

METHODS FOR THE DETECTION AND IDENTIFICATION OF FUNGAL SPECIES

Field of the Invention

The present invention is generally in the field of the detection and identification nucleic acids in a sample. More specifically, it relates to the detection and identification of fungal species in a sample by detecting and identifying genomic DNA of said fungal species, in particular of fungal species that are involved in fungal sepsis. The detection and identification of such fungal nucleic acids in the sample then allows the diagnosis of fungal sepsis in a subject from which said sample has been obtained.

Background of the invention

Sepsis is a serious medical condition with high mortalities that requires rapid treatment. Each year, according to the Centers for Disease Control and Prevention (CDC), at least 1.7 million adults in the U.S. develop sepsis, and nearly 270,000 die as a result. While conventionally sepsis is associated with bacterial infection, over the recent years, fungal infections and fungal sepsis have been reported to be increasing and are associated with considerable morbidity and mortality. Particularly problematic is that many fungal infections go undiagnosed. Fungal sepsis is therefore often diagnosed too late or not at all to the detriment of the patients. It is estimated that the risk of death from sepsis is as high as 30% and increases in cases of severe sepsis up to 50% and septic shock up to 80%.

A further complication is that the current gold standard methods for the detection of fungal sepsis pathogens (blood culture and microbiology tests) and the following diagnosis take 3 to 7 days. It has been estimated that the mortality rate among septic shock patients increases by 7.6% for each hour appropriate antimicrobial treatment is delayed. In many cases of suspected sepsis, empirical treatment with broad-spectrum antibiotics is therefore immediately started without waiting for the result of pathogen identification. In some of these cases, inappropriate antibiotic selection does not only annul the effects of chemotherapy but also promotes the emergence of drug-resistant bacteria. This is highly undesired and has long-lasting consequences in that it promotes the occurrence of multi-resistant bacteria and fungi strains.

While invasive fungal infections are predominantly caused by *Candida* species, with candidemia being a highly common health care-associated bloodstream infection, increasingly immunocompromised patient populations have given rise to the emergence of fungal infections with other fungal species such as *Aspergillus species*, *Pneumocystis jiroveci*, *Cryptococcus*, *Zygomycetes*, *Fusarium species*, and *Scedosporium species*. At least 17 *Candida* species have been reported to cause severe infections in humans, but 5 species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) represent more than 90%. *C. albicans* has historically been the predominant pathogen, but to date other *Candida* species, such as *C. glabrata*, *C. parapsilosis* and *C. tropicalis* become more and more common in septic infections. As these species differ significantly in their susceptibility to certain compounds, such as triazoles and echinocandins, knowledge of the epidemiology is imperative for selection of appropriate empirical therapy.

There is thus need in the art for methods that allow rapid detection and identification of causative agents for fungal sepsis which allow the distinction between common and related fungal species involved in fungal sepsis such as to allow appropriate antimicrobial therapy without delay.

Summary of the Invention

The present invention meets this need by providing a method and kit that allow for the rapid detection and identification of a variety of fungal species in a sample simultaneously and thus allow avoiding the current laborious and time-consuming detection methods.

In a first aspect, the present invention is thus directed to a method for the detection of the presence and/or amount of two or more target nucleic acids in a sample, wherein the two or more target nucleic acids are of fungal pathogens involved in fungal sepsis, the method comprising:

i) contacting the sample with a combination of two or more oligonucleotide amplification primer sets under conditions that allow hybridization of the oligonucleotide primers to the target nucleic acids of the fungal pathogens, wherein the two or more primer sets are directed to different target nucleic acids of at least two different fungal pathogen species involved in fungal sepsis and are independently selected from the group consisting of:

- (1) a *Candida albicans* primer set targeting a nucleotide sequence in the rDNA of *Candida albicans*;
- (2) a *Candida tropicalis* primer set targeting a nucleotide sequence in the rDNA of *Candida tropicalis*;
- (3) a *Candida glabrata* primer set targeting a nucleotide sequence in the rDNA of *Candida glabrata*;
- (4) a *Candida parapsilosis* primer set targeting a nucleotide sequence in the rDNA of *Candida parapsilosis*;
- (5) a *Candida krusei* primer set targeting a nucleotide sequence in the rDNA of *Candida krusei*;
- (6) a *Candida guilliermondii* primer set targeting a nucleotide sequence in the rDNA of *Candida guilliermondii*;
- (7) a *Candida auris* primer set targeting a nucleotide sequence in the rDNA of *Candida auris*;
- (8) a *Cryptococcus neoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus neoformans*;
- (9) a *Cryptococcus deneoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus deneoformans*;
- (10) a *Cryptococcus gattii* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus gattii*;
- (11) a *Fusarium spp.* primer set targeting a nucleotide sequence in the rDNA of *Fusarium spp.*;
- (12) a *Trichosporon spp.* primer set targeting a nucleotide sequence in the rDNA of *Trichosporon spp.*

Trichosporon spp.;

(13) a *Schizosaccharomyces pombe* primer set targeting a nucleotide sequences in the genomic DNA of *Schizosaccharomyces pombe*; and

(14) a *Candida spp.* primer set targeting a nucleotide sequence in the rDNA of *Candida spp.*; and

ii) amplifying the target nucleic acid(s) hybridized to any one or more of the oligonucleotide primers by PCR; and

iii) detecting the presence and/or amount of the target nucleic acid(s) in the sample by contacting the PCR amplified sample of step ii) with combination of oligonucleotide detection probes comprising at least one probe specific for each of the amplicons produced by the amplification of the target nucleic acids in step ii) under conditions that allow the hybridization of the probes to their specific target nucleic acid sequences and determining the presence and/or amount of probe:target hybrids.

In various embodiments of these methods according to the invention,

- (1) the target region of rDNA of *Candida albicans* is of ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is of ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is of ITS1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is of ITS2 region thereof,
- (5) the target region of rDNA of *Candida krusei* is of ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is of ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is of ITS2 region thereof;
- (8) the target region of rDNA of *Cryptococcus neoformans* is of IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is of IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is of IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is of 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is of 18S region thereof;
- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is of Cytochrome B gene region; and/or
- (14) the target region of rDNA of *Candida spp.* is of 18S region thereof.

In various embodiments, at least one primer of the *Candida albicans* primer set, the *Candida tropicalis* primer set, the *Candida parapsilosis* primer set, the *Candida krusei* primer set, the *Candida guilliermondii* primer set, and the *Candida auris* primer set comprises the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof. In various embodiments,

- (1) at least one primer of the *Candida albicans* primer set comprises the nucleotide sequence of SEQ ID NO: 2 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 2 over its entire length, or a complement thereof;
- (2) at least one primer of the *Candida tropicalis* primer set comprises the nucleotide sequence of SEQ ID NO: 3 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 3 over

its entire length, or a complement thereof;

(3) at least one primer of the *Candida parapsilosis* primer set comprises the nucleotide sequence of SEQ ID NO: 4 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 4 over its entire length, or a complement thereof;

(4) at least one primer of the *Candida krusei* primer set comprises the nucleotide sequence of SEQ ID NO: 5 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5 over its entire length, or a complement thereof;

(5) at least one primer of the *Candida guilliermondii* primer set comprises the nucleotide sequence of SEQ ID NO: 6 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 6 over its entire length, or a complement thereof; and

(6) at least one primer of the *Candida auris* primer set comprises the nucleotide sequence of SEQ ID NO: 7 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 7 over its entire length, or a complement thereof. In these embodiments (1) to (6) the other primer of the primer pair may a primer that comprises the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof.

In various embodiments, the *Candida glabrata* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 8 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 8 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 9 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 9 over its entire length, or a complement thereof.

In various embodiments, the *Cryptococcus neoformans* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 10 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 10 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 11 over its entire length, or a complement thereof.

In various embodiments, the *Cryptococcus deneoformans* primer set comprises a primer having a nucleotide sequence of SEQ ID NO: 12 or the nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 12 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 13 over its entire length, or a complement thereof.

In various embodiments, the *Cryptococcus gattii* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 14 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 14 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 15 over its entire length, or a complement thereof.

In various embodiments, the *Fusarium spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 16 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 17 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 17 over its entire length, or a complement thereof.

In various embodiments, the *Trichosporon spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 18 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 18 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 19 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 19 over its entire length, or a complement thereof.

In various embodiments, the *Schizosaccharomyces pombe* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 20 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 20 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 21 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 21 over its entire length, or a complement thereof.

In various embodiments the *Candida spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 22 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 22 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 23 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 23 over its entire length, or a complement thereof.

In various embodiments, any two or more of the primer sets described herein above may be used in a single multiplexing method. In various embodiments, the methods of the invention thus use 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 primer sets as described herein. These primer sets are preferably different in that they are directed against different species or families of organisms and allow specific detection and identification thereof.

It is understood that the more general primer sets disclosed herein, namely those directed to *Candida spp.*, *Trichosporon spp.*, and *Fusarium spp.*, are specific for the respective family of fungi, but cannot distinguish between the different species belonging to that family. However, their inclusion may still be preferable, as a positive signal with a *Candida spp.* primer set, while all specific *Candida* primer sets are negative indicates that the sample comprises a *Candida* species other than those specifically tested.

In various embodiments, the combination of oligonucleotide detection probes comprises two or more probes selected from:

- (1) a *Candida albicans* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida albicans*;

- (2) a *Candida tropicalis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida tropicalis*;
- (3) a *Candida glabrata* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida glabrata*;
- (4) a *Candida parapsilosis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida parapsilosis*;
- (5) a *Candida krusei* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida krusei*;
- (6) a *Candida guilliermondii* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida guilliermondii*;
- (7) a *Candida auris* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida auris*;
- (8) a *Cryptococcus neoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus neoformans*;
- (9) a *Cryptococcus deneoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus deneoformans*;
- (10) a *Cryptococcus gattii* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus gattii*;
- (11) a *Fusarium spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Fusarium spp.*;
- (12) a *Trichosporon spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Trichosporon spp.*;
- (13) a *Schizosaccharomyces pombe* probe having a nucleotide sequence specific to a target region in the genomic DNA of *Schizosaccharomyces pombe*; and
- (14) a *Candida spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida spp.*

In various embodiments,

- (1) the target region of rDNA of *Candida albicans* is in the ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is in the ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is in the ITS 1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is in the ITS2 region thereof;
- (5) the target region of rDNA of *Candida krusei* is in the ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is in the ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is in the ITS2 region thereof;
- (8) the target region of rDNA of *Cryptococcus neoformans* is in the IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is in the IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is in the IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is in the 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is in the 18S region thereof

- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is in the Cytochrome B gene region; and
- (14) the target region of rDNA of *Candida spp.* is in the 18S region thereof.

In various embodiments,

- (1) the *Candida albicans* probe comprises the nucleotide sequence of SEQ ID NO: 24 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 24 over its entire length, or a complement thereof;
- (2) the *Candida tropicalis* probe comprises the nucleotide sequence of SEQ ID NO: 25 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 25 over its entire length, or a complement thereof;
- (3) the *Candida glabrata* probe comprises the nucleotide sequence of SEQ ID NO: 30 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 30 over its entire length, or a complement thereof;
- (4) the *Candida parapsilosis* probe comprises the nucleotide sequence of SEQ ID NO: 26 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 26 over its entire length, or a complement thereof;
- (5) the *Candida krusei* probe comprises the nucleotide sequence of SEQ ID NO: 27 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 27 over its entire length, or a complement thereof;
- (6) the *Candida guilliermondii* probe comprises the nucleotide sequence of SEQ ID NO: 28 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 28 over its entire length, or a complement thereof;
- (7) the *Candida auris* probe comprises the nucleotide sequence of SEQ ID NO: 29 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 29 over its entire length, or a complement thereof;
- (8) the *Cryptococcus neoformans* probe comprises the nucleotide sequence of SEQ ID NO: 32 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 32 over its entire length, or a complement thereof;
- (9) the *Cryptococcus deneoformans* probe comprises the nucleotide sequence of SEQ ID NO: 33 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 33 over its entire length, or a complement thereof;
- (10) the *Cryptococcus gattii* probe comprises the nucleotide sequence of SEQ ID NO: 34 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 34 over its entire length, or a complement thereof;
- (11) the *Fusarium spp.* probe comprises the nucleotide sequence of SEQ ID NO: 35 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 35 over its entire length, or a complement thereof;
- (12) the *Trichosporon spp.* probe comprises the nucleotide sequence of SEQ ID NO: 36 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 36 over its entire length, or a

complement thereof;

(13) the *Schizosaccharomyces pombe* probe comprises the nucleotide sequence of SEQ ID NO: 37 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 37 over its entire length, or a complement thereof; and

(14) the *Candida spp.* probe comprises the nucleotide sequence of SEQ ID NO: 31 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 31 over its entire length, or a complement thereof.

In various embodiments, the combination of primer sets comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or at least 14 different primer sets. The combination of primer sets may be selected from the group consisting of:

(1) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;

(2) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set and *Candida auris* primer set;

(3) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set and *Candida krusei* primer set;

(4) *Candida albicans* primer set, *Candida tropicalis* primer set and *Candida glabrata* primer set;

(5) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set;

(6) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;

(7) *Candida albicans* primer set and *Candida tropicalis* primer set; and

(8) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set and *Cryptococcus gattii* primer set.

The combination of probes may comprise at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or at least 14 different probes. In various embodiments, the different probes are each specific for a different pathogenic fungi species.

The combination of probes may be selected from the group consisting of:

(1) *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida guilliermondii* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;

(2) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis*

probe, *Candida krusei* probe, *Candida guilliermondii* probe and *Candida auris* probe;

(3) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe and *Candida krusei* probe;

(4) *Candida albicans* probe, *Candida tropicalis* probe and *Candida glabrata* probe;

(5) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe;

(6) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;

(7) *Candida albicans* probe and *Candida tropicalis* probe; and

(8) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe and *Cryptococcus gattii* probe.

The method of the invention may be a method for the diagnosis of fungal sepsis in a subject. The subject may be a human subject. In various embodiments, the sample is a blood, serum or plasma sample. In these methods for the diagnosis of fungal sepsis in a subject, the subject may be diagnosed with fungal sepsis, if one or more of the target nucleic acids are detected in the sample. Depending on the diagnosis and the detected microorganism an appropriate therapy may be selected, typically an antifungal agent that is effective against the identified pathogen.

In another aspect, the present invention relates to a kit for carrying out the method of the invention. Such kits typically comprise two or more amplification primer sets and two or more detection probes, wherein (A) the two or more amplification primer sets are directed to different target nucleic acids of at least two different fungal pathogen species involved in fungal sepsis and are independently selected from the group consisting of:

(1) a *Candida albicans* primer set targeting a nucleotide sequence in the rDNA of *Candida albicans*;
(2) a *Candida tropicalis* primer set targeting a nucleotide sequence in the rDNA of *Candida tropicalis*;
(3) a *Candida glabrata* primer set targeting a nucleotide sequence in the rDNA of *Candida glabrata*;
(4) a *Candida parapsilosis* primer set targeting a nucleotide sequence in the rDNA of *Candida parapsilosis*;

(5) a *Candida krusei* primer set targeting a nucleotide sequence in the rDNA of *Candida krusei*;

(6) a *Candida guilliermondii* primer set targeting a nucleotide sequence in the rDNA of *Candida guilliermondii*;

(7) a *Candida auris* primer set targeting a nucleotide sequence in the rDNA of *Candida auris*;

(8) a *Cryptococcus neoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus neoformans*;

(9) a *Cryptococcus deneoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus deneoformans*;

(10) a *Cryptococcus gattii* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus gattii*;

- (11) a *Fusarium spp.* primer set targeting a nucleotide sequence in the rDNA of *Fusarium spp.*;
 - (12) a *Trichosporon spp.* primer set targeting a nucleotide sequence in the rDNA of *Trichosporon spp.*;
 - (13) a *Schizosaccharomyces pombe* primer set targeting a nucleotide sequences in the genomic DNA of *Schizosaccharomyces pombe*; and
 - (14) a *Candida spp.* primer set targeting a nucleotide sequence in the rDNA of *Candida spp.*; and
- (B) the two or more detection probes comprise at least one probe specific for each of the amplicons produced by amplification of the target nucleic acids by each of the two or more primer pairs.

In various embodiments of the kits of the invention, the primer sets and probes are defined as described above in relation to the described methods.

The invention further features the use of the kit of the invention for the diagnosis of fungal sepsis in a subject. The subject and the sample analyzed may be as described above for the methods of the invention. Also encompassed are methods for the diagnosis of fungal sepsis in a subject, wherein said kits are used, preferably to allow practicing the methods of the invention.

Brief Description of the Drawings

Figure 1 shows the test results of a test panel of 14 primer set/probe combinations for 14 different pathogenic fungi. In these experiments, PCR was used for amplification of genomic DNA of the respective target.

Detailed Description

The present invention is based on the inventors' surprising finding that pathogens that are involved in and responsible for fungal infection and fungal sepsis and human patients can be rapidly detected and identified using a multiplex diagnostic assay that uses a panel of primers and probes that allow simultaneous detection and identification of fungal pathogens in a given sample. It has been found that the developed methods and assays allow specific detection of numerous fungal pathogens in a sample without suffering from high background signals, cross-reactivity or any other interference that may hamper nucleic acid amplification and detection.

The methods of the invention are therefore for the detection of the presence and/or amount of two or more target nucleic acids in a sample, wherein the two or more target nucleic acids are of fungal pathogens involved in fungal sepsis. While the described methods may qualitatively detect the presence or absence of a given target, with the presence being confirmed if the detected signal exceeds a pre-determined threshold value, they are similarly suitable to quantitatively determine the target in a sample and thus provide information on the concentration levels of the target in the sample. Accordingly, the methods can detect either only the presence or additionally also the amount of two or more target nucleic acids in sample.

"Two or more" and "at least two", as used interchangeably herein, refer to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,

12, 13, 14 or more. It is understood that if used in relation to a specific target nucleic acid, it relates not to the absolute number of identical target molecules but rather to different types of target molecules. For example, "two or more target nucleic acids" of at least two different fungal pathogens may comprise target nucleic acids of *C. albicans* and *C. glabrata*. The same applies if this term is used in relation to amplification primer sets and probes.

The sample used in the methods of the invention may be any type of sample and includes tissue and body fluid samples, such as those obtained from a subject, as well as cultured samples. The sample may thus be a blood, serum or plasma sample or a cultured blood sample or any other cultured sample. The sample may be subjected to various processing steps before being used in the methods of the invention, including common isolation and/or purification steps, for examples steps to extract DNA from cells that may be present in the sample.

The methods of the invention generally comprise the steps of amplification of the target nucleic acid(s) and the subsequent detection of the amplicons. For the amplification, the two or more oligonucleotide amplification primer sets are contacted with the sample, (suspected of) containing the target nucleic acid(s) under conditions that allow the hybridization of the respective primers to their target. The hybridization conditions are selected such that they allow the specific hybridization of the primer to its complementary target sequence. The hybridization conditions are typically selected such that they are strict enough to avoid unspecific interactions or interactions with nucleic acids that are not sufficiently, preferably not fully, complementary to the target-specific region of the primer. This means that in various embodiments, hybridization to nucleic acids that are not fully complementary but comprise one or more mismatches does not occur or does at least not stably occur under the selected hybridization conditions. These hybridization conditions therefore allow to minimize unspecific interaction and thus serve to reduce background signals in the following amplification.

The primers generally comprise a target-specific region, i.e. a sequence stretch that is fully complementary to the intended target sequence. In various embodiments, the primers consist of such a target-complementary region, i.e. the full length of the primer is designed and intended to hybridize to the target. The primers are typically used in pairs. Such primer pair generally comprises a forward and a reverse primer that are designed such that they flank the sequence of the target to be amplified. "Complementary", as used herein in relation to nucleic acid interaction, relates to conventional Watson-Crick base pairing.

The primer pairs are selected from the following primers pairs:

- (1) a *Candida albicans* primer set targeting a nucleotide sequence in the rDNA of *Candida albicans*;
- (2) a *Candida tropicalis* primer set targeting a nucleotide sequence in the rDNA of *Candida tropicalis*;
- (3) a *Candida glabrata* primer set targeting a nucleotide sequence in the rDNA of *Candida glabrata*;
- (4) a *Candida parapsilosis* primer set targeting a nucleotide sequence in the rDNA of *Candida parapsilosis*;

- (5) a *Candida krusei* primer set targeting a nucleotide sequence in the rDNA of *Candida krusei*;
- (6) a *Candida guilliermondii* primer set targeting a nucleotide sequence in the rDNA of *Candida guilliermondii*;
- (7) a *Candida auris* primer set targeting a nucleotide sequence in the rDNA of *Candida auris*;
- (8) a *Cryptococcus neoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus neoformans*;
- (9) a *Cryptococcus deneoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus deneoformans*;
- (10) a *Cryptococcus gattii* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus gattii*;
- (11) a *Fusarium spp.* primer set targeting a nucleotide sequence in the rDNA of *Fusarium spp.*;
- (12) a *Trichosporon spp.* primer set targeting a nucleotide sequence in the rDNA of *Trichosporon spp.*;
- (13) a *Schizosaccharomyces pombe* primer set targeting a nucleotide sequences in the genomic DNA of *Schizosaccharomyces pombe*; and
- (14) a *Candida spp.* primer set targeting a nucleotide sequence in the rDNA of *Candida spp.*

It is understood that, for example, a “*Candida albicans* primer set” refers to an oligonucleotide amplification primer set, as defined herein, that targets a region in the nucleic acid of *Candida albicans*. The selected target is generally selected such that it is specific for the indicated species to avoid cross-reactivity with other species. In the afore-mentioned example this means that the *Candida albicans* primer set recognizes and binds to a *Candida albicans* target sequence but does not hybridize to nucleic acid regions in the DNA of other *Candida* species, at least no under the hybridization conditions chosen or at least with significantly lower affinity.

“rDNA”, as used herein, refers to ribosomal DNA. In contrast, “genomic DNA” refers to the general genomic DNA of an organism.

It is understood that those primer pairs for which no species information is given, such as *Fusarium spp.*, *Trichosporon spp.* and *Candida spp.* are not specific for a certain species but detect to rDNA of several, but not necessarily all, members of this family of organisms, but have little or no cross-reactivity with species of other families. As explained above, such primer sets are used to detect numerous species of the respective family without discriminating such as to cover also those species for which no specific primer set is included. In various embodiments, this may either serve as a confirmation that a species of a given family is indeed present, i.e. in case one of the specific primer pairs for this species also gives a positive signal, or that a species of that family is present but is none of those species for which specific primer sets have been included. The specific primer sets have been selected based on the relevance and importance of the respective fungal species in fungal sepsis. The 11 specific fungal pathogens for which specific primer sets have been described herein do together cover the pathogens found in more than 90% of fungal sepsis cases.

In various embodiments of these methods according to the invention,

- (1) the target region of rDNA of *Candida albicans* is of ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is of ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is of ITS1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is of ITS2 region thereof;
- (5) the target region of rDNA of *Candida krusei* is of ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is of ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is of ITS2 region thereof;
- (8) the target region of rDNA of *Cryptococcus neoformans* is of IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is of IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is of IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is of 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is of 18S region thereof;
- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is of Cytochrome B gene region; and/or
- (14) the target region of rDNA of *Candida spp.* is of 18S region thereof.

“ITS”, as used herein, refers to the internal transcribed spacer region of rDNA. “IGS” refers to ribosomal intergenic spacer. The 26S and 18S regions are those regions of the rDNA that encode the 26S subunit of the ribosome and the 18S subunit of the ribosome, respectively. The cytochrome B gene is a gene that encodes for the mitochondrial protein cytochrome B and is found in the genomic DNA. The above target regions have been selected to ensure sufficient specificity for the respective species or family and avoid cross-reactivity with the other listed primer pairs.

In various embodiments, at least one primer of the *Candida albicans* primer set, the *Candida tropicalis* primer set, the *Candida parapsilosis* primer set, the *Candida krusei* primer set, the *Candida guilliermondii* primer set, and the *Candida auris* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof. The primer of SEQ ID NO:1 has sufficient complementarity to the target nucleic acids of all these species to form a target hybrid under the reaction conditions. The desired specificity of the detection is then brought about by the second primer of the pair, as described below.

The determination of the identity of nucleotide sequences is achieved by a sequence comparison. This comparison or alignment is typically based on the BLAST algorithm well-established and known in the art (See, e.g., Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990): "Basic local alignment search tool", J. Mol. Biol. 215:403-410, and Altschul, Stephan F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Hheng Zhang, Webb Miller, and David J. Lipman (1997): "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs"; Nucleic Acids Res., 25, S.3389-3402) and is in principle carried out by aligning stretches of nucleotides in the nucleotide

sequences with each other. Another algorithm available in the art is the FASTA algorithm. Sequence comparisons (alignments), in particular multiple sequence comparisons, can be generated using computer programs. Commonly used are for example the Clustal series (See, e.g. Chenna et al. (2003): "Multiple sequence alignment with the Clustal series of programs," Nucleic Acid Research 31, 3497-3500), T-Coffee (See, e.g., Notredame et al. (2000): "T-Coffee: A novel method for multiple sequence alignments", J. Mol. Biol. 302, 205-217) or programs based thereon or the respective algorithms. Further possible are sequence comparisons (alignments) with the computer program Vector NTI® Suite 10.3 (Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA, USA) with the pre-set standard parameters, the AlignX-module of which is based on ClustalW. If not explicitly defined otherwise, sequence identity is determined using the BLAST algorithm.

Such a comparison allows determining the identity of two sequences and is typically expressed in % identity, i.e. the portion of identical nucleotides in the same or corresponding positions. If not explicitly stated otherwise, the sequence identities defined herein relate to the percentage over the entire length of the respective sequence, i.e. typically the reference sequence. If the reference sequence is 20 nucleotides in length, a sequence identity of 90 % means that 18 nucleotides in a query sequence are identical while 2 may differ.

In various embodiments, in particular in those in which the first primer of the primer pair comprises or consists of the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof,

- (1) at least one primer of the *Candida albicans* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 2 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 2 over its entire length, or a complement thereof;
- (2) at least one primer of the *Candida tropicalis* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 3 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 3 over its entire length, or a complement thereof;
- (3) at least one primer of the *Candida parapsilosis* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 4 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 4 over its entire length, or a complement thereof;
- (4) at least one primer of the *Candida krusei* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 5 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5 over its entire length, or a complement thereof;
- (5) at least one primer of the *Candida guilliermondii* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 6 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 6 over its entire length, or a complement thereof; and/or
- (6) at least one primer of the *Candida auris* primer set comprises or consists of the nucleotide sequence of SEQ ID NO: 7 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 7 over its entire length, or a complement thereof.

In these embodiments (1) to (6), if the first primer of the primer pair is a primer that comprises or consists the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof, the primer of embodiment (1) to (6) is the respective second primer of the primer pair. It is understood that if, in such embodiments, the first primer is a complement of SEQ ID NO:1, the second primer is also the complement of the respective indicated sequence.

In various embodiments, the *Candida glabrata* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 8 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 8 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 9 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 9 over its entire length, or a complement thereof. As described above, if one of these primer sequences is the complement of the indicated sequence, the other is also a complement of the given sequence. This similarly applies to all other primer pairs described below.

In various embodiments, the *Cryptococcus neoformans* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 10 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 10 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 11 over its entire length, or a complement thereof.

In various embodiments, the *Cryptococcus deneoformans* primer set comprises a primer having a nucleotide sequence of SEQ ID NO: 12 or the nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 12 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 13 over its entire length, or a complement thereof.

In various embodiments, the *Cryptococcus gattii* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 14 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 14 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 15 over its entire length, or a complement thereof.

In various embodiments, the *Fusarium spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 16 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 17 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 17 over its entire length, or a complement thereof.

In various embodiments, the *Trichosporon spp.* primer set comprises a primer having the nucleotide

sequence of SEQ ID NO: 18 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 18 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 19 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 19 over its entire length, or a complement thereof.

In various embodiments, the *Schizosaccharomyces pombe* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 20 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 20 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 21 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 21 over its entire length, or a complement thereof.

In various embodiments the *Candida spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 22 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 22 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 23 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 23 over its entire length, or a complement thereof.

In various embodiments, any two or more of the primer sets described herein above may be used in a single multiplexing method. In various embodiments, the methods of the invention thus use at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 primer sets as described herein. These primer sets are preferably different in that they are directed against different species or families of organisms and allow specific detection and identification thereof. In various embodiments, primer sets that are directed to the same target but are directed against different target regions of the same target may also be included. However, the methods of the invention are preferably designed such that at least two different pathogenic fungal species can be detected, i.e. at least two primer sets directed to different fungal pathogen species are included.

It is understood that the more general primer sets disclosed herein, namely those directed to *Candida spp.*, *Trichosporon spp.*, and *Fusarium spp.*, are specific for the respective family of fungi, but cannot distinguish between the different species belonging to that family. However, their inclusion may still be preferable, as a positive signal with a *Candida spp.* primer set, while all specific *Candida* primer sets are negative indicates that the sample comprises a *Candida* species other than those specifically tested.

The combination of primer sets used in the methods of the invention may be selected from the groups comprising or consisting of:

- (1) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;
- (2) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida*

parapsilosis primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set and *Candida auris* primer set;

(3) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set and *Candida krusei* primer set;

(4) *Candida albicans* primer set, *Candida tropicalis* primer set and *Candida glabrata* primer set;

(5) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set;

(6) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;

(7) *Candida albicans* primer set and *Candida tropicalis* primer set; and

(8) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set and *Cryptococcus gattii* primer set.

Any two or more of these primer set combinations (1) to (8) may be combined or supplemented by any one or more of the other primer sets described herein. These primer set combinations (1) to (8) preferably comprise the primers with the specific nucleotide sequences disclosed herein.

In various embodiments, the primer set for *Candida albicans* disclosed herein as comprising the nucleic acid sequences of SEQ ID Nos:1 and 2 may be combined with further second primers for the other *Candida* species, namely those of SEQ ID Nos. 3-7 as well as with the primer set for *C. glabrata*, i.e. the primers with the sequences set forth in SEQ ID Nos. 8 and 9. In various embodiments, the primers of SEQ ID Nos. 1, 2 and any one, two or more of 3, 4, 5, 6, and 7 are included. These are in various embodiments further combined with those of SEQ ID Nos. 8 and 9. In various embodiments, the primers of SEQ ID Nos. 1 and any two of 3-7 are combined, optionally further combined with those of SEQ ID Nos. 8 and 9. Such primer set combinations are specifically suited for methods for diagnosis of fungal sepsis caused by *Candida* species. In order to further cover not specifically named *Candida* species the *Candida spp.* primer set, i.e. of SEQ ID Nos. 22 and 23, may be additionally included in all aforementioned embodiments.

In the next step of the method, the amplification reaction is carried out. In this reaction the hybridized primer:target complexes are recognized by a polymerase and the respective target sequence amplified. The preferred method of amplification in the methods of the invention is PCR. PCR is a well-known and established technique and commonly known and practiced by those skilled in the art. Said amplification step generates a multitude of amplicons of all target nucleic acids present in the sample for which also a suitable primer set is included by thermocycling the reaction mixture in the presence of a thermostable DNA polymerase to allow multiple cycles of primer annealing and elongation.

The primer sets have been designed such that they have under the conditions of the described methods no significant cross-reactivity and thus allow specific detection of one or more, preferably 2, 3, 4, 5, 6, 7,

8, 9, 10, or 11 species for which primer sets are described herein. These primer sets may therefore be used in combination to allow multiplexing. In the multiplexing methods of the invention, in various embodiments, at least 2, preferably 3 or more of the species-specific primer sets are used. These may be further combined with 1, 2 or 3 of the family-specific primer sets, i.e. the *Candida*, the *Fusarium* or the *Trichosporon* primer sets. All these primer sets disclosed herein below with their nucleic acid sequences have been found to allow simultaneous use without significant interference or cross-reactivity.

The primers used in the methods of the invention may comprise further portions in addition to their target-complementary nucleotide sequence necessary for target recognition and binding. These portions may include further nucleotide sequences that do not base-pair with the target and serve different purposes, for example providing ligation sites or restriction enzyme sites or for interaction with enzymes/proteins, such as the polymerase, or non-nucleotide moieties, such as labeling or coupling moieties, including biotin and various chromophores or fluorophores. In various embodiments, one of the primers of a given primer pair is biotinylated on its 5' end.

In a following step of the methods of the invention, the presence and/or amount of the target nucleic acid(s) in the sample is detected by contacting the amplified sample / reaction mixture containing the amplicons of any target nucleic acid that may have been present in the sample with a combination of oligonucleotide detection probes comprising at least one probe specific for each of the targets of the amplification primer sets used. These probes are generally specific for the target nucleic acid sequence as well as the amplicons produced by the amplification of the target nucleic acids in that they hybridize to these (amplified) target nucleic acid sequences. This hybridization is again carried out under conditions that are sufficiently strict to avoid unspecific hybridization or prevent hybridization to nucleic acids that carry one or more mismatches. The thus formed probe:target hybrids are then detected and optionally quantified to determine the presence and/or amount of the respective target nucleic acids in the sample. Various detection methods are known in the art and can be chosen by those skilled in the art based on preference. Common detection methods include radio-labeled, fluorophore- or chromophore-labeled probes that can then be detected by a read-out of the radioactivity, the fluorescence or color, for example by using available instruments, such as scintillation counters and spectrometers. Other options include the use of probes coupled to a solid support, such as a nanoparticle or bead, including magnetic beads, or so-called micro-discs. One system that may advantageously be used for probe:target complex detection is the π Code™ system of PlexBio™.

In various embodiments, the combination of oligonucleotide detection probes comprises two or more probes selected from:

- (1) a *Candida albicans* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida albicans*;
- (2) a *Candida tropicalis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida tropicalis*;

- (3) a *Candida glabrata* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida glabrata*;
- (4) a *Candida parapsilosis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida parapsilosis*;
- (5) a *Candida krusei* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida krusei*;
- (6) a *Candida guilliermondii* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida guilliermondii*;
- (7) a *Candida auris* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida auris*;
- (8) a *Cryptococcus neoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus neoformans*;
- (9) a *Cryptococcus deneoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus deneoformans*;
- (10) a *Cryptococcus gattii* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus gattii*;
- (11) a *Fusarium spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Fusarium spp.*;
- (12) a *Trichosporon spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Trichosporon spp.*;
- (13) a *Schizosaccharomyces pombe* probe having a nucleotide sequence specific to a target region in the genomic DNA of *Schizosaccharomyces pombe*; and
- (14) a *Candida spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida spp.*

It is understood that the probes are selected in dependence of the primer sets used for amplification such that for each potential target a probe is used. Accordingly, the method uses suitable primer pair-probe combinations. The target regions for the probes are generally selected such that they are within the amplified sequence stretch, i.e. lie in the region that is amplified by the use of the primers in an amplification reaction. It is preferably that the to be detected sequence does not overlap with the primer binding sites, but rather lies between the sequence stretches bound by the forward and reverse primers. As a consequence, thereof, the target region of the probes used is the same as that to which the primers are directed. Therefore, in various embodiments in accordance with the preferred primer target regions,

- (1) the target region of rDNA of *Candida albicans* is in the ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is in the ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is in the ITS 1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is in the ITS2 region thereof;
- (5) the target region of rDNA of *Candida krusei* is in the ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is in the ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is in the ITS2 region thereof;

- (8) the target region of rDNA of *Cryptococcus neoformans* is in the IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is in the IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is in the IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is in the 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is in the 18S region thereof
- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is in the Cytochrome B gene region; and
- (14) the target region of rDNA of *Candida spp.* is in the 18S region thereof.

The probes used in the methods of the invention may comprise further portions in addition to their target-complementary nucleotide sequence necessary for target recognition and binding. These portions may include further nucleotide sequences that do not base-pair with the target and serve different purposes, for example providing ligation sites or restriction enzyme sites or for interaction with enzymes/proteins, such as the polymerase, or non-nucleotide moieties, such as labeling or coupling moieties, including biotin and various chromophores or fluorophores. In various embodiments, such additional portions may provide for a linker function that allows coupling with a non-nucleotide moiety. For this purpose, these nucleotides or nucleotide sequences, for example dT sequences of 2 to 20 nucleotides in length, may be further modified with a functional group or a spacer including a functional group, such as an amino group. This may then allow coupling to various detectable labels, such as pi-code microdiscs (ex PlexBio, TW).

Probe sequences may be designed in accordance with the above-described principles. It is further important that the probe sequences are again sufficiently specific to avoid cross-reactivity between different targets. Therefore, within the amplified sequence stretches sufficiently different target regions for probe binding need to be identified that allow preferential binding of a specific probe for the respective target over binding of any other probe directed to a different target. Herein disclosed are probe sequences that have been designed accordingly and can be used in combination without the risk of cross-reactivity and thus false positives and undesired interference with each other.

In various embodiments,

- (1) the *Candida albicans* probe comprises the nucleotide sequence of SEQ ID NO: 24 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 24 over its entire length, or a complement thereof;
- (2) the *Candida tropicalis* probe comprises the nucleotide sequence of SEQ ID NO: 25 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 25 over its entire length, or a complement thereof;
- (3) the *Candida glabrata* probe comprises the nucleotide sequence of SEQ ID NO: 30 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 30 over its entire length, or a complement thereof;
- (4) the *Candida parapsilosis* probe comprises the nucleotide sequence of SEQ ID NO: 26 or a

nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 26 over its entire length, or a complement thereof;

(5) the *Candida krusei* probe comprises the nucleotide sequence of SEQ ID NO: 27 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 27 over its entire length, or a complement thereof;

(6) the *Candida guilliermondii* probe comprises the nucleotide sequence of SEQ ID NO: 28 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 28 over its entire length, or a complement thereof;

(7) the *Candida auris* probe comprises the nucleotide sequence of SEQ ID NO: 29 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 29 over its entire length, or a complement thereof;

(8) the *Cryptococcus neoformans* probe comprises the nucleotide sequence of SEQ ID NO: 32 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 32 over its entire length, or a complement thereof;

(9) the *Cryptococcus deneoformans* probe comprises the nucleotide sequence of SEQ ID NO: 33 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 33 over its entire length, or a complement thereof;

(10) the *Cryptococcus gattii* probe comprises the nucleotide sequence of SEQ ID NO: 34 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 34 over its entire length, or a complement thereof;

(11) the *Fusarium spp.* probe comprises the nucleotide sequence of SEQ ID NO: 35 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 35 over its entire length, or a complement thereof;

(12) the *Trichosporon spp.* probe comprises the nucleotide sequence of SEQ ID NO: 36 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 36 over its entire length, or a complement thereof;

(13) the *Schizosaccharomyces pombe* probe comprises the nucleotide sequence of SEQ ID NO: 37 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 37 over its entire length, or a complement thereof; and

(14) the *Candida spp.* probe comprises the nucleotide sequence of SEQ ID NO: 31 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 31 over its entire length, or a complement thereof.

For the determination of the sequence identity, the same as described above in relation to the primers applies.

The combination of probes may comprise at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or at least 14 different probes. In various embodiments, the different probes are each specific for a different pathogenic fungi species. As described above, the different probes are selected such that at least one probe for each potentially

amplified target, i.e. those targets for which primers have been used/included, is present to ensure that each target that has successfully been amplified is subsequently detected. The respective preferred combinations of probes are thus equivalent to the preferred combinations of primer sets disclosed herein. Accordingly, the combination of probes may be selected from the group comprising or consisting of:

- (1) *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida guilliermondii* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;
- (2) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida guilliermondii* probe and *Candida auris* probe;
- (3) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe and *Candida krusei* probe;
- (4) *Candida albicans* probe, *Candida tropicalis* probe and *Candida glabrata* probe;
- (5) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe;
- (6) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;
- (7) *Candida albicans* probe and *Candida tropicalis* probe; and
- (8) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe and *Cryptococcus gattii* probe.

The method of the invention may be a method for the diagnosis of fungal sepsis in a subject. Specifically, the invention also covers a method for the diagnosis of fungal sepsis in a subject that comprises a first step of obtaining a sample from said subject and then subjecting said sample to the methods of the invention for the detection of the presence and/or amount of two or more target nucleic acids in a sample, wherein the two or more target nucleic acids are of fungal pathogens involved in fungal sepsis. The respective analysis steps are carried out *in vitro*, i.e. using the sample obtained from the subject.

The subject may be a human subject. In various embodiments, the sample is a blood, serum or plasma sample. Alternatively, other sample types may be used, including tissue or other body fluid samples, although blood, plasma and serum samples are the most common.

In these methods for the diagnosis of fungal sepsis in a subject, the subject may be diagnosed with fungal sepsis, if one or more of the target nucleic acids are detected in the sample. In other words, if in the methods for the detection of two or more target nucleic acids in a sample described herein one or more of said targets is successfully identified, this may in a further step then lead to diagnosing the subject with fungal infection and fungal sepsis. It is understood that for the final diagnosis of fungal sepsis other factors may also be considered, including medical conditions, such as fever and the like.

Depending on the diagnosis and the detected microorganism an appropriate therapy may be selected, typically an antifungal agent that is effective against the identified pathogen. For all fungal pathogens specifically described herein, appropriate antifungal treatment options exist and suitable medicaments are available in the art. The skilled person can thus readily select an appropriate antifungal agent depending on the results of the inventive methods.

The invention further features kits for carrying out the methods of the invention. Such kits typically comprise a number of agents and reagents useful and necessary to perform the methods of the invention. In various embodiments, the kits thus comprise at least the primer sets and corresponding probes disclosed herein, i.e. two or more amplification primer sets and two or more detection probes. The primers and probes are preferably those that have been described in relation to the methods herein.

In addition to the primers and probes, the kit may comprise a number of further agents that may be selected from, without limitation, a polymerase, buffers, mononucleotides, and all other agents necessary for a PCR reaction and subsequent isolation/purification of the amplicons.

The invention further features the use of these kits for the diagnosis of fungal sepsis in a subject, i.e. for use in the methods of diagnosis described herein above. This means that also encompassed are methods for the diagnosis of fungal sepsis in a subject, wherein said kits are used, preferably to allow practicing the methods of the invention. The subject and the sample analyzed may be as described above for the methods of the invention.

All embodiments disclosed herein in relation to the methods of the invention similarly apply to the kits and uses disclosed and vice versa.

Primers and probes described above are listed below:

Primers:

SEQ ID NO:	Sequence name	Sequence (from 5' to 3')
1	Candida_spp_ITS2_F	CAACAACGGATCTCTTGGTTCTC
2	Candida_albi_ITS2_R	AGGTCAAAGTTTGAAGATATACGTG
3	Candida_trop_ITS2_R	CTACCTGATTTGAGGTCAAAGTTATG
4	Candida_para_ITS2_R	CCTGATTTGAGGTCTGAATTTGG
5	Candida_krus_ITS2_R	GTCTCGCAACACTCGCTY
6	Candida_guil_ITS2_R	ATATCCCGCCACACCATTC
7	Candida_auri_ITS2_R	TTCGTGCAAGCTGTAATTTTGTG
8	Candida_glab_ITS1_F	AGACATCTCTGGGGAGGAC
9	Candida_glab_ITS1_R	CCCCCAACGAACAAAAGAATAG

10	Crypto_neof_IGS1_F	TGACTCTAGGAGGGGTCTGAG
11	Crypto_neof_IGS1_R	GAACCTTTCAAGTCCTCCGCAAC
12	Crypto_deneo_IGS1_F	TCCAAACTTATCCATWCACATTAC
13	Crypto_deneo_IGS1_R	AAATGCTTATCTATCYGTCCAACC
14	Crypto_gatt_IGS1_F	SCAGGTTATCGCAAGTTGG
15	Crypto_gatt_IGS1_R	TGYCCATCCCCTCTTGAC
16	Fusarium_spp_26S_F	GCCTTCCGAGTTCCTG
17	Fusarium_spp_26S_R	CCAAGCCCAAGTCTGGTC
18	Tricho_spp_18S_F	GACTTGGAGGAACGGTCTG
19	Tricho_spp_18S_R	GATCCAGTCAAAAAATAACGTGGA
20	S.pomb_cytb_F	ATATGATTGATGCTCCTGAACCTTC
21	S.pomb_cytb_R	CATTTGCATGGAAAGCTCTTAATAAG
22	Candida_spp_18S_F	TGGTTCTATTKTGTTGGTTTCTAGG
23	Candida_spp_18s_R	AGACTACGACGGTATCTGATCA

Probes:

SEQ ID NO:	Sequence name	Sequence (from 5' to 3')
24	Probe_C.albi_ITS2	CTAACCAAAAACATTGCTTGC
25	Probe_C.trop_ITS2	AGGTTTGTTTGAAAGAATT
26	Probe_C.para_ITS2	TGCTTGAAAGAAAGGCG
27	Probe_C.krus_ITS2	CGAGCGAACTAGACTTT
28	Probe_C.guil_ITS2	GTCGACCTCTCAATGTA
29	Probe_C.auri_ITS2	GATGTCTTCTCACCATC
30	Probe_c.glab_ITS1	CTCTCTATTCCAAAGGAGG
31	Probe_Candida_spp_18S	ATCATTACGATGGTCCTA
32	Probe_Cr.neof_IGS1	GGGTCATTGATATTCTGTAA
33	Probe_Cr.deneofo_IGS1	CAGTAGTATGCCAGTG
34	Probe_Cr.gatt_IGS1	ACGCATCTTAGCCAAG
35	Probe_Fu.spp_26S	GAGTGATCGAAAGATG
36	Probe_Tr.spp_18s	AAGACTCAGACAAACAG
37	Probe_S.pomb_cytb	CCAGAAGTATGAAATATTG

Examples

Example 1: Fungal sepsis panel and testing thereof

A panel of primer/probe combinations was tested using DNAs of different fungal species. For each PCR reaction genomic DNA (2 pg) of one fungus was used as a template. The full multiplex primer mixture was used for each conditions. Said primer mixture comprised primers for *Candida albicans*; *Candida*

tropicalis, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida guilliermondii*, *Candida auris*, *Cryptococcus neoformans*, *Cryptococcus deneoformans*, *Cryptococcus gattii*, *Fusarium spp.*, *Trichosporon spp.*, *Schizosaccharomyces pombe*, and *Candida spp.* having the nucleic acid sequences set forth in SEQ ID Nos. 1-23. PCR conditions were 94°C for 1 min; 40x cycles of 94°C for 30 sec, 60°C for 1 min; and 72°C for 10 min.

In order to make the amplicon detectable in a subsequent step of the method, one of the primers for each target is conjugated with biotin at the 5' end as shown in the list below (5Biosg):

SEQ ID NO:	Sequence name	Sequence (from 5' to 3')	Modification
1	Candida_spp_ITS2_F	CAACAACGGATCTCTTGGTTCTC	not applicable
2	Candida_albi_ITS2_R	AGGTCAAAGTTTGAAGATATACGTG	/5Biosg/
3	Candida_trop_ITS2_R	CTACCTGATTTGAGGTCAAAGTTATG	/5Biosg/
4	Candida_para_ITS2_R	CCTGATTTGAGGTGGAATTTGG	/5Biosg/
5	Candida_krus_ITS2_R	GTCTCGCAACACTCGCTY	/5Biosg/
6	Candida_guil_ITS2_R	ATATCCCGCCACACCATTC	/5Biosg/
7	Candida_auri_ITS2_R	TTCGTGCAAGCTGTAATTTTGTG	/5Biosg/
8	Candida_glab_ITS1_F	AGACATCTCTGGGGAGGAC	not applicable
9	Candida_glab_ITS1_R	CCCCCAACGAACAAAAGAATAG	/5Biosg/
10	Crypto_neof_IGS1_F	TGACTCTAGGAGGGGTCTGAG	not applicable
11	Crypto_neof_IGS1_R	GAACCTTTCAAGTCTCCGCAAC	/5Biosg/
12	Crypto_deneo_IGS1_F	TCCAACTTATCCATWCACATTAC	/5Biosg/
13	Crypto_deneo_IGS1_R	AAATGCTTATCTATCYGTCCAACC	not applicable
14	Crypto_gatt_IGS1_F	SCAGGTTATCGCAAGTTGG	/5Biosg/
15	Crypto_gatt_IGS1_R	TGYCCATCCCCCTCTTGAC	not applicable
16	Fusarium_spp_26S_F	GCCTTCCGAGTTCCCTG	not applicable
17	Fusarium_spp_26S_R	CCAAGCCCAAGTCTGGTC	/5Biosg/
18	Tricho_spp_18S_F	GACTTGAGGAACGGTCTG	/5Biosg/
19	Tricho_spp_18S_R	GATCCAGTCAAAAAATAACGTGGA	not applicable
20	S.pomb_cytb_F	ATATGATTGATGCTCCTGAACCTTC	/5Biosg/
21	S.pomb_cytb_R	CATTTGCATGGAAAGCTCTTAATAAG	not applicable
22	Candida_spp_18S_F	TGGTTCTATTKTGTGTTGTTCTAGG	/5Biosg/
23	Candida_spp_18s_R	AGACTACGACGGTATCTGATCA	not applicable

The probes (SEQ ID NO 24-37) of individual targets were modified with adding a short oligo (dT) fragment at the 5' end. The short oligo (dT) fragment serves as a linker between the pi-Code microdiscs (ex PlexBio, TW) and the probe sequence. In addition, the 5' end of the oligo (dT) fragment was modified with an amino group with a (C6) spacer arm as shown below (5AmMC6).

Probes:

SEQ ID NO:	Sequence name	Sequence (from 5' to 3')	Modification
24	Probe_C.albi_ITS2	TTTTTCTAACCAAAAACATTGCTTGC	/5AmMC6/
25	Probe_C.trop_ITS2	TTTTTTAGGTTTGTGTTGAAAGAATT	/5AmMC6/
26	Probe_C.para_ITS2	TTTTTTTTTGTCTGAAAGAAAGGCG	/5AmMC6/
27	Probe_C.krus_ITS2	TTTTTTTTTCGAGCGAACTAGACTTT	/5AmMC6/
28	Probe_C.guil_ITS2	TTTTTTTTTGTGCGACCTCTCAATGTA	/5AmMC6/
29	Probe_C.auri_ITS2	TTTTTTTGATGTCTTCTCACCAATC	/5AmMC6/
30	Probe_c.glab_ITS1	TTTTTCTCTCTATTCCAAAGGAGG	/5AmMC6/
31	Probe_Candida_spp_18S	TTTTTTTATCATTACGATGGTCCTA	/5AmMC6/
32	Probe_Cr.neof_IGS1	TTTTTGGGTCATTGATATTCTGTAA	/5AmMC6/
33	Probe_Cr.deneofo_IGS1	TTTTTTTTTCAGTAGTATGCCCAGTG	/5AmMC6/
34	Probe_Cr.gatt_IGS1	TTTTTTTTTTACGCATCTTAGCCAAG	/5AmMC6/
35	Probe_Fu.spp_26S	TTTTTTTTTTGAGTGATCGAAAGATG	/5AmMC6/
36	Probe_Tr.spp_18s	TTTTTTTTTAAGACTCAGACAAACAG	/5AmMC6/
37	Probe_S.pomb_cytb	TTTTTCCAGAAGTATGAAATATTTG	/5AmMC6/

For probe hybridization, multiplex π Code™ probes (ex PlexBio™) were prepared in advance. The probe mixture comprised probes for each of the targets of the primer sets having the nucleotide sequences set forth in SEQ ID Nos. 24-37. The mixture was added into each well of a 96-well plate for later use.

20 μ l of each PCR product was denatured at 95°C for 5 min and chilled on ice for 2 min. The denatured PCR product was then added to an individual well with the probe mixture and probe hybridization was done in an automated IntelliPlex 1000 device (PlexBio™). Detection of the formed probe:target hybrids was done by putting the plate in an automated BioPlex 100 detection device (PlexBio™) and the data obtained collected and subsequently analyzed.

The results of this analysis are shown in Figure 1. Each sample was detected specifically by the corresponding probe(s), for example, *Candida albicans* gDNA was detected by the specific probe Probe_C.albi_ITS2 and the pan-*Candida* probe Probe_Candida_spp_18S. The strain of *Cryptococcus neoformans* is a hybrid strain, it contains chromosomes of both *C.neoformans* and *C.deneoformans*, therefore, both probes (Probe_Cr.neof_IGS1 and Probe_Cr.deneofo_IGS1) were positive. There is no cross-reaction among different fungal species. Every well had all the different piCode-probes, only the specific probe(s) showed strong positive signal, other probes just showed background signals with MFI ~ 2000. The probe of *Candida albicans* has no cross-reaction with *Candida dubliniensis*, although these two strains are very similar and some commercially available test assays claim that they cannot discriminate these two species. SAPE Control probe is a probe with random artificial sequence conjugated with Biotin at the 3' end, which is supposed to be positive after the test. Blank Control probe

is with the same artificial sequence which is supposed to be negative since no amplicons are supposed to hybridize on it.

The primers used in the panel were found not to interfere with each other. The same applies to the probes used. Each sample only needed one round of PCR to obtain the amplicon.

CLAIMS

1. Method for the detection of the presence and/or amount of two or more target nucleic acids in a sample, wherein the two or more target nucleic acids are of fungal pathogens involved in fungal sepsis, the method comprising:

i) contacting the sample with a combination of two or more oligonucleotide amplification primer sets under conditions that allow hybridization of the oligonucleotide primers to the target nucleic acids of the fungal pathogens, wherein the two or more primer sets are directed to different target nucleic acids of at least two different fungal pathogen species involved in fungal sepsis and are independently selected from the group consisting of:

- (1) a *Candida albicans* primer set targeting a nucleotide sequence in the rDNA of *Candida albicans*;
 - (2) a *Candida tropicalis* primer set targeting a nucleotide sequence in the rDNA of *Candida tropicalis*;
 - (3) a *Candida glabrata* primer set targeting a nucleotide sequence in the rDNA of *Candida glabrata*;
 - (4) a *Candida parapsilosis* primer set targeting a nucleotide sequence in the rDNA of *Candida parapsilosis*;
 - (5) a *Candida krusei* primer set targeting a nucleotide sequence in the rDNA of *Candida krusei*;
 - (6) a *Candida guilliermondii* primer set targeting a nucleotide sequence in the rDNA of *Candida guilliermondii*;
 - (7) a *Candida auris* primer set targeting a nucleotide sequence in the rDNA of *Candida auris*;
 - (8) a *Cryptococcus neoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus neoformans*;
 - (9) a *Cryptococcus deneoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus deneoformans*;
 - (10) a *Cryptococcus gattii* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus gattii*;
 - (11) a *Fusarium spp.* primer set targeting a nucleotide sequence in the rDNA of *Fusarium spp.*;
 - (12) a *Trichosporon spp.* primer set targeting a nucleotide sequence in the rDNA of *Trichosporon spp.*;
 - (13) a *Schizosaccharomyces pombe* primer set targeting a nucleotide sequences in the genomic DNA of *Schizosaccharomyces pombe*; and
 - (14) a *Candida spp.* primer set targeting a nucleotide sequence in the rDNA of *Candida spp.*;
- and

ii) amplifying the target nucleic acid(s) hybridized to any one or more of the oligonucleotide primers by PCR; and

iii) detecting the presence and/or amount of the target nucleic acid(s) in the sample by contacting the PCR amplified sample of step ii) with combination of oligonucleotide detection probes comprising at least one probe specific for each of the amplicons produced by the amplification of the target nucleic

acids in step ii) under conditions that allow the hybridization of the probes to their specific target nucleic acid sequences and determining the presence and/or amount of probe:target hybrids.

2. The method according to Claim 1, wherein:

- (1) the target region of rDNA of *Candida albicans* is of ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is of ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is of ITS1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is of ITS2 region thereof;
- (5) the target region of rDNA of *Candida krusei* is of ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is of ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is of ITS2 region thereof;
- (8) the target region of rDNA of *Cryptococcus neoformans* is of IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is of IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is of IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is of 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is of 18S region thereof;
- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is of Cytochrome B gene region; and
- (14) the target region of rDNA of *Candida spp.* is of 18S region thereof.

3. The method according to Claim 1 or 2, wherein:

at least one primer of the *Candida albicans* primer set, the *Candida tropicalis* primer set, the *Candida parapsilosis* primer set, the *Candida krusei* primer set, the *Candida guilliermondii* primer set, and the *Candida auris* primer set comprises the nucleotide sequence of SEQ ID NO: 1 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 1 over its entire length, or a complement thereof.

4. The method according to any one of Claims 1-3, wherein:

- (1) at least one primer of the *Candida albicans* primer set comprises the nucleotide sequence of SEQ ID NO: 2 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 2 over its entire length, or a complement thereof;
- (2) at least one primer of the *Candida tropicalis* primer set comprises the nucleotide sequence of SEQ ID NO: 3 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 3 over its entire length, or a complement thereof;
- (3) at least one primer of the *Candida parapsilosis* primer set comprises the nucleotide sequence of SEQ ID NO: 4 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 4 over its entire length, or a complement thereof;
- (4) at least one primer of the *Candida krusei* primer set comprises the nucleotide sequence of SEQ ID NO: 5 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 5 over its entire length, or a complement thereof;
- (5) at least one primer of the *Candida guilliermondii* primer set comprises the nucleotide sequence

of SEQ ID NO: 6 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 6 over its entire length, or a complement thereof; and

(6) at least one primer of the *Candida auris* primer set comprises the nucleotide sequence of SEQ ID NO: 7 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 7 over its entire length, or a complement thereof.

5. The method according to any one of Claims 1-4, wherein

(1) the *Candida glabrata* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 8 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 8 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 9 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 9 over its entire length, or a complement thereof;

(2) the *Cryptococcus neoformans* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 10 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 10 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 11 over its entire length, or a complement thereof;

(3) the *Cryptococcus deneoformans* primer set comprises a primer having a nucleotide sequence of SEQ ID NO: 12 or the nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 12 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 13 over its entire length, or a complement thereof;

(4) the *Cryptococcus gattii* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 14 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 14 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 15 over its entire length, or a complement thereof;

(5) the *Fusarium spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 16 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 17 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 17 over its entire length, or a complement thereof;

(6) the *Trichosporon spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 18 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 18 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 19 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 19 over its entire length, or a complement thereof;

(7) the *Schizosaccharomyces pombe* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 20 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 20 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID

NO: 21 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 21 over its entire length, or a complement thereof; and

(8) the *Candida spp.* primer set comprises a primer having the nucleotide sequence of SEQ ID NO: 22 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 22 over its entire length, or a complement thereof, and a primer having the nucleotide sequence of SEQ ID NO: 23 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 23 over its entire length, or a complement thereof.

6. The method according to any one of Claims 1-5, wherein the combination of oligonucleotide detection probes comprises two or more probes selected from:

- (1) a *Candida albicans* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida albicans*;
- (2) a *Candida tropicalis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida tropicalis*;
- (3) a *Candida glabrata* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida glabrata*;
- (4) a *Candida parapsilosis* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida parapsilosis*;
- (5) a *Candida krusei* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida krusei*;
- (6) a *Candida guilliermondii* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida guilliermondii*;
- (7) a *Candida auris* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida auris*;
- (8) a *Cryptococcus neoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus neoformans*;
- (9) a *Cryptococcus deneoformans* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus deneoformans*;
- (10) a *Cryptococcus gattii* probe having a nucleotide sequence specific to a target region in the rDNA of *Cryptococcus gattii*;
- (11) a *Fusarium spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Fusarium spp.*;
- (12) a *Trichosporon spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Trichosporon spp.*;
- (13) a *Schizosaccharomyces pombe* probe having a nucleotide sequence specific to a target region in the genomic DNA of *Schizosaccharomyces pombe*; and
- (14) a *Candida spp.* probe having a nucleotide sequence specific to a target region in the rDNA of *Candida spp.*

7. The method according to Claim 6, wherein:

- (1) the target region of rDNA of *Candida albicans* is in the ITS2 region thereof;
- (2) the target region of rDNA of *Candida tropicalis* is in the ITS2 region thereof;
- (3) the target region of rDNA of *Candida glabrata* is in the ITS 1 region thereof;
- (4) the target region of rDNA of *Candida parapsilosis* is in the ITS2 region thereof;
- (5) the target region of rDNA of *Candida krusei* is in the ITS2 region thereof;
- (6) the target region of rDNA of *Candida guilliermondii* is in the ITS2 region thereof;
- (7) the target region of rDNA of *Candida auris* is in the ITS2 region thereof;
- (8) the target region of rDNA of *Cryptococcus neoformans* is in the IGS1 region thereof;
- (9) the target region of rDNA of *Cryptococcus deneoformans* is in the IGS1 region thereof;
- (10) the target region of rDNA of *Cryptococcus gattii* is in the IGS1 region thereof;
- (11) the target region of rDNA of *Fusarium spp.* is in the 26S region thereof;
- (12) the target region of rDNA of *Trichosporon spp.* is in the 18S region thereof;
- (13) the target region of genomic DNA of *Schizosaccharomyces pombe* is in the Cytochrome B gene region; and
- (14) the target region of rDNA of *Candida spp.* is in the 18S region thereof.

8. The method according to any one of Claim 6 or 7, wherein:

- (1) the *Candida albicans* probe comprises the nucleotide sequence of SEQ ID NO: 24 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 24 over its entire length, or a complement thereof;
- (2) the *Candida tropicalis* probe comprises the nucleotide sequence of SEQ ID NO: 25 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 25 over its entire length, or a complement thereof;
- (3) the *Candida glabrata* probe comprises the nucleotide sequence of SEQ ID NO: 30 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 30 over its entire length, or a complement thereof;
- (4) the *Candida parapsilosis* probe comprises the nucleotide sequence of SEQ ID NO: 26 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 26 over its entire length, or a complement thereof;
- (5) the *Candida krusei* probe comprises the nucleotide sequence of SEQ ID NO: 27 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 27 over its entire length, or a complement thereof;
- (6) the *Candida guilliermondii* probe comprises the nucleotide sequence of SEQ ID NO: 28 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 28 over its entire length, or a complement thereof;
- (7) the *Candida auris* probe comprises the nucleotide sequence of SEQ ID NO: 29 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 29 over its entire length, or a complement thereof;
- (8) the *Cryptococcus neoformans* probe comprises the nucleotide sequence of SEQ ID NO: 32 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 32 over its entire length, or

a complement thereof;

(9) the *Cryptococcus deneoformans* probe comprises the nucleotide sequence of SEQ ID NO: 33 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 33 over its entire length, or a complement thereof;

(10) the *Cryptococcus gattii* probe comprises the nucleotide sequence of SEQ ID NO: 34 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 34 over its entire length, or a complement thereof;

(11) the *Fusarium spp.* probe comprises the nucleotide sequence of SEQ ID NO: 35 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 35 over its entire length, or a complement thereof;

(12) the *Trichosporon spp.* probe comprises the nucleotide sequence of SEQ ID NO: 36 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 36 over its entire length, or a complement thereof;

(13) the *Schizosaccharomyces pombe* probe comprises the nucleotide sequence of SEQ ID NO: 37 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 37 over its entire length, or a complement thereof; and

(14) the *Candida spp.* probe comprises the nucleotide sequence of SEQ ID NO: 31 or a nucleotide sequence having at least 90% sequence identity to SEQ ID NO: 31 over its entire length, or a complement thereof.

9. The method according to any one of Claims 1-8, wherein the combination of primer sets comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or at least 14 different primer sets.

10. The method according to any one of Claims 1 to 9, wherein the combination of primer sets is selected from the group consisting of:

(1) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;

(2) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida guilliermondii* primer set and *Candida auris* primer set;

(3) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set and *Candida krusei* primer set;

(4) *Candida albicans* primer set, *Candida tropicalis* primer set and *Candida glabrata* primer set;

(5) *Candida albicans* primer set, *Candida tropicalis* primer set, *Candida glabrata* primer set, *Candida parapsilosis* primer set, *Candida krusei* primer set, *Candida auris* primer set, *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus gattii* primer set;

(6) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set, *Cryptococcus*

gattii primer set, *Fusarium spp.* primer set and *Trichosporon spp.* primer set;

(7) *Candida albicans* primer set and *Candida tropicalis* primer set; and

(8) *Cryptococcus neoformans* primer set, *Cryptococcus deneoformans* primer set and *Cryptococcus gattii* primer set;

11. The method according to any one of Claims 1 to 10, wherein combination of probes comprises at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or at least 14 different probes, wherein preferably the different probes are each specific for a different pathogenic fungi species.

12. The method according to any one of Claims 1 to 11, wherein the combination of probes is selected from the group consisting of:

(1) *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida guilliermondii* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;

(2) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida guilliermondii* probe and *Candida auris* probe;

(3) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe and *Candida krusei* probe;

(4) *Candida albicans* probe, *Candida tropicalis* probe and *Candida glabrata* probe;

(5) *Candida albicans* probe, *Candida tropicalis* probe, *Candida glabrata* probe, *Candida parapsilosis* probe, *Candida krusei* probe, *Candida auris* probe, *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe;

(6) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe, *Cryptococcus gattii* probe, *Fusarium spp.* probe and *Trichosporon spp.* probe;

(7) *Candida albicans* probe and *Candida tropicalis* probe; and

(8) *Cryptococcus neoformans* probe, *Cryptococcus deneoformans* probe and *Cryptococcus gattii* probe.

13. Kit for carrying out the method of any one of claims 1 to 12, wherein the kit comprises two or more amplification primer sets and two or more detection probes, wherein

(A) the two or more amplification primer sets are directed to different target nucleic acids of at least two different fungal pathogen species involved in fungal sepsis and are independently selected from the group consisting of:

(1) a *Candida albicans* primer set targeting a nucleotide sequence in the rDNA of *Candida albicans*;

(2) a *Candida tropicalis* primer set targeting a nucleotide sequence in the rDNA of *Candida tropicalis*;

(3) a *Candida glabrata* primer set targeting a nucleotide sequence in the rDNA of *Candida*

glabrata;

(4) a *Candida parapsilosis* primer set targeting a nucleotide sequence in the rDNA of *Candida parapsilosis*;

(5) a *Candida krusei* primer set targeting a nucleotide sequence in the rDNA of *Candida krusei*;

(6) a *Candida guilliermondii* primer set targeting a nucleotide sequence in the rDNA of *Candida guilliermondii*;

(7) a *Candida auris* primer set targeting a nucleotide sequence in the rDNA of *Candida auris*;

(8) a *Cryptococcus neoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus neoformans*;

(9) a *Cryptococcus deneoformans* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus deneoformans*;

(10) a *Cryptococcus gattii* primer set targeting a nucleotide sequence in the rDNA of *Cryptococcus gattii*;

(11) a *Fusarium spp.* primer set targeting a nucleotide sequence in the rDNA of *Fusarium spp.*;

(12) a *Trichosporon spp.* primer set targeting a nucleotide sequence in the rDNA of *Trichosporon spp.*;

(13) a *Schizosaccharomyces pombe* primer set targeting a nucleotide sequences in the genomic DNA of *Schizosaccharomyces pombe*; and

(14) a *Candida spp.* primer set targeting a nucleotide sequence in the rDNA of *Candida spp.*;
and

(B) the two or more detection probes comprise at least one probe specific for each of the amplicons produced by amplification of the target nucleic acids by each of the two or more primer pairs.

14. The method according to any one of claims 1 to 12, wherein the method is a method for the diagnosis of fungal sepsis in a subject.

15. The method according to claim 14, wherein the subject is a human.

16. The method according to claim 14 or 15, wherein if one or more of the target nucleic acids are detected in the sample, the subject is diagnosed with fungal sepsis.

17. Use of the kit of claim 13 for the diagnosis of fungal sepsis in a subject.