New Strategy for Rapid Diagnosis and Characterization of Fungal Infections: The Example of Corneal Scrapings

Pablo Goldschmidt1*, Sandrine Degorge1, Patricia Che Sarria2, Djida Benallaoua1, Oudy Semoun1, Vincent Borderie1, Laurent Laroche1, Christine Chaumeil1

1 Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts, Paris, France, 2 Laboratoire Jean Dausset, Hôpital Saint Louis, Paris, France

Abstract

Purpose: The prognosis of people infected with Fungi especially immunocompromised depends on rapid and accurate diagnosis to capitalize on time administration of specific treatments. However, cultures produce false negative results and nucleic-acid amplification techniques require complex post-amplification procedures to differentiate relevant fungal types. The objective of this work was to develop a new diagnostic strategy based on real-time polymerase-chain reaction high-resolution melting analysis (PCR-HRM) that a) detects yeasts and filamentous Fungi, b) differentiates yeasts from filamentous Fungi, and c) discriminates among relevant species of yeasts.

Methods: PCR-HRM detection limits and specificity were assessed with a) isolated strains; b) human blood samples experimentally infected with Fungi; c) blood experimentally infected with other infectious agents; d) corneal scrapings from patients with suspected fungal keratitis (culture positive and negative) and e) scrapings from patients with suspected bacterial, viral or Acanthamoeba infections. The DNAs were extracted and mixed with primers diluted in the MeltDoctor® HRM Master Mix in 2 tubes, the first for yeasts, containing the forward primer CandUn (5’CATGCCTGTTGAGCGTC) and the second for filamentous Fungi, containing the forward primer FilamUn (5’TGCCTGTCCGAGCGTCAT) and FungUn. Molecular probes were not necessary. The yields of DNA extraction and the PCR inhibitors were systematically monitored.

Results: PCR-HRM detected 0.1 Colony Forming Units (CFU)/µl of yeasts and filamentous Fungi, differentiated filamentous Fungi from yeasts and discriminated among relevant species of yeasts. PCR-HRM performances were higher than haemoculture and sensitivity and specificity was 100% for culture positive samples, detecting and characterizing Fungi in 7 out 10 culture negative suspected fungal keratitis.

Conclusions: PCR-HRM appears as a new, sensitive, specific and inexpensive test that detects Fungi and differentiates filamentous Fungi from yeasts. It allows direct fungal detection from clinical samples and experimentally infected blood in less than 2.30 h after DNA extraction.

Introduction

The frequency of fungal infections has been increasing for the last 30 years due to viral or iatrogenic immunodeficiencies, the efficiency in treating bacterial infections, the development of indwelling devices, and the massive use of contact lenses.[1–8] The incidence of fungal keratitis (keratomycosis) is also on the rise, and filamentous Fungi are the most frequently reported pathogens. [3,8] From yeasts, Candida albicans is the most frequently associated with disease. However, C. glabrata, C. tropicalis, C. krusei, and C. parapsilosis have gained greater significance.[1,2,4,8]

Fungal infection management requires timely diagnosis for rapid onset of treatments, but approximately one half of the samples remain culture negative and/or negative by fungal antigen detection using immunosorbent assays (ELISA).[9–10] Improved detection performances were reported by amplifying fungal genomic regions (polymerase chain reactions, PCRs). [11,12] However, the classic PCRs do not differentiate filamentous Fungi from yeasts and require post amplification procedures (restriction enzyme digestion and analysis; single-base extension; hybridization probes or molecular sequencing).[11–15] The “gold standard” for fungal characterization is DNA sequencing, but this method is laborious, expensive and cannot be performed routinely for daily diagnosis. [15]

The real-time Taqman PCR using fluorogenic labelled Taqman-probes facilitates the detection and partial characterization of Fungi but requires a series of expensive labelled probes (each probe detects a single fungal type or one species per reaction). [12,16–17]. Because the first-line therapy is different for filamentous Fungi and yeasts as well as for different yeasts, rapid and accurate
The availability of improved fluorescent DNA binding dyes with highly predictable saturation properties allows precise assessment of sequence length by High Resolution Melting real-time PCR (PCR-HRM). [21,22] Recently, a diagnosis test based on PCR-HRM technology was reported for vaginal samples, detecting and identifying \( \text{\textit{C. albicans}} \) at species level. [23] Nevertheless, it was unable to differentiate \( \text{\textit{C. albicans}} \) from filamentous \( \text{\textit{Fungi}} \) and did not detect and characterize \( \text{\textit{S. cerevisiae}} \) and \( \text{\textit{T. candida}} \).

The goal of the present work is to develop a new test able to detect in 1 run the equivalent of at least 1 fungal colony forming unit (CFU) per reaction. In addition this molecular approach should simultaneously differentiate yeasts from filamentous \( \text{\textit{Fungi}} \) and discriminate among relevant species of yeasts in clinical samples and in blood experimentally infected with fungal suspensions.

**Materials and Methods**

Investigations were conducted according to the principles expressed in the Declaration of Helsinki (http://www.wma.net/e/policy/ and were approved by the Institutional Review Board of the Centre Hospitalier National des Quinze-Vings (CHNO), Ministry of Public Health, Paris-France. Written informed consent was obtained from all participants for the use of each sample. Forms with written consent were drafted according to the requirements of the CHNO Review Board and the National Health Authorities were double checked, validated and signed by the physician in charge of the sampling and sent to the laboratory. The preliminary studies were performed with characterized strains isolated from patients presenting corneal ulcers in the National Eye Hospital in Paris (CHNO des Quinze-Vings) or from strains isolated from blood stream infections (generous gift from Dr Christophe Hennequin’s laboratory, CHU Saint-Antoine, Paris, France).

One colony of each fungal species was scraped from the surface after 48 h of culture on Sabouraud’s dextrose agar, suspended in Phosphate Buffer Solution (PBS) and replaced. To reduce the over representation of fungal DNA from non viable organisms, one colony was scraped from the second dish 48 hours later, suspended in PBS and tenfold diluted. Each dilution was divided in several aliquots; three were plated on Sabouraud’s dextrose agar to assess the number of colonies (equivalent CFU/ml) and the others kept as calibrators. For each series of experiments the PCR-HRM detection limits were validated with serial dilutions of fungal suspensions diluted in PBS and simultaneously titrated by plating.

Blood samples were collected in 10 ml citrate tubes from vein puncture of healthy subjects and transported to the laboratory within 1 h. After white cell count to assess the cell load of inoculums (white cell counts >12,000/\( \mu \text{l} \) were excluded) randomized aliquots were spiked with different titrated fungal suspensions. Negative controls consisted in non infected blood or leukocyte suspensions from the same individuals.

For each fungal strain, haemoculture bottles were inoculated with 10 ml of saline or blood spiked or not with \( \text{\textit{Fungi}} \) and incubated up to 12 days before discarded. Fungal isolates were using flanking vector primers. [12,15] Bacteria were cultured and characterized with routine diagnosis tests; \( \text{\textit{Acanthamoeba}} \) and \( \text{\textit{Herpesviridae}} \) were detected by real-time PCR. [24,25]

Corneal scrapings from 38 patients, 13 with proven fungal culture positive, 10 with suspected fungal keratitis culture negative and 15 with non suspected fungal keratitis (bacterial, viral or \( \text{\textit{Acanthamoeba}} \)) were tested masked. Sampling from patients presenting corneal ulcers and requiring microbiological diagnosis was performed by deep corneal scraping by certified ophthalmologists with sterile stainless steel blades after rinsing of fluorescein and topical anaesthetic from the eye surface. [24] Slides with aliquots of scrapings were fixed and stained (Giemsa pH 7.4) for direct microscopic examination and the presence of \( \text{\textit{Fungi}} \) was confirmed by Grocott’s methenamine silver reaction. The second aliquots were cultured within 30 minutes after collection up to 30 days before discarded as culture negative and remnants of blades were frozen dry at \(-80^\circ \text{C}\) for further molecular diagnosis. PCR-HRM was carried out after thawing and addition of 200 \( \mu \text{l} \) of sterile Phosphate Buffer Solution (PBS) to the tubes containing the dry blades.

The DNA extraction was carried out in a vertical safety laminar flow cabinet in a dedicated room. To monitor the extraction yields and the absence of PCR inhibitors the internal control (IC) consisting of 5 \( \mu \text{g} \) of a whole virus preparation of seal herpes virus (gift from G. J. van Doornum, Dept. of Virology Erasmus MC, Rotterdam, The Netherlands) was added to 200 \( \mu \text{l} \) of each suspension (scraping, blood, leukocytes or saline) before extraction (final concentration of 1000 to 2000 viral particles/ml). [24,25] In order to obtain spheroplasts, each specimen (sample + IC) was mixed with tris-EDTA buffer and 10 \( \mu \text{l} \) recombinant lyticase (Sigma-Aldrich, France)/100 \( \mu \text{l} \) of suspension and incubated at 37°C for 60 min. After incubation, the suspensions were vortexed thoroughly and 100 \( \mu \text{l} \) were used for DNA extraction using the MagNA Pure compact nucleic acid isolation kit \( \text{\textregistered} \) as described by the manufacturer in the MagNA Pure Compact automate \( \text{\textregistered} \) (Roche Diagnostics, Meylan, France) and eluted in 100 \( \mu \text{l} \) of elution buffer. To monitor the DNA extraction yields and the PCR inhibitors the seal herpes virus internal control (IC) was amplified in an independent real-time PCR run. [24,25] The primer sequences were respectively: 5’GGGCGAATACACA-GATTGA ATC and 5’GGGGTTCCAACGTACCAA and VIC-TTTTATGTTGTCGCCACCACATCT GGATC-TAMRA for the probe. Amplification and detection of the IC was carried out in a separate tube containing 18.5 \( \mu \text{l} \) of the TaqMan \( \text{\textregistered} \) Fast Universal PCR Mastermix (2X no Ampereace \( \text{\textregistered} \) UNG) (Applied Biosystems-France ABI Ref. 4352042), the forward and the reverse primers (0.5 \( \mu \text{M} \) each) with or without the fluorophore-labelled TaqMan \( \text{\textregistered} \) probe (0.5 \( \mu \text{M} \)). This solution was mixed with 5 \( \mu \text{l} \) of the DNA eluted in DNA and RNA-free solution. The PCR cycling program consisted of one cycle at 95°C for 20 sec and 45 cycles at 95°C for 3 sec and 30 sec at 60°C. [24]

Kits (stable at \(-20^\circ \text{C}\) for at least 12 weeks) for fungal detection consist of 2 tubes, the first for detection, semi quantification and identification of yeasts; the second for detection and semi quantification of filamentous \( \text{\textit{Fungi}} \). Each tube contains 10 \( \mu \text{l} \) of MeltDoctor \( \text{\textregistered} \) HRM Master Mix (MDHRM) (ABI Applied Biosystems-France Ref 4415440), and 1 \( \mu \text{l} \) of the forward and 1 \( \mu \text{l} \) of the reverse primer, each at 300 \( \text{nM} \) (final concentration).

For the detection, quantification and characterization of yeasts and Filamentous \( \text{\textit{Fungi}} \) the primers were selected in a region bracketing significant polymorphisms of multicopy ribosomal genes of the \( \text{\textit{18S}} \) ribosomal RNA gene. The Primer 1: HRM CandidUn1:5’CATGCGCTGTTGATGCGT (conserved sequences of yeasts,) and the Primer 2: HRM FungUn: 5’TCCGTCGCTTATGATGCT (conserved regions of all
Table 1. High-resolution melting analysis (PCR-HRM) detection limits (fungal spore suspensions titrated by plating) and discrimination among fungal species using the primers CandUn + FungUn and FilamUn + FungUn.

| Ref | Strain                   | HRM detection limit (CFU/ml) | Differential profiles                                                                 |
|-----|--------------------------|-----------------------------|---------------------------------------------------------------------------------------|
| I   | a: Candida tropicalis    | ≤0.1                        | ≥1                                                                                   |
| b   | Candida parapsilosis     | ≤0.1                        | ≥1                                                                                   |
| c   | Candida albicans         | ≤0.1                        | ≥1                                                                                   |
| d   | Candida glabrata         | ≤0.1                        | ≥1                                                                                   |
| e   | Candida krusei           | ≤0.1                        | ≥1                                                                                   |
| f   | Saccharomyces cervisiae  | ≤0.1                        | ≥1                                                                                   |
| g   | Trichosporon             | ≤0.1                        | ≥1                                                                                   |
| h   | Aspergillus nidulans     | ≥5                          | ≤0.1                                                                                 |
| i   | Aspergillus niger        | ≥5                          | ≤0.1                                                                                 |
| j   | Penicillium picum        | ≥5                          | ≤0.1                                                                                 |
| k   | Aspergillus sp.          | ≥5                          | ≤0.1                                                                                 |
| l   | Fusarium solani          | ≥5                          | ≤0.1                                                                                 |

Summary: The primers CandUn and FungUn were used to develop a high-resolution melting analysis (PCR-HRM) method for the detection and discrimination of fungal species. The method allowed obtaining profiles for the different yeasts according to the sizes of amplicons (alignment of sequences according to EMBL data library). The amplicon sizes (nucleotides bracketed by the primers CandUn + FungUn) for Candida tropicalis and Candida parapsilosis were ≤0.1 CFU/ml, whereas Candida albicans and Candida glabrata showed a detection limit of ≥1 CFU/ml.

Results

Preliminary experiments were performed to assess the best conditions for extraction of DNA from spores: a- heat for 10 min at 94°C; b- proteinase K at 37°C for 60 min and heat at 94°C for 10 min; c- proteinase K at 37°C for 60 min, heat at 94°C for 10 min and extraction with the MagNA Pure compact nucleic acid isolation kit. The PCR-HRM method was used to detect and discriminate among fungal species, allowing for the development of a diagnostic tool for fungal infections.
MagNA Pure Compact® automate (Roche Diagnostics, Meylan, France); d- shaked in presence of beads and extraction with MagNA Pure; e- shaked in presence of beads with or without proteinase K at 37°C for 60 min, heat at 94°C for 10 min and extraction with Magna Pure; f- shaked in presence of beads with or without lyticase at 37°C for 60 min and heat at 94°C for 10 min; g- shaked in presence of beads with lyticase at 37°C for 60 min, heat at 94°C for 10 min and extracted with Magna Pure; or h- lyticase at 37°C for 60 min, heat at 94°C for 10 min and extraction with Magna Pure. The highest fungal DNA extraction rates were obtained using the procedures g or h (results not shown).

The detection limits have been obtained by dilution of fresh titrated fungal suspensions. PCR-HRM with the primers CandUn + FungUn detected 0.1 CFU/µl of Candida albicans, C. krusei, C. glabrata, C. tropicalis, Saccharomyces cerevisiae and Trichosporon and 1 CFU/µl of filamentous Fungi suspended in PBS. As shown in Table 1 PCR-HRM detection capacities were repeatedly higher for yeasts (10 to 100 times) using the set CandUn + FungUn, and more than 10 times higher for filamentous Fungi using the set FilamUn + FungUn (Aspergillus nubilus, A. niger, A. versicolor, A. terreus, Penicillium picum and Fusarium solani). These results suggest that the optimization of fungal detection requires the simultaneous amplification of DNA extracts in 2 tubes, the first with the set CandUn + FungUn and the second with FilamUn + FungUn. Under these conditions, the PCR-HRM coefficient of variation for the interassay reproducibility in the complete linear range of detection [10⁵ to 10⁻¹ colony forming units (CFU)/ml] for 5 runs was less than 10%. The patterns of the first derivative (difference plot) permitted differentiation of yeasts from filamentous Fungi and the divergence between amplicon sizes of closely related species allowed PCR-HRM to easily discriminate among yeasts (Figure 1). According to the amplicon sizes bracketed by the set of primers FilamUn + FungUn (generally ≥194 nucleotides for Fusarium sp. versus ≤192 for most species of Aspergillus) the melting curve shapes were repeatedly different for Fusarium solani (Figure 2).

Sensitivity and specificity of PCR-HRM (while comparing with corneal scraping cultures) was of 100%. PCR-HRM allowed rapid diagnosis of keratomycosis differentiating clinical relevant species of yeasts, and produced negative results for all the samples obtained from patients with non suspected fungal keratitis and for all the negative controls [DNA extracted from 10⁶ CFU/ml of Bacteria, 10⁶ PFU (plaque forming units)/ml of Herpes simplex virus type 1 or 10⁵ PFU/ml of Herpes simplex virus type 2, and 10⁵ Acanthamoeba cysts/ml suspended in saline] (Table 2). Human cells (10⁶ human epithelial cells or fibroblasts) did not interfere with the PCR-HRM performances, confirming the in-silico specificity predictions. In patients with clinically suspected fungal keratitis (samples 1; 3; 5; 6; 8; 10; 12; 13; 17; 18; 20; 23; 24) culture was positive in 55% and images evoking Fungi were detected in 65% of the clinical samples by direct microscopic examination (Table 2). In 4 out 10 patients with clinically suspected fungal keratitis and culture negative (samples 29–38), Fungi were detected by direct microscopic examination of deep corneal scrapings (filaments in 2;
budding yeasts in 1 and pseudohypha in 1) (samples 29; 31; 32 and 36). In addition, for these same 10 culture negative patients, PCR-HRM detected and characterized Fungi in 7 out of 10, including the 4 detected by direct microscopic examination. The relative high number of cornea negative cultures could be partially the result of residual eye drop preservatives carried with the samples from the eye surface. PCR-HRM was negative for all the controls carried out with the samples obtained from the air, surfaces, reactants and the blood of healthy donors (total blood or buffy coats) (Table 2).

Table 3 shows the recovery and detection time for haemoculture bottles spiked with Fungi. For fungal inoculums containing 100 CFU/bottle, the time for positivity was ranged between 16 and 36 hours of incubation for yeasts and between 36–48 hours for filamentous Fungi. Positivity was obtained after 24 to 48 hours of incubation of blood spiked with yeasts and after 24 to 72 hours with filamentous Fungi. For inoculums of 10 CFU/bottle the time for positivity was ranged between 24–48 hours for yeasts and between 48 and 72 hours for filamentous Fungi. For yeasts suspended in blood cultures were positive after 24–48 hours and after 24–72 h for filamentous Fungi. Only C. albicans, Trichosporon and Penicillium piccum could be detected for inoculums containing 1 CFU per bottle (after 96 h of culture) in saline. Inoculums containing 1 CFU per bottle or less of 7 different yeasts or 4 filamentous Fungi suspended in blood were negative. PCR-HRM detected 100% of the samples containing the equivalent of 0.1 CFU/ml of yeasts and filamentous Fungi and generated reproducible melt-curves. When challenged against profiles obtained from referenced strains run in parallel the melting profiles obtained with all the samples allowed differentiating yeasts from filamentous Fungi and discriminating among 7 different strains of yeasts. PCR-HRM performances were equivalent for Fungi suspended in saline or blood at concentrations significantly lower (10 times or more) than the detection limits of fungal cultures (Table 3).

**Discussion**

The automatic melting analysis of fungal sequences amplified with 2 sets of primers diluted in a mix containing a DNA intercalating dye (SYTO9) allowed rapid detection of Fungi. The differences in amplicon sizes between species were suited to fungal differentiation of yeasts from filamentous Fungi and to speciation among yeasts. By adapting the existing real-time PCR instrumentation for data acquisition it was possible to carry out reproducible diagnosis in less than 2.30 h after DNA extraction, with detection limits of at least 0.1 CFU of filamentous Fungi and yeasts per μl of sample with no need for molecular probes (radioactive, enzymatic or fluorogenic) or post amplification procedures (sequencing, amplicon restriction enzyme analysis, etc.). Corneal samples could
Table 2. Comparison of direct microscopic examination, culture and high-resolution melting analysis performances (PCR-HRM) on corneal scrapings obtained from patients with keratitis.

| Sample Number | Sample Type | Diagnostic method | PCR (Herpes simplex and Acanthamoeba) | HRM results and profiles |
|---------------|-------------|-------------------|----------------------------------------|--------------------------|
| 1             | yeasts      | Candida glabrata  | NEG                                    | Candida glabrata          |
| 2             | GNR         | Pseudomonas aeruginosa | NEG                                      | NEG                      |
| 3             | budding yeasts | Candida albicans | NEG                                    | Candida albicans          |
| 4             | GPC         | Staphylococcus aureus | NEG                                      | NEG                      |
| 5             | yeasts      | Candida kruzei     | NEG                                    | Candida kruzei            |
| 6             | Yeasts; pseudohypha | Saccharomyces cervisiae | NEG                                      | S. cervisiae              |
| 7             | GPR         | Corynebacteria     | NEG                                    | NEG                      |
| 8             | filaments   | Aspergillus nidulans | NEG                                      | Filamentous Fungi         |
| 9             | LY; AEC     | Candida tropicalis | NEG                                    | Candida tropicalis        |
| 10            | filaments   | Fusarium solani   | NEG                                    | Fusarium sp.              |
| 11            | LY; AEC     | Candida parapsilosis | NEG                                      | Candida parapsilosis      |
| 12            | GPC         | Staphylococcus epidermidis | NEG                                      | NEG                      |
| 13            | GPC         | Escherichia coli  | NEG                                    | NEG                      |
| 14            | GPR         | Acinetobacter baumannii | NEG                                      | NEG                      |
| 15            | yeasts      | Trichosporon      | NEG                                    | POS; Trichosporon        |
| 16            | yeasts      | Candida parapsilosis | NEG                                      | Candida parapsilosis      |
| 17            | yeasts      | Streptococcus agalactiae | NEG                                      | NEG                      |
| 18            | FF          | Aspergillus niger | NEG                                    | Filamentous Fungi         |
| 19            | GPR         | Fusarium solani   | NEG                                    | Fusarium sp.              |
| 20            | GPC         | Pseudomonas aeruginosa | NEG                                      | NEG                      |
| 21            | GPR         | Streptococcus pneumoniae | NEG                                      | NEG                      |
| 22            | AEC         | Aspergillus niger | NEG                                    | Filamentous Fungi         |
| 23            | GPR         | Fusarium solani   | NEG                                    | Fusarium sp.              |
| 24            | GPC         | Propionibacterium acne | NEG                                      | NEG                      |
| 25            | GPR         | Staphylococcus epidermidis | NEG                                      | NEG                      |
| 26            | GPC         | Pseudomonas aeruginosa | NEG                                      | NEG                      |
| 27            | GPR         | Streptococcus pneumoniae | NEG                                      | NEG                      |
| 28            | filaments   | NEG                | NEG                                    | Filamentous Fungi         |
| 29            | budding yeasts | NEG                | NEG                                    | Candida albicans          |
| 30            | NEG         | NEG                | NEG                                    | NEG                      |
| 31            | filaments   | NEG                | NEG                                    | Fusarium sp.              |
| 32            | budding yeasts | NEG                | NEG                                    | Candida albicans          |
| 33            | NEG         | NEG                | NEG                                    | NEG                      |
| 34            | NEG         | NEG                | NEG                                    | Filamentous Fungi         |
| 35            | NEG         | NEG                | NEG                                    | NEG                      |
| 36            | filaments   | NEG                | NEG                                    | Filamentous Fungi         |
| 37            | NEG         | NEG                | NEG                                    | Filamentous Fungi         |
| 38            | NEG         | NEG                | NEG                                    | Candida albicans          |

Controls

| Controls | Sample Type | PCR (Herpes simplex and Acanthamoeba) | HRM results and profiles |
|----------|-------------|----------------------------------------|--------------------------|
| 39       | NEG         | $10^6$ Human epithelial cells | NEG                                      | NEG                      |
| 40       | NEG         | Distilled water | NEG                                      | NEG                      |
| 41       | NEG         | Saline | NEG                                      | NEG                      |
| 42       | NEG         | DNA extraction reactants | NEG                                      | NEG                      |
| 43       | NEG         | $10^6$ Human fibroblasts | NEG                                      | NEG                      |
| 44       | NEG         | Transport media | NEG                                      | NEG                      |
be readily assayed after DNA extraction without interference from other DNAs found in the specimens.

Fungal culture performances depend on the type of agent, the fungal load and the mass of material that can be processed, the microbiology laboratory capacities and the presence of antiseptic or antifungal in the samples. Moreover, cultures rely on the ability of the organism to grow ex vivo and may require long incubation periods (may become positive late in the course of the infection), are time consuming and prone to contamination. [1,4,11].

In 89% of patients with suspected fungal keratitis PCR-HRM was able to detect *Fungi* differentiating filamentous *Fungi* and yeasts. Interestingly, in all patients with positive PCR-HRM the corneal infiltrates were dramatically reduced after antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown). Local and topical antifungal treatments, suggesting that PCR-HRM signals represented true positive infections (results not shown).

**Table 2.** Cont.

| Sample Number | Diagnostic method | Culture | PCR (Herpes simplex and Acanthamoeba) | HRM results and profiles |
|---------------|-------------------|---------|--------------------------------------|--------------------------|
| 45            | NEG               | Laminar-flow air control | NEG | NEG |
| 46            | NEG               | 10⁶ Human leukocytes ** | NEG | NEG |
| 47            | NEG               | Whole blood donor 1     | NEG | NEG |
| 48            | NEG               | Whole blood donor 2     | NEG | NEG |
| 49            | NEG               | Whole blood donor 3     | NEG | NEG |
| 50            | NEG               | Whole blood donor 4     | NEG | NEG |
| 51            | NEG               | 10⁷ Human leukocytes     | NEG | NEG |
| 52            | NEG               | 10⁷ Human leukocytes     | NEG | NEG |
| 53            | NEG               | 10⁷ Human leukocytes     | NEG | NEG |
| 54            | NEG               | 10⁷ Human leukocytes     | NEG | NEG |
| 55            | NEG               | Air                   | NEG | NEG |
| 56            | NEG               | Laminar flow (air)     | NEG | NEG |
| 57            | NEG               | Laminar flow (surface) | NEG | NEG |
| 58            | NEG               | DNA extraction reactants| NEG | NEG |
| 59            | NEG               | Laminar flow (surface) | NEG | NEG |
| 60            | NEG               | Air (laboratory)       | NEG | NEG |
| 61            | NEG               | Laminar flow (air)     | NEG | NEG |
| 62            | NEG               | DNA extraction reactants| NEG | NEG |

HRM: high-resolution melting analysis; **: after Giemsa (pH: 7.4) and Grocott staining; POS: Positive; NEG: Negative; FF: Filamentous Fungi; GNR: Gram-negative rods; GPR: Gram-positive rods; GPC: Gram-positive cocci; LY: lymphocytes; AEC: Altered epithelial cells; HSV1: Herpes-simplex virus type 1; HSV2: Herpes-simplex virus type 2; AC: Acanthamoeba; **: DNA was extracted from 6 different individuals and tested separately; #:#: controls were extracted and tested for each run; *: the leukocytes from 4 different donors were extracted and tested separately. Air and working surface samples were obtained placing open tubes for 15 minutes under the laminar flow or in the laboratory working table. Surface samples were collected with cotton devices humidified with saline by gentle swabbing the working surface. DNA extraction procedures were conducted in duplicate in each series of experiments for the DNA extraction reactants.

doi:10.1371/journal.pone.0037660.t002
| Fungal load (log CFU/ml) | Fluid | Diagnosis method | Fungal Culture # Result Hours for positivity | HRM # Result Profile |
|-------------------------|-------|-----------------|---------------------------------------------|----------------------|
| **Candida albicans**    |       |                 |                                             |                      |
| 0                      | S     | POS             | 16                                          | POS POS C. albicans  |
| 1                      | S     | POS             | 24                                          | POS POS C. albicans  |
| 0.1                    | S     | POS             | 96                                          | POS POS C. albicans  |
| **Candida krusei**      |       |                 |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS C. krusei    |
| 1                      | S     | POS             | 48                                          | POS POS C. krusei    |
| 0.1                    | S     | NEG             | 96                                          | POS POS C. krusei    |
| **Candida glabrata**    |       |                 |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS C. glabrata  |
| 1                      | S     | POS             | 48                                          | POS POS C. glabrata  |
| 0.1                    | S     | NEG             | 96                                          | POS POS C. glabrata  |
| **Candida tropicalis**  |       |                 |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS C. tropicalis|
| 1                      | S     | POS             | 48                                          | POS POS C. tropicalis|
| 0.1                    | S     | NEG             | 96                                          | POS POS C. tropicalis|
| **Candida parapsilosis**|      |                 |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS C. parapsilosis|
| 1                      | S     | NEG             | 36                                          | POS POS C. parapsilosis|
| 0.1                    | S     | NEG             | –                                            | POS POS C. parapsilosis|
| **Saccharomyces cerevisiae** | |      |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS S. cerevisiae|
| 1                      | S     | NEG             | 36                                          | POS POS S. cerevisiae|
| 0.1                    | S     | NEG             | –                                            | POS POS S. cerevisiae|
| **Trichosporon**        |       |                 |                                             |                      |
| 0                      | S     | POS             | 24                                          | POS POS Trichosporon|
| 1                      | S     | NEG             | 36                                          | POS POS Trichosporon|
| 0.1                    | S     | NEG             | –                                            | POS POS Trichosporon|
| **Aspergillus niger**   |       |                 |                                             |                      |
| 0                      | S     | POS             | 36                                          | POS POS Filamentous Fungi|
| 1                      | S     | NEG             | 36                                          | POS POS Filamentous Fungi|
| 0.1                    | S     | NEG             | –                                            | POS POS Filamentous Fungi|
For diagnosis of candidemia in subjects with haematological malignancies or various forms of immunodeficiency a real-time PCR targeting the 18 S rRNA gene and requiring a series of labelled molecular probes, yielded positive results in 58.3% of blood culture-positive samples and detected in blood the genomes of Candida earlier than culture. [37] In this series 27% of whole-blood were PCR positive compared to 15% of haemoculture (92% of correlation of positives). Other studies indicate that numerous pairs of primers and labelled probes were required for each sample to identify 72% of species of positive cultures. [38] For vaginal specimens it was reported a test based on PCR-HRM technology, but it was unable to differentiate yeasts from filamentous fungi. [22] However, the strategy developed here is different because PCR-HRM is able to detect and differentiate yeasts and filamentous fungi in one run with detection limits of 0.1 CFU/ml or less. In addition to differentiating filamentous fungi from yeasts, PCR-HRM discriminated among relevant clinical species of yeasts in less than 2.30 hours after DNA extraction. This only required upgrading the available real-time PCR software of the thermocyclers used in the routine microbiology laboratory.

Because PCR-HRM was able to produce consistent results without the need for synthesizing and labelling molecular probes and without post amplification procedures (restriction enzymes, electrophoresis, gel analysis, hybridisation, sequencing reactants) the cost for reagents for testing one DNA extract could be reduced to less than 2 USD (2 primers and HRM mix). Compared to classic PCR (amplification followed by electrophoreses and/or hybridization and/or sequencing) this new PCR-HRM has the additional advantage of minimizing risks for false positive results due to cross contamination, because the targeted sequence amplification, the signal detection, and the DNA melting analyses are carried out in closed tubes. Moreover, PCR-HRM minimizes risks for false negative results because the yields of extraction of the DNA and the potential interference of PCR inhibitors are systematically monitored in each run and for all the samples. According to the results obtained in this study, if the future runs generate reproducible melt-curves over time in different settings, a reference database could be built to store PCR-HRM calculations and shapes of the melting profiles for each family or species to be challenged against profiles. Larger prospective multicentric trials testing different types of samples (clinical and environmental) are necessary to validate PCR-HRM usefulness as a diagnosis tool and for environmental studies.

Acknowledgments

We thank the Centre de Ressources Biologiques du Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts, 75012 Paris, France, for providing biological samples.

Author Contributions

Conceived and designed the experiments: PG SD. Performed the experiments: PG SD DB OS. Analyzed the data: PG SD DB OS CC. Contributed reagents/materials/analysis tools: PG PB LL CC. Wrote the paper: PG.
References

1. Edmond MB, Wallace S, McClish D, Pfaffer MA, Jones RN, et al. (1999) Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis 29: 239–44.

2. Ascoglu S, Rex JH, de Paauw B, Bennett JE, Bille J, et al. (2002) Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer, Mycoses Study Group of the National Institute of Allergy and Infectious Diseases. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 34: 7–14.

3. Szczotka-Flynn LB, Pearlman E, Ghanmoun M (2010) Microbial contamination of contact lenses, lens care solutions and their accessories: a literature review. Eye Contact Lens 36: 116–29.

4. Mattern KA, Carter R, Crippa F, Wald A, Corey L (2002) Epidemiology and outcome of mould infections in hematopoietic stem cell transplant recipients. Clin Infect Dis 34: 909–17.

5. Pagano L, Caira M, Gandoni A, Oliediani M, Fanchi L, et al. (2006) The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 91: 1068–75.

6. Horvath L, George B, Murray C, Harrizzo L, Hospenthal D (2004) Direct comparison of the BACTEC 9240 and BacT/ALERT 3D automated blood culture systems for Candida growth detection. J Clin Microbiol 42: 115–8.

7. Rosa C, Araujo R, Rodrigues A, Pinto-de-Sousa M, Pina-Vaz C (2011) Detection of Aspergillus species in BACTEC blood cultures. J Med Microbiol 60: 1467–71.

8. Hall BJ, Jones I (2010) Contact lens cases: the missing link in contact lens safety. Eye Contact Lens 36: 101–5.

9. Allan EK, Jordanides NE, McLintock LA, Copland M, Devaney M, et al. (2005) Development of novel real-time PCR assays for detection and differentiation of 11 medically important Aspergillus and Candida species in clinical specimens. J Clin Microbiol 43: 5660–4.

10. Maertens J, Verhaegen J, van Eldere J, Boogaerts M (2001) Multilocus DNA reaction and slot blot hybridization. Diagn Microbiol Infect Dis 38: 207–12.

11. Jordanides NE, Allan EK, McLintock LA, Copland M, Devaney M, et al. (2005) Comparison of SYT09 and SYBR Green I for real-time polymerase chain reaction and melting curve analysis of panfungal amplicons. J Mol Diagn 12: 91–101.

12. Goldschmidt P, Rostane H, Saint-Jean C, Batellier L, Arouchef C, et al. (2006) High-throughput identification and quantification of Candida species using high resolution melt analysis of panfungal amplicons. J Mol Diagn 12: 91–101.

13. Fidel Jr P, Vazquez J, Sobel J (1999) Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. Clin Microbiol Rev 12: 80–96.

14. van Doornum G, Guldemeester J, Osterhaus A, Nieters H (2003) Diagnosis of herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 41: 576–80.

15. Sevzaranag R, Buı U, Limaye, Cookson B (2003) Rapid identification of commonly encountered Candida species directly from blood culture bottles. J Clin Microbiol 41: 5660–4.

16. van Den Bosch H, Dromer F, Improvisi I, Lozano-Chiu M, Rex H, et al. (1998) Antifungal drug resistance in pathogenic fungi. Med Mycol 36(suppl): 119–28.

17. Sullivan D, Haynes K, Bille J, Boerlin P, Rodero L, et al. (1997) Widespread geographic distribution of oral Candida dubliniensis strain in human immunodeficiency virus-infected individuals. J Clin Microbiol 35: 960–4.

18. Rico P, Vazquez J, Sobel J (1999) Candida albicans: review of epidemiology, pathogenesis, and clinical disease with comparison to C. glabrata. Clin Microbiol Rev 12: 80–96.

19. Hsu M, Chen K, Lo H, Chen Y, Liao M, et al. (2003) Species identification of Aspergillus fumigatus DNA in clinical samples from neutropenic patients. J Clin Microbiol 41: 1011–1018.

20. Hsu M, Chen K, Lo H, Chen Y, Liao M, et al. (2005) Species identification of medically important fungi by use of realtime LightCycler PCR for determination and quantification of Aspergillus fumigatus DNA in clinical samples from neutropenic patients. J Clin Microbiol 41: 1011–1018.

21. Arancia S, Carattoli A, La Valle R, Cassone A, De Bernardinis F (2006) Use of 65 kDa mannoprotein gene primers in real time PCR identification of Candida albicans in biological samples. Mol Cell Probes 20: 263–268.

22. Fidel Jr P, Vazquez J, Sobel J (1999) Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. Clin Microbiol Rev 12: 80–96.

23. Tamer I, Gündüz F, Demirtürk B, Ziya U, Sönmez F, et al. (2011) Development of a LightCycler PCR assay for determination and quantification of Aspergillus fumigatus DNA in clinical samples from neutropenic patients. J Clin Microbiol 49: 721–725.

24. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

25. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

26. Selvarangan R, Buı U, Limaye, Cookson B (2003) Rapid identification of commonly encountered Candida species directly from blood culture bottles. J Clin Microbiol 41: 5660–4.

27. Vanden Bossche H, Dromer F, Improvisi I, Lozano-Chiu M, Rex H, et al. (1998) Antifungal drug resistance in pathogenic fungi. Med Mycol 36(suppl): 119–28.

28. Sullivan D, Haynes K, Bille J, Boerlin P, Rodero L, et al. (1997) Widespread geographic distribution of oral Candida dubliniensis strain in human immunodeficiency virus-infected individuals. J Clin Microbiol 35: 960–4.

29. Fidel Jr P, Vazquez J, Sobel J (1999) Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. Clin Microbiol Rev 12: 80–96.

30. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

31. Spiess B, Buchheidt D, Baust C, Skladny H, Seifarth W, et al. (2003) Development of a LightCycler PCR assay for determination and quantification of Aspergillus fumigatus DNA in clinical samples from neutropenic patients. J Clin Microbiol 41: 1011–1018.

32. Hsu M, Chen K, Lo H, Chen Y, Liao M, et al. (2005) Species identification of medically important fungi by use of realtime LightCycler PCR. J. Med. Microbiol 52: 1071–1076.

33. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

34. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

35. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

36. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

37. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

38. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

39. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.

40. Timpani G, Gavaldà J, et al. (2006) Detection of fungal DNA by real-time polymerase chain reaction: evaluation of 2 methodologies in experimental pulmonary aspergillosis. Diagn. Microbiol. Infect. Dis 56: 307–309.