Short Communication

Correspondence
Peter-Michael Rath
rath@uni-essen.de

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Faster genetic identification of medically important aspergilli by using gellan gum as gelling agent in mycological media

Dirk Schmidt and Peter-Michael Rath

Institut für Medizinische Mikrobiologie, Universitätsklinikum Essen, Hufelandstrasse 55, D-45122 Essen, Germany

Using gellan gum as a substitute for agar-agar in a mycological medium and sequencing of the ITS 1 and 2 regions resulted in an accurate identification of *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus terreus* and *Aspergillus ustus* within 24 h of subculture.

Introduction

Members of the genus *Aspergillus* are well-known as opportunistic pathogens especially in immunocompromised patients (Denning, 1998). A fast and accurate species identification is desirable, as certain species are associated with higher mortality, and some show resistance to antifungal agents; for example, *Aspergillus terreus* is resistant to amphotericin B (Sutton *et al.*, 1999). Conventional identification, which is commonly based on macro- and micro-morphological features, is time-consuming and experience-dependent. Another approach could be sequence analysis of the internal transcribed spacer (ITS) regions which are part of the rDNA gene cluster. These regions are known to be highly variable and have been reported to be of great value for the genetic identification of fungi (Iwen *et al.*, 2002). PCR with fungal DNA directly extracted from solid media containing agar as a gelling agent usually fails as agar inhibits PCR in a concentration-dependent manner (Gibb & Wong, 1998). In order to remedy this flaw, we substituted agar with gellan gum (Rath & Schmidt, 2001). Thus we were able to perform DNA extraction, PCR and sequence analysis within a single day.

Methods

We investigated 26 reference strains of six different species obtained from international culture collections (*Aspergillus fumigatus* CBS 180.76, CBS 154.89, ATCC 32617, CBS 133.61 and CBS 152.89; *Aspergillus flavus* CBS 113.49, ATCC 32611, CBS 130.61, CBS 131.54 and ATCC 32612; *Aspergillus niger* CBS 121.55, CBS 733.88, CBS 112.30 and ATCC 10578; *Emmericella nidulans* CBS 542.83, CBS 565.70, CBS 100.20, CBS 240.90 and ATCC 64747; *A. terreus* CBS 116.46 and CBS 469.81; *Aspergillus ustus* CBS 596.65, CBS 239.90, CBS 209.92, CBS 133.55 and IHM 9696). In addition, one isolate of *A. niger* and three isolates of *A. terreus* had been recovered from patients suffering from cystic fibrosis. All strains were subcultured on a solid medium contain-

ing 30 g Sabouraud dextrose broth (Oxoid), 1 g magnesium chloride and 7.5 g gellan gum (GELRITE; Roth) l⁻¹. After 24 h incubation at 35 °C, mycelial blocks of approximately 1 cm² were transferred to a microtube and homogenized by using a mini pestle. Five hundred microlitres of extraction buffer (0.2 M Tris/HCl, pH 8.0; 0.5 M NaCl; 10 mM EDTA; 1 mg proteinase K ml⁻¹) was added and the mixture was incubated for 1 h at 60 °C. DNA was extracted with an equal volume of phenol/chloroform and further precipitation in 0.7 vol. 2-propanol and 0.2 vol. 5 M ammonium acetate. The pellet was resuspended in TE buffer (100 mM Tris/HCl, pH 7.9; 10 mM EDTA) and stored at 4 °C until further use.

The primers which were used for amplifying the ITS 1 (5-8S: 5'-CGCTGCGTTCTTCATCG-3'; SR6R: 5'-AAGTAT AAGTCGTAA CAA GG-3') and ITS 2 (5-8 SR: 5'-TCGATG AAG AAC GCA GCG-3'; LR1: 5'-GGTGGTTTCTTCTTC-3') region have previously been described elsewhere (Gams & Meyer, 1998; W. Meyer, personal communication). Primers had been synthesized by MWG-Biotech. The PCR assay was performed with 5 µl DNA in a total volume of 100 µl including 50 µl mineral oil. The assay mixture contained 10 µl GeneAmp 10× PCR buffer (PE Applied Biosystems), 1.5 mM magnesium chloride, 0.8 µl of a dNTP mixture (Biozym Diagnostik), 1% (v/v) dimethyl sulfoxide, 100 pmol of each of the respective primers and 2.5 U AmpliTaq DNA polymerase LD (PE Applied Biosystems) per 100 µl. PCR was carried out in an MJ Research PTC-150/16 thermocycler (Biozym). After initial denaturation at 95 °C for 3 min, 30 cycles were performed, consisting of a denaturation step at 95 °C for 30 s, an annealing step at 54 °C for 60 s and an extension step at 72 °C for 120 s, with a final extension step at 72 °C for 5 min following the last cycle. For the amplification of the ITS 2 region the same PCR set-up was used, except that the annealing temperature was reduced to 50 °C. Amplification products were separated in a 1% (w/v) agarose gel for 30 min at 150 V.

Prior to sequencing, all amplification products were purified using the QIAquick PCR Purification Kit (Qiagen). For the sequencing PCR assay the 7-Deaza-dGTP Cy5/Cy5.5 Dye Primer Sequencing Kit (Visible Genetics) was used, following the protocol provided by the manufacturer. For sequencing, the same primers were used as mentioned above. All of these had been 5'-end labelled using Cy5 and Cy5.5, respectively (MWG-Biotech). The assay was carried out in an MJ Research PTC-100 thermocycler (Biozym) with the following setup: initial denaturation at 95 °C for 2 min, followed by 35 cycles consisting of a denaturation step at 95 °C for 30 s, an annealing step at 54 °C for 30 s and an extension step at 72 °C for 60 s, with a final extension step at 72 °C for 5 min.

Abbreviation: ITS, internal transcribed spacer.

DNA sequencing was performed with a Long-Read Tower DNA sequencer (Visible Genetics) according to the protocol supplied by the manufacturer. Consensus regions of each pair of sequences of both regions were created using the BioEdit Sequence Alignment Editor version 5.0.9. Sequence comparisons were performed using a non-gapped, advanced BLAST search on the DNA Database of Japan (DDBJ) homepage. Identification of the sequences was made using the highest bit score and e-value of listed species.

Results and Discussion

Identification of the fungi after alignments of each of the consensus sequences of both regions is shown in Table 1. Whenever two or more species of the same strain are listed, these species presented with identical bit scores and e-values.

Most strains of the species *A. fumigatus*, *A. terreus*, *A. ustus* and *E. nidulans* were correctly identified. In contrast, results obtained for strains of the species *A. flavus* and *A. niger* differed from the data obtained by the culture collections or different species gave the same e-value and bit scores. These results are in agreement with data presented by another group who previously met the same problems when comparing sequences of the ITS 1 and ITS 2 regions (Henry *et al.*, 2000). For example, most of the *E. nidulans* strains were identified as *Emericella quadrilineata*. Furthermore, it was not possible to discriminate between closely related species of the *A. flavus* cluster, and between *A. niger* and *Arthrotrichomyces* spp. and *Gliocladium cibotii*, respectively, by sequence analysis, irrespective of which of the two ITS regions was analysed.

Table 1. Identification of fungi after alignments of the consensus sequences of the ITS 1 and ITS 2 regions

Species*	Origin	ITS 1 region	ITS 2 region
<i>Aspergillus fumigatus</i>	CBS 180.76	<i>Neosartorya fischeri</i>	<i>Aspergillus fumigatus</i>
<i>Aspergillus fumigatus</i>	CBS 154.89	<i>Aspergillus fumigatus</i>	<i>Aspergillus fumigatus</i>
<i>Aspergillus fumigatus</i>	ATCC 32617	<i>Neosartorya fischeri</i>	<i>Aspergillus fumigatus</i>
<i>Aspergillus fumigatus</i>	CBS 133.61	<i>Aspergillus fumigatus</i>	<i>Aspergillus fumigatus</i>
<i>Aspergillus fumigatus</i>	CBS 152.89	<i>Aspergillus fumigatus</i>	<i>Aspergillus fumigatus</i>
<i>Aspergillus flavus</i>	CBS 113.49	<i>Aureobasidium mansonii</i> ; <i>Aspergillus flavus</i>	<i>Aspergillus parasiticus</i>
<i>Aspergillus flavus</i>	ATCC 32611	<i>Aspergillus flavus</i>	<i>Aspergillus tamarii</i>
<i>Aspergillus flavus</i>	CBS 130.61	<i>Aspergillus oryzae</i> ; <i>Aspergillus flavus</i>	<i>Aspergillus oryzae</i> ; <i>Aspergillus flavus</i>
<i>Aspergillus flavus</i>	CBS 131.54	<i>Aspergillus toxicarius</i> ; <i>Aspergillus sojae</i> ; <i>Aspergillus parasiticus</i>	<i>Aspergillus sojae</i> ; <i>Aspergillus parasiticus</i> ; <i>Aspergillus toxicarius</i>
<i>Aspergillus flavus</i>	ATCC 32612	<i>Aureobasidium mansonii</i> ; <i>Aspergillus flavus</i> ; <i>Aspergillus oryzae</i>	<i>Aspergillus oryzae</i> ; <i>Aspergillus flavus</i>
<i>Aspergillus niger</i>	CBS 121.55	<i>Aspergillus niger</i> ; <i>Aspergillus wentii</i> ; <i>Aspergillus awamori</i>	<i>Aspergillus niger</i> ; <i>Arthrotrichomyces</i> spp.
<i>Aspergillus niger</i>	CBS 733.88	<i>Aspergillus spec.</i>	<i>Gliocladium cibotii</i> ; <i>Arthrotrichomyces</i> spp.; <i>Aspergillus niger</i>
<i>Aspergillus niger</i>	CBS 112.30	<i>Aspergillus niger</i>	<i>Aspergillus niger</i> ; <i>Arthrotrichomyces</i> spp.
<i>Aspergillus niger</i>	ATCC 10578	<i>Aspergillus foetidus</i> ; <i>Aspergillus tubingensis</i> ; <i>Gliocladium cibotii</i>	<i>Gliocladium cibotii</i>
<i>Aspergillus niger</i>	CF patient	<i>Aspergillus phoenicis</i> ; <i>Aspergillus foetidus</i> ; <i>Aspergillus tubingensis</i>	<i>Gliocladium cibotii</i>
<i>Emericella nidulans</i>	CBS 542.83	<i>Emericella quadrilineata</i>	<i>Emericella nidulans</i> ; <i>Emericella quadrilineata</i>
<i>Emericella nidulans</i>	CBS 565.70	<i>Emericella nidulans</i>	<i>Emericella nidulans</i> ; <i>Emericella quadrilineata</i>
<i>Emericella nidulans</i>	CBS 100.20	<i>Emericella nidulans</i>	<i>Emericella nidulans</i> ; <i>Emericella quadrilineata</i>
<i>Emericella nidulans</i>	CBS 240.90	<i>Emericella nidulans</i>	<i>Emericella quadrilineata</i> ; <i>Emericella nidulans</i>
<i>Emericella nidulans</i>	ATCC 64747	<i>Emericella nidulans</i>	<i>Emericella nidulans</i>
<i>Aspergillus terreus</i>	CBS 116.46	<i>Aspergillus terreus</i>	<i>Aspergillus terreus</i>
<i>Aspergillus terreus</i>	CBS 469.81	<i>Aspergillus terreus</i>	<i>Aspergillus terreus</i>
<i>Aspergillus terreus</i>	CF patient	<i>Aspergillus terreus</i>	<i>Aspergillus terreus</i>
<i>Aspergillus terreus</i>	CF patient	<i>Aspergillus terreus</i>	<i>Aspergillus terreus</i>
<i>Aspergillus terreus</i>	CF patient	<i>Aspergillus terreus</i>	<i>Aspergillus terreus</i>
<i>Aspergillus ustus</i>	CBS 596.65	<i>Aspergillus ustus</i>	<i>Aspergillus ustus</i>
<i>Aspergillus ustus</i>	CBS 239.90	<i>Aspergillus ustus</i>	<i>Aspergillus ustus</i>
<i>Aspergillus ustus</i>	CBS 209.92	<i>Aspergillus ustus</i>	<i>Aspergillus ustus</i>
<i>Aspergillus ustus</i>	CBS 133.55	<i>Aspergillus ustus</i>	<i>Aspergillus ustus</i>
<i>Aspergillus ustus</i>	IHEM 9696	<i>Aspergillus ustus</i>	<i>Aspergillus ustus</i>

*Data from the culture collections or based on macro- and micromorphological characteristics.

Since the analysis of the ITS 2 region gave no significant additional information about the species investigated here, we do not think that it is necessary to perform the time- and cost-intensive analysis of both regions in all cases, as was recommended by Henry *et al.* (2000). The analysis of other regions might be more useful for distinguishing between species, especially in the *A. niger* and *A. flavus* clusters.

Disregarding these limitations, the procedure described here represents progress for the identification of medically important aspergilli. Since there is no inhibition of PCR reactions by gellan gum, DNA from 12–24-h-old cultures can be extracted directly. This significantly reduces the time between sampling and identification. Additionally, the amount of DNA that is extracted is sufficient for PCR and sequence analysis, thus making enhancing by cloning unnecessary.

In conclusion, a fast and accurate genetic identification of medically important *Aspergillus* species is possible using gellan gum as substitute for agar in mycological media, although a cautious interpretation of the results is necessary.

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