Development of A DNA Chip For the Determination of Molds in Indoor and On Material Samples

Kathrin Leppchen-Fröhlich[1], Ina Prade[1], Caroline Rothe[2], Mareen Müller[2], Werner Brabetz[2], Michael Meyer[1]

[1]Research Institute of Leather and Plastic Sheeting (FILK) gGmbH, Meißner Ring 1-5, 09599 Freiberg, Germany, phone +49 3731 366-125, fax , e-mail: michael.meyer@filkfreiberg.de
[2]Biotype Diagnostic GmbH, Moritzburger Weg 67, 01109 Dresden, Germany, phone +49 351 8838 400, fax +49 351 8838 403, www.biotype.de

Abstract

Moisture damage can provoke a high mould contamination on organic materials. These contaminations in interiors of homes or vehicles may induce infections, intoxication and sensitisation. Apart from the health related aspects, the fungus growth destroys infested materials and causes substantial economic damage.
Genera and species of fungi are usually determined following macroscopic and microscopic characteristics. However, several days to weeks are required until a fungus forms its typical features. Moreover, these features are often ambiguous, because preservatives and other additives of the materials compromise the morphology of the fungus.
Here we present a DNA chip for rapid identification of fungi from indoors and interior materials. Our system replaces the classic identification of molds in order to establish new standards in the field of material testing.
The two stage test system is based on a multiplex polymerase chain reaction (PCR) combined with a subsequent detection of PCR products using the Arrayed Ligation Reaction (ALR) chip technology. To date, the application allows the detection of 42 fungal species, 8 genera and 11 clusters.
Compared to the time consuming conventional and previously used molecular biological methods, the ALR chip technology is an easy to handle, routine capable diagnostic providing reliable differentiation of fungal species in short time. In particular, the determination of several fungal species occurring in mixed culture can be realized.

Keywords: Fungus; Genotyping; DNA chip; Polymerase chain reaction; Arrayed Ligation Reaction

1. Introduction

Interior mold contamination received increasing attention in the last decades not only because of the risks for human health. Especially for the leather, textile and wood industry it is a crucial economic problem. The growth of mold fungi leads to various damages and material destruction. The contamination usually occurs from the surrounding air often in combination with increased water activity (aw). Fabrication-associated sources are steam, water logging or process-related steps. Particularly damp, chrome tanned wet blue leather is susceptible to fungal damage. According to guidelines of the Robert Koch Institute (RKI) and the Federal Environment Agency, a meaningful assessment of health burden or material damage requires the differentiation of the fungal species. Additionally, the contamination with specific microorganisms can help to identify the source (for example organic waste, pets, surrounding agricultural companies).

The identification of a given microorganism is therefore a fundamental step for a technical and medical evaluation of fungal damage and for quality and process control during manufacturing.

Routine diagnostic is almost exclusively based on microscopy and microbiological culture of the fungus and requires the isolation of an abundant amount of fungal material. The microorganism is then cultivated as a pure culture and identified based on specific morphologically or biochemically characteristics. Thus, the conventional diagnosis is time-consuming (days to weeks), requires experienced mycological experts and species identification is often limited.

The presented study intended to develop a DNA-based method for a rapid determination of mold contamination in indoor air samples as well as on materials including leather and textiles. The test system combines the detection and identification of a multiplex-PCR product using species-specific probes within an ALR microarray. ALR is a chip-based method for genotyping DNA mutations and polymorphisms using DNA ligases (Case-Green et al. 2003). In this method, sequence-specific scavenger-oligonucleotide are immobilized in discrete areas on a transparent, planar carrier. Single stranded genomic DNA fragments from a multiplex-PCR will be hybridized with the scavenger-oligonucleotides together with a solution of detection-oligonucleotides. These detection-oligonucleotides are fluorescently labeled at the 3'-end and phosphorylated at the 5'-end, and bind downstream of the scavenger-oligonucleotides. A DNA ligase covalently bridges the scavenger and the detection-oligonucleotides. After several washing steps to remove the PCR products, the result of the genotyping can be read with a monochromatic fluorescence scanner or a CCD camera. Compared to simple DNA hybridization chips, the ALR is characterized by universal reaction conditions and a higher specificity to distinguish small DNA sequence differences.

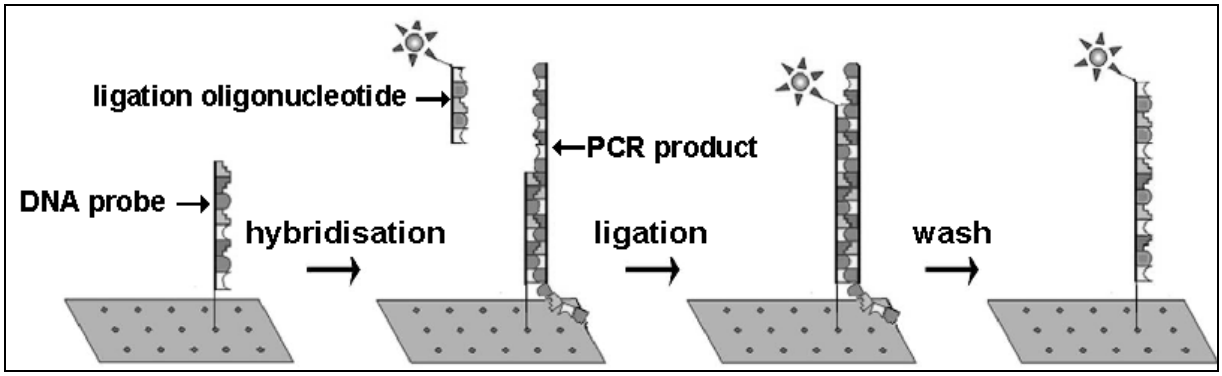

Fig. 1. Schematic representation of the ALR.

2. Material and Methods

Microorganisms. Based on the practical significance and frequency of occurrence on indoor materials, 81 fungus strains were obtained from the Leibniz Institute - German Collection of Microorganisms and Cell Culture (DSMZ) and the Centraalbureau voor Schimmelcultures (CBS), and used as reference strains.

DNA extraction. The fungal strains were cultured for 14 days according to instructions of the provider. DNA was extracted with ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research

Corp.). In short, mycelium and fungal spores were collected in lysis buffer, pretreated with R-Zymolyase (Zymo Research Corp.) for 30 min and mechanically disrupted in a bead mill at maximum speed for 5 min. The resulting solution was centrifuged, mixed with DNA binding buffer and transferred to a DNA collection tube. The DNA was eluated after several washing steps.

ITS and beta-tubulin amplification. rDNA ITS region and beta-tubulin region II were amplified in a multiplex-PCR with the following fungus-specific primers: ITS1-2W (5′-TCCGTWGGTGAACCWGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), bTUB-584-f (5′-ATCTCTGGYGAGCACGGYCT-3′) and bTUB-922-r (5′-GGACTGRCCGAARACGAAGTT-3′). The multiplex-PCR was performed with 1 µl of template DNA or a negative control (water) in a total reaction volume of 25 µl under the following conditions: 94°C for 4 min; 35 cycles of denaturation (94°C, 30 s), annealing (60°C, 60 s), and extension (72°C, 60 s); and a final extension at 72°C for 7 min. The PCR products were purified with NucleoSpin® Extract II (Macherey-Nagel GmbH & Co.KG).

Probe design and microarray fabrication. Species- and genus-specific oligonucleotide probes were designed using ITS and beta-tubulin sequences and alignment tool from Bioedit, ARB (Ludwig et al., 2004) and SILVA software (Pruesse et al., 2007). Probe specificity was confirmed with BLAST-Tool (Basic Local Alignment Search Tool; Cambridge, UK, www.blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA microarray was synthesized with 113 different oligonucleotide probes and controls. Probe signals were synchronized by adapting probe concentration (10-100µm) during the spotting of the DNA chip.

Sample preparation and DNA amplification. In total, 41 unknown DNA samples and 27 contaminated material specimens were analyzed with the chip. DNA from specimens was prepared after mechanical and chemical destruction of small material samples using a bead mill and the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp.). 1 µl isolated DNA was amplified in a multiplex PCR as described above using ITS (ITS-2W and ITS4) and beta-tubulin (bTUB-584-f and bTUB-922-r) primers and purified with NucleoSpin® Extract II (Macherey-Nagel GmbH & Co.KG). Afterwards PCR products were fragmented for 15 min at 37°C with DNA glycosylase and denatured for 10 min at 95°C.

Arrayed Ligation Reaction (ALR) and image analysis. The DNA microarray chip was placed in a heating block at 58°C. PCR products were mixed with ALR reaction buffer (Mycotype® Basidio^{QS} kit, Biotype Diagnostic GmbH) and loaded on the chip. The hybridization reaction was carried out at 58°C for 20 min. The DNA chip was washed with 95°C ddH2O for 1 min, gently slewed for 2 min in a 50°C 0.3 % (w/v) Alconox solution (1/3 Vol. 1 % (w/v) Alconox and 2/3 Vol. 95°C ddH2O) and immersed three times for ca. 1 min in fresh 95°C ddH2O. After drying with gaseous nitrogen, fluorescence signals of the chip were detected with a fluorescence scanner (Ditabis Microarray Scanner MarS, DITABIS AG, Germany). Image processing and calculation of the signal intensities were performed with MycoProof Basidio Software (Biotype Diagnostic GmbH).

3. Results and Discussion

Optimization of a method for the quantitative extraction sufficiently pure genomic DNA. To enable a broad practical application, the DNA isolation was optimized for several materials and air samples collected indoor. Established test systems and standard laboratory

procedures for DNA extraction were tested for materials such as adhesive film specimens, swabs, eluates with spores and mycelia, filter papers, gelatin filters for air sampling and various leather and textile samples. As an example, different methods for the isolation of DNA from leather samples were compared by inoculating and incubating 10^6 fungal spores on leather specimens. The number of spores was taken from the literature and represents the average spore concentration in interior dust (Arbeitskreis „Qualitätssicherung – Schimmelpilze in Innenräumen“, 2001 & 2004). Spores and mycelia were collected and DNA isolation was performed according to the manuals of the manufacturers. As a result, a comprehensive user information for DNA isolation from various materials and gelatin filters for air sampling has been developed and integrated in the Mycotype® BasidioQS manual (Biotype Diagnostic GmbH).

Simultaneous amplification of the rDNA gene region and the beta-tubulin gene of all targeted species in a multiplex polymerase chain reaction. The universal amplification of all targeted fungi was tested with the primer pair ITS1 / ITS4 (White et al. 1990). Since these primers did not amplify all fungi of interest, the forward primer was redesigned (ITS-2W, 5′-TCCGTWGGTGAACCWGCGG-3′). It appears that the ITS region is not sufficient to differentiate closely related species of a genus. Another, more discriminatory DNA region (beta-tubulin gene, region II) was included in the study. The amplification and sequencing of the beta-tubulin gene (primer pair bt3 LM / Bt10 LM; Myllys et al. 2001) of selected Aspergillus and Penicillium species revealed more sequence difference between the fungi compared to the ITS region. Based on the DNA sequences of the reference strain collection and NCBI data, a new primer pair bTUB-584-f and bTUB-922-r was generated specifically for the targeted fungi. The multiplex PCR was established and optimized with the primer pairs regarding buffer composition, primer concentration, annealing temperature and time. It could be shown that all relevant mold species show a PCR product of the ITS region and / or the beta-tubulin region (Fig. 2). The PCR products were purified and sequenced.

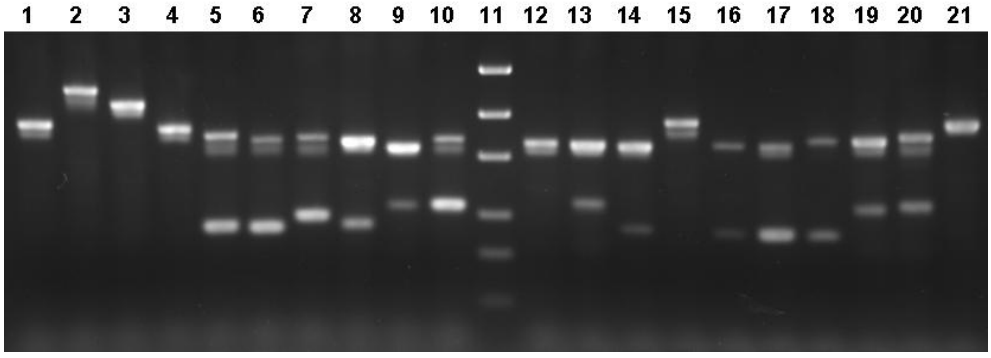

Fig. 2. Example of a gelelectrophoretic separation of multiplex-PCR products from 20 reference molds. Lanes: 1: *Absidia glauca*, 2: *Absidia corymbifera*, 3: *Acremonium alternatum*, 4: *Alternaria alternata*, 5: *Alternaria citri*, 6: *Alternaria tenuissima*, 7: *Acremonium strictum*, 8: *Aspergillus caesiellus*, 9: *Aspergillus candidus*, 10: *Botrytis cinerea*, 11: DNA marker, 12: *Chaetomium globosum*, 13: *Cladosporium sphaerospermum*, 14: *Cladosporium cladosporioides*, 15: *Engyodontium album*, 16: *Exophiala dermatitidis*, 17: *Fusarium culmorum*, 18: *Fusarium oxysporum*, 19: *Fusarium solani*, 20: *Hormoconis resinae*, 21: *Geotrichum candidum*.

Design and testing of functional and specific gene probes for the ALR (Arrayed Ligation Reaction). To identify the fungi of interest, species- and genus-specific probes were designed

based on the ITS and beta-tubulin region of the reference strains. Initially, ITS and beta-Tubulin sequences were aligned to highlight specific and identical sequence segments using BioEdit, ARB (Ludwig et al., 2004) and Silva (Pruesse et al., 2007) software. Individual probes were selected according to the largest possible sequence difference to the sequences of other fungi. Each set of oligonucleotides was tested for cross-reactivity with a BLAST search (Basic Local Alignment Search Tool, Cambridge, UK: www.blast.ncbi.nlm.nih.gov/Blast.cgi). For some species, such as Aspergillus species, no sufficiently specific probes could be derived. Sequence analyses of other target genes did not allow a further differentiation of these fungal species. This is in line with several studies showing that certain types of fungi are identical and indistinguishable both in sequence and in morphological and metabolic characteristics (Geiser et al. 2007, Houbraken et al. 2007, Samson et al. 2007, Varga et al. 2007). These species were grouped into clusters. Finally, at least two specific fungal probes for 42 species, 8 genera and 11 clusters were generated and spotted on a DNA chip.

All 113 probes were tested for specificity and functionality using the ALR-chip technology. The ITS and beta-tubulin region from all 82 reference fungal species was amplified with the optimized multiplex-PCR, purified and tested individually in the ALR using the DNA chip. The evaluation of the specific probe for the fungus Wallemia sebi is shown as an example (Fig. 3). It was found that all 113 probes are functional and specific in the ALR.

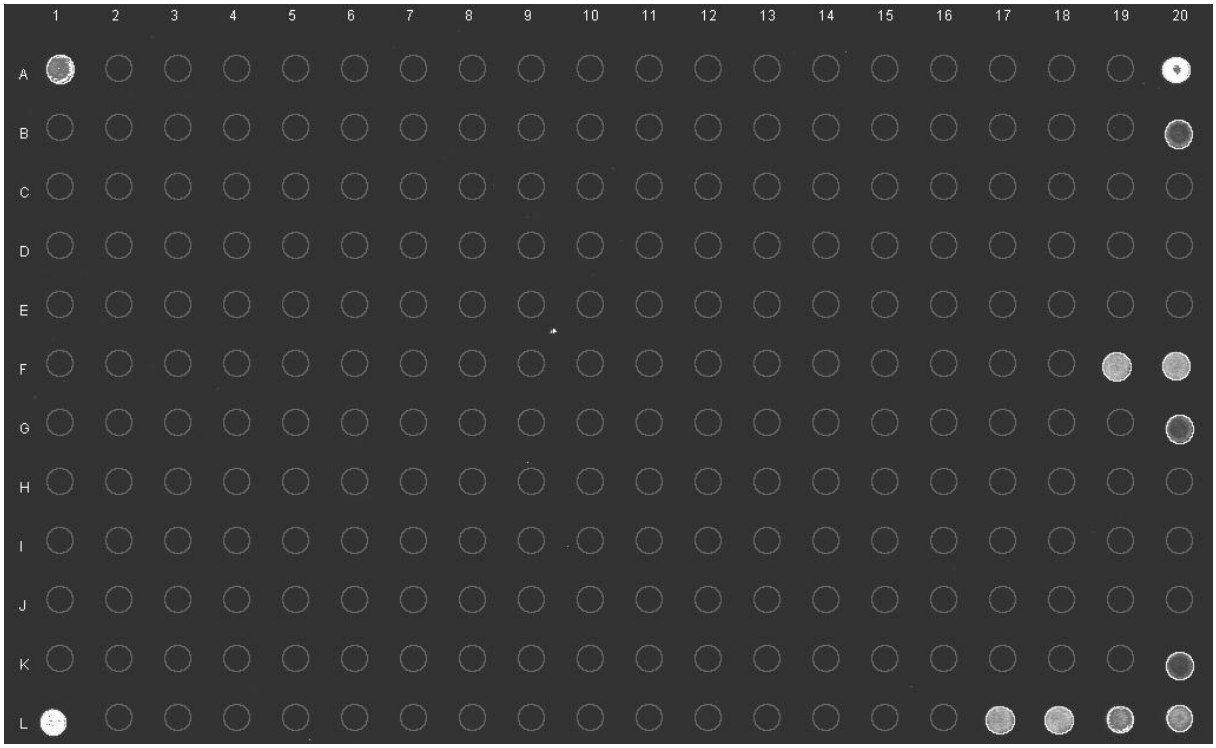

Fig. 3. Scanned fluorescence image after hybridization of DNA from *Wallemia sebi* on the DNA chip. Fluorescence signals confirm the specificity of the *Wallemia sebi* probes (F19, F20, L17, L18). Positive controls appear in A1, A20, B20, G20, K20, L1, L19, L20 indicating a successful PCR and ligation reaction, and the presence of a fungal species. Negative control: A2, B1.

Evaluation of the DNA chip and identification of microorganisms on material samples.

41 anonymous DNA samples (blind samples) were used to validate the functionality of the ALR chip. 38 of 41 test samples were identified correctly, wherein three samples did not show a PCR product.

Practicability of the ALR-chip technology was further tested with DNA extracted from 27 samples. Results were compared to conventional methods (macroscopic and microscopic analysis, sequencing).

The chip allowed the detection and identification of all samples within one day. Furthermore, the detection limit of the DNA chip was found to be as low as 100-200 pg genomic fungal DNA. Additionally, in contrast to conventional methods, it was possible to identify mixed samples containing several fungal species. Simultaneously occurring fungal species in a sample can be differentiated with the DNA chip in a single step. It revealed that the developed DNA chip is a fast and reliable tool with a high success rate. The chip can be standardized and used for diagnostic purpose. Prerequisite for a successful diagnosis is, however, an amplifiable DNA from the sample.

4. Conclusion

The new developed test system (Figure 2) includes a two stage molecular biological laboratory screening test, which consists of a multiplex polymerase chain reaction (PCR) and the species and genus specific identification by the ALR chip technology. The application is based on the detection of 42 fungal species, 8 genera and 11 clusters. Compared to the time consuming conventional and previously used molecular biological methods (e.g. rDNA ITS sequencing), the ALR chip technology is an easy to handle, routine capable diagnostic providing reliable differentiation of fungal species in less time. In particular, the identification of several fungal species occuring in mixed cultures can be realized.

5. Acknowledgements

The ZIM Project No. KF2049512AJ0 was funded by the AiF-Pojekt GmbH on behalf of the German Ministry of Industry and Technology (BMWi) upon a resolution of the German Bundestag. We thank for the granted support.

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