As negative control, a similarly sized non-fluorescent adjacent area was excised from each specimen and processed in parallel.

DNA extraction and PCR

A small amount (45 ul) of lysis buffer and 8 µl proteinase K (NucleoSpin Tissue XS kit) were added to the excised samples and incubated at 56°C for 24 h. Subsequently, 5 IU lyticase (Sigma-Aldrich, St. Louis, USA) was added and incubated at 37°C for 45 min followed by DNA extraction using the NucleoSpin Tissue XS kit according to the manufacturer's recommendations (Macherey & Nagel) and elution with 20 µl BE buffer. The first internal transcribed spacer (ITS-1) region was PCR amplified from genomic DNA using the panfungal primer set ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-2 (5′-GCTGCGTTCTTCATCGATGC-3′) [16,17], GoTaq Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA) and the following thermal protocol involving initial incubation for 5 min at 95°C, followed by 40 cycles of (i) denaturation for 1 min at 94°C, (ii) annealing for 2 min at 52°C, and (iii) synthesis for 2 min at 72°C. DNA extracted from fungi cultured from each specimen was included as a positive control. All PCR reactions were repeated three times. Amplification products were visualized using gel electrophoresis and ethidium bromide stain on horizontal 2% agarose gels (Bioline, Luckenwalde, Germany) in 0.5 M Tris-borate-EDTA-running buffer.

Sequence analysis

PCR amplicons were purified using the NucleoSpin Extract II system (Macherey-Nagel) and sequenced (Seqlab, Goettingen, Germany) using primers ITS-1 and ITS-2. The sequences were compared to sequences listed in the GenBank database and compared to fungal sequences using the BLAST program [18]. Sequence alignments were conducted using MEGA4 [19]. All derived fungal sequences were deposited into GenBank (for accession numbers see Table 1).

Table 1 Species identification by ITS-1 sequencing of cultured fungi and of 10 laser-microdissected hyphal strands per specimen.

Specimen	Culture	LCM	No. of amplicons positive in LCM	No. of genotypes	Accession numbers
S1	*A. fumigatus*	*A. fumigatus*	7/10	1	GU992278
S2	*A. fumigatus*	*A. fumigatus*	8/10	1	GU992278
	A. niger		0/10		GU992283
	L. corymbifera		0/10		GU992286
S3	*A. fumigatus*	*A. fumigatus*	9/10	4	GU992276, GU992277, GU992278*, GU992279
	L. corymbifera		0/10		GU992286
S4	*A. fumigatus*	*A. fumigatus*	6/10	3	GU992276*, GU992278,
	R. microsporus		0/10		GU992279 GU992284
S5	*A. fumigatus*	*A. fumigatus*	7/10	1	GU992280
	R. oryzae		0/10		GU992285
		Cladosporium spp.	2/10	1	GU992282
S6	*A. terreus*	*A. terreus*	8/10	1	GU992281

*Accession number of *A. fumigatus* cultured and microdissected.

Results

Fungi recovered in culture from the six specimens were identified according to morphology and sequence analysis as *A. fumigatus* in S1 and concurrent infections of *Lichtheimia corymbifera*, *A. fumigatus* and *Aspergillus niger* in S2 (Table 1). Simultaneous infections of *A. fumigatus* were detected with *L. corymbifera* in S3, *Rhizopus microsporus* in S4 and *Rhizopus oryzae* in S5, respectively. Only *Aspergillus terreus* was identified in samples of S6.

PCR and sequencing of DNA extracted from LCM samples identified *A. fumigatus* in 7 of 10 samples from S1 and in 8 of 10 microdissected hyphal strands, respectively (Table 1). *A. fumigatus* was identified in 9 of 10, 6 of 10 and 7 of 10 microdissected hyphal strands of S3, S4, and S5, respectively (Fig. 2). *Cladosporium* spp. was sequenced from two amplicons of samples of S5. *A. terreus* was identified in 8 of 10 microdissected cells of S6. No other fungal or amplifiable analysable DNA was detected in samples S1 to S6. The PCR reactions were repeated three times and gave reproducible electrophoresis results. Sequence comparison of the amplified ITS-1 region identified five sequence variants with base variations in two positions (Fig. 3) within strains of *A. fumigatus*. Four different *A. fumigatus* variants were detected in laser-microdissected hyphal strands in S3, while three different variants *A. fumigatus* were found in S4 (Table 1). FM staining of FFPE tissue sections identified no melanin-containing fungi in specimen S5.

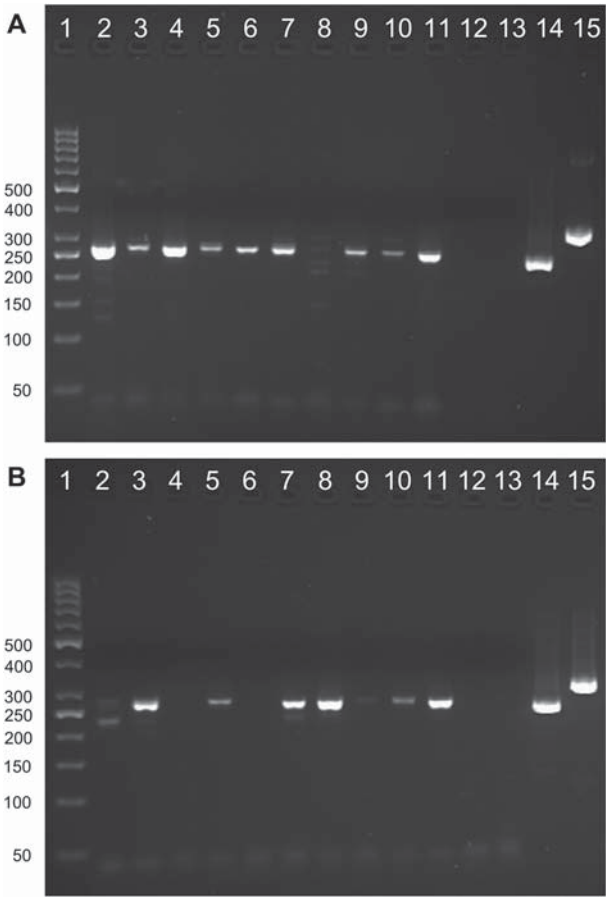

Fig. 2 Gel electrophoresis (2% TBE-agarose gel) showing amplified DNA from laser-microdissected fluorescently labelled single fungal hyphae of specimen S3 (A) and S4 (B), respectively. Lane 1, 50-bp marker. Lanes 2–11, DNA amplified from laser-microdissected hyphal strands by primers ITS1 and ITS2. Lanes 12, non fluorescent, laser-microdissected tissue from the same specimens as negative controls of LCM and DNA extraction. Lane 13, non-template control. Lanes 14 and 15, amplified DNA of cultured fungi as positive controls. Lane 14, *A. fumigatus*. Lane 15, *L. corymbifera* (A) and *R. microsporus* (B), respectively.

Discussion

Accurate species diagnosis is essential in IFI to provide appropriate treatment, as fungal species vary in their susceptibility to antimycotic agents [3,20]. In our study we tested whether PCR amplification of the ITS-1 region from laser-microdissected hyphae from Blankophor-stained cryosections followed by sequencing could be used to identify fungal species from clinical specimens. We obtained fungal ITS-1 sequences from 60–90% of the samples per specimen, confirming the general applicability of our method for genetic species identification. However, as no PCR products could be obtained from 40% of the samples in specimen S4, a sufficient number of LCM samples is needed to achieve a reliable assessment of the infectious fungal species.

By sequencing DNA from individual hyphae from ten independent fungi-positive histological lesions, we confirmed the culture results of *A. fumigatus* and *A. terreus*, respectively, and demonstrated that the approach can differentiate between them. However, we found discrepancies in culture results and LCM-based diagnosis in four of six specimens. *A. niger* was cultured from one specimen, but the fungus could not be detected by LCM and sequencing. Moreover, although zygomycetes (*Lichtheimia* and *Rhizopus*) were detected in four of six specimens by culture, these results could not be confirmed in accompanying LCM samples. These differing results could be due to false-positive culture results caused by contamination or non-invasive colonization. Alternatively, by analyzing ten different lesions per specimen with LCM we might have missed single lesions caused by zygomycetes in a mixed invasive infection.

Furthermore, recent publications have demonstrated that sequencing of the ITS-1 region might not be the optimal method for species differentiation for all fungi because of ITS sequence heterogeneity and other yet unknown factors might hamper amplification or sequencing [21,22]. Thus, failure to identify *Lichtheimia* and *Rhizopus* in the

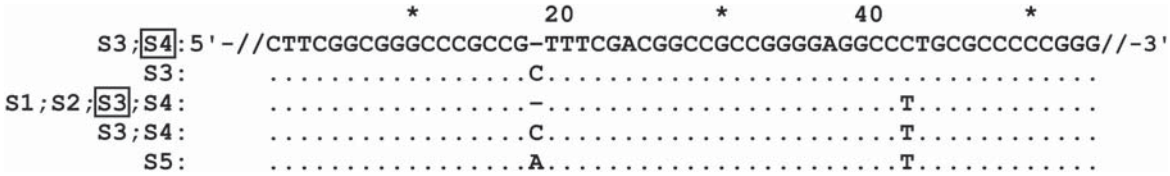

Fig. 3 Sequence alignment of partial ITS-1 regions of microdissected *Aspergillus fumigatus* hyphae. Five different sequence variants were identified due to single nucleotide insertions after position 72 and point mutations at position 96 of the ITS-1 region of *A. fumigatus* (nucleotide positions refer to ATCC36607; [24]). Four different variants were detected in specimen S3, while three different variants were detected in S4. Boxed: Sequence variants identified in cultured *A. fumigatus* of specimens with different variants in LCM.

specimens included in this study might also be partially due to the restricted applicability of ITS sequencing for species identification in certain fungi. Finally, capture of sufficient numbers of nuclei for subsequent PCR amplification might be more difficult in zygomycete hyphae due to the lack of septation (M. E. Brandt, personal communication).

Nevertheless, the LCM-based species results in this study suggest that *A. fumigatus*, in birds, had a dominant role in all cases with concurrent infections in some cases. This information could be critical for the treatment of a patient with IFI [20]. In one specimen we identified *Cladosporium* spp. by sequencing in two of 10 microdissected samples. However, FM staining of the accompanying histologic section could not confirm this result, since no melanin-containing fungi were observed. This implies that contamination of the sample during the downstream processing or due to inhaled spores of *Cladosporium* spp. may have occured. However, it should also be noted that FM staining of dematiaceous fungi stain may require prolonged incubation times and due to faint staining, may not be reliable in all cases [14,23].

Interestingly, comparison of ITS-1 sequences obtained from LCM samples identified three and four different *A. fumigatus* strains in specimen S3 and S4, respectively. The sequences showed single nucleotide variants in two areas known for high ITS-1 sequence variations in *Aspergillus* spp. [24]. Our results confirm previous studies showing that multiple strains of *A. fumigatus* can be recovered from healthy birds and birds with fungal pneumonia [1,25]. Because of limited intraspecies variations, genotyping of *A. fumigatus* strains by sequence analysis of the ITS-1 region alone is not conclusive enough for large scale strain discrimination [26]. However, when combined with strain typing methods such as multilocus sequence typing or microsatellite genotyping [27], our newly developed method may be used for reliable intralesional strain identification in basic fungal research. Moreover, if tissue specimens are available, the method may also be useful for confirmation of the primary infectious fungus directly from biopsies or archived material, i.e., in unclear cases or for retrospective epidemiological studies. Although our study is based mainly on avian lung specimens, we demonstrate that the method is also applicable to mammalian samples. Thus, the technique should be easily transferable to human specimens, and may be used in support of the histological confirmation of IFI in humans.

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