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(54) **CANDIDA PARAPSILOSIS  
OLIGONUCLEOTIDES, DETECTION  
METHOD, AND KIT THEREOF**

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(57) **ABSTRACT**

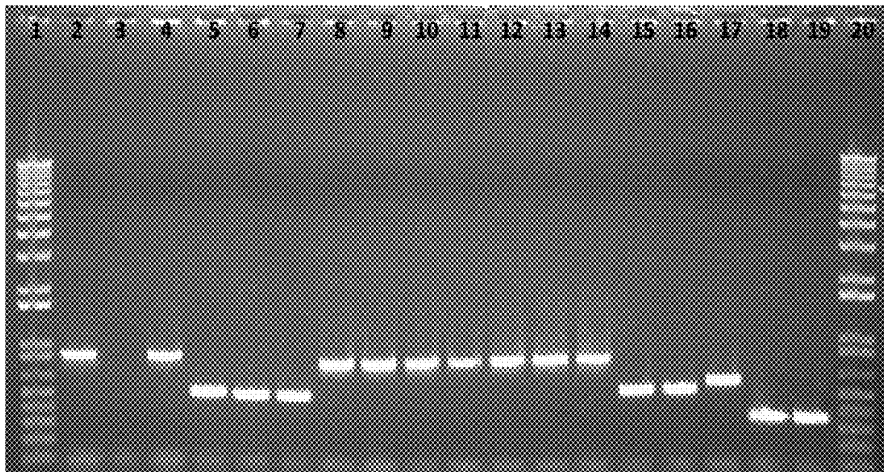
(22) Filed: **Oct. 22, 2014**

**Related U.S. Application Data**

(60) Provisional application No. 61/894,974, filed on Oct.  
24, 2013.

The invention discloses an in vitro method for the identifica-  
tion of *Candida parapsilosis*, the sequences associated to said  
identification, as well as diagnosis kits for identifying *Can-  
dida parapsilosis*.

FIG. 1



FIGs. 2A-2C

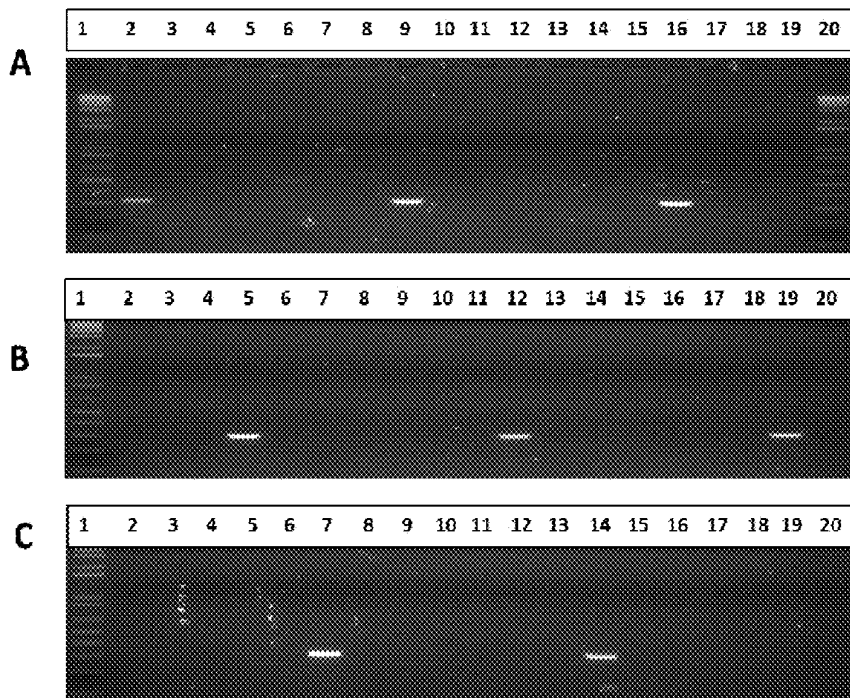


FIG. 3A

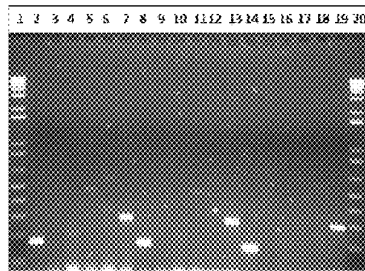


FIG. 3B

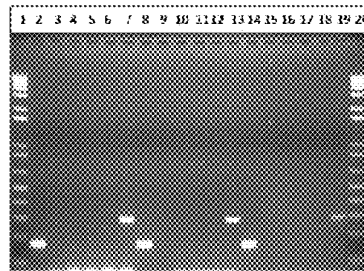


FIG. 3C

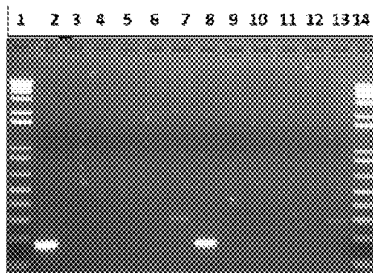


FIG. 3D

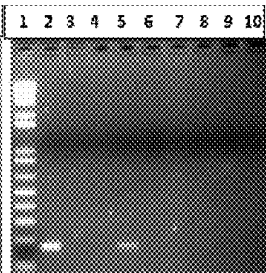


FIG. 3E

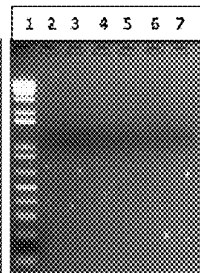
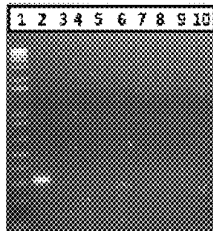
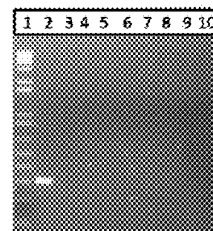


FIG. 4A



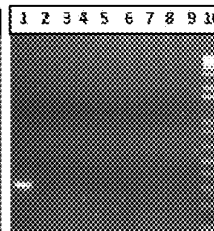
100 nM

FIG. 4B



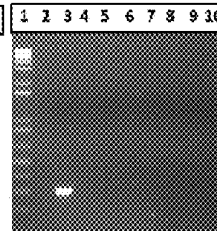
200 nM

FIG. 4C



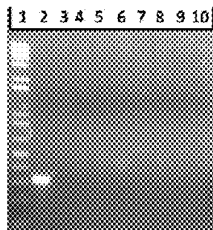
400 nM

FIG. 4D



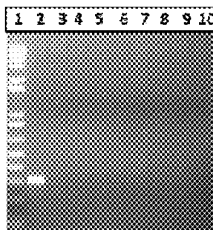
500 nM

FIG. 4E



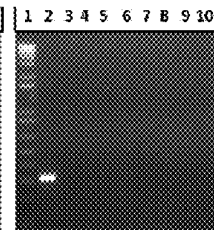
600 nM

FIG. 4F



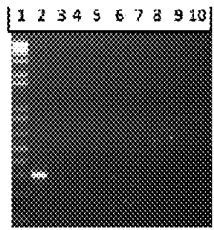
800 nM

FIG. 4G

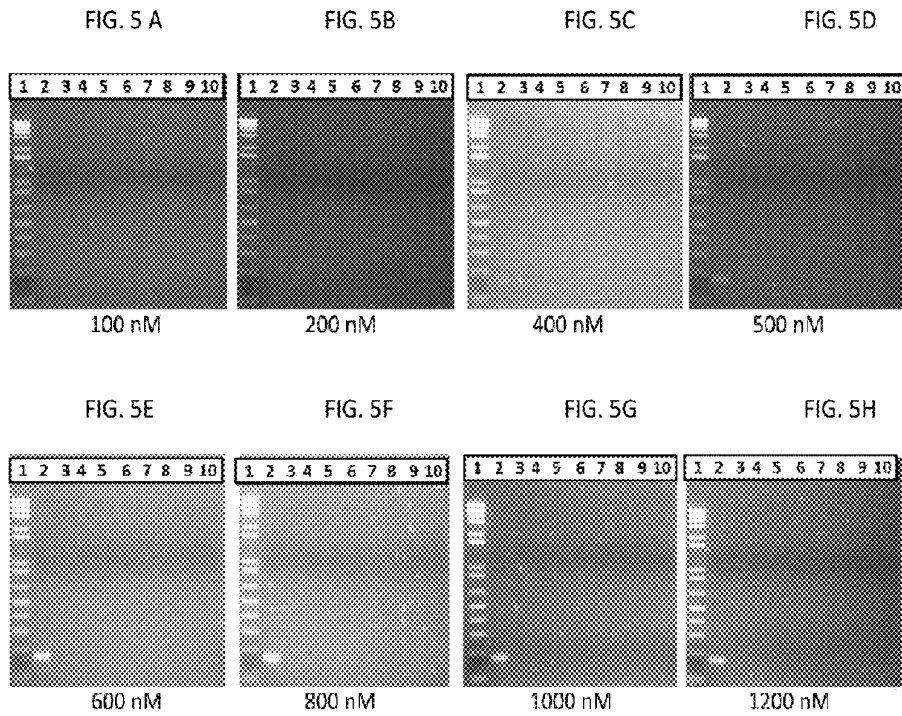


1000 nM

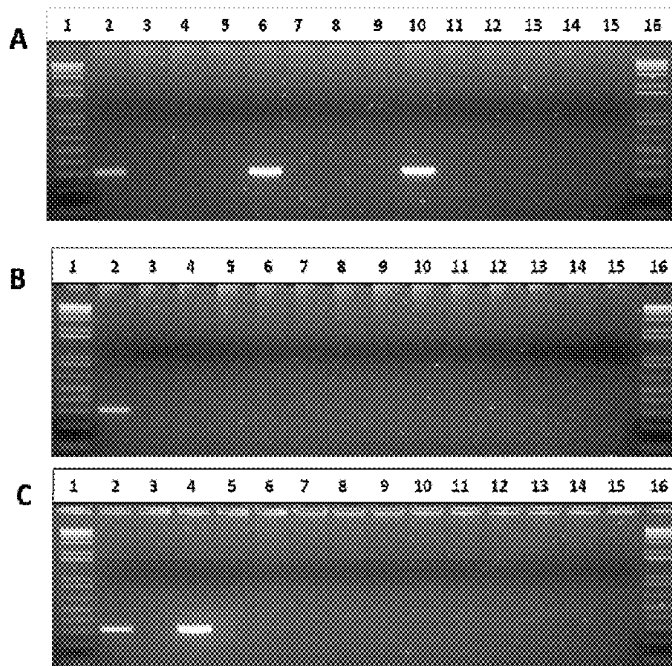
FIG. 4H



1200 nM



FIGs. 6A-6C



FIGs. 7A-7B

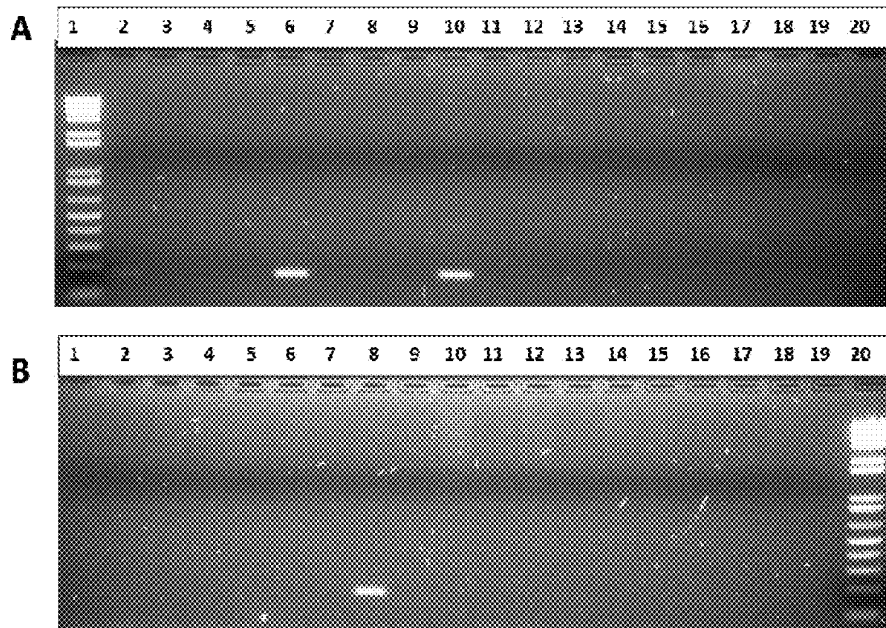


FIG. 8



FIG. 9A

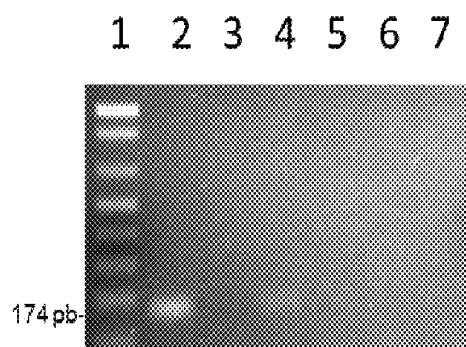
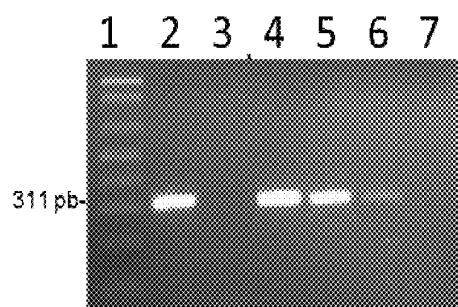


FIG. 9B



**CANDIDA PARAPSILOSIS  
OLIGONUCLEOTIDES, DETECTION  
METHOD, AND KIT THEREOF**

CROSS-REFERENCE TO RELATED  
APPLICATIONS

**[0001]** This application claims the benefit of priority to U.S. Provisional Application No. 61/894,974 filed Oct. 24, 2013, the contents of which are incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

**[0002]** The present invention belongs to the biotechnology field, especially to methods for detecting infectious diseases.

BACKGROUND OF THE INVENTION

**[0003]** The incidence of hospital infections by opportunistic fungal pathogens has increased substantially in the last two decades, especially among patients immuno-suppressed or serious underlying diseases. *Candidas* are the most common fungal pathogens affecting humans. Several epidemiologic studies around the world report that the invasive infections with *Candidas* have increased. Therefore, for example the Center for Disease Control and Prevention (CDC) is requiring sensitive, specific and rapid detection and identification methods for this kind of fungi.

**[0004]** Although more than 100 *Candida* species are known, only four are responsible for about 95% hematological infections and 95-97% of invasive infections caused by *Candida* in US hospitals.

**[0005]** In the case of hematological infections the most frequent species are: *Candida albicans* (45.6%), *Candida glabrata* (26%), *Candida parapsilosis* (15.7%) and *Candida tropicalis* (8.1%). These proportions vary depending on the patient's condition, but are the same four species causing 95% of overall candidiasis.

**[0006]** Current detection methods are imprecise and take several days for determining the kind of *Candida* in biological samples. This provokes that the patient's treatment is inadequate and the mortality in hospitals is increased as well as health care costs. Molecular detection methods based on ITS or rDNA sequences usually have a high incidence of false positive or negative results because of the close phylogenetic relation among the different *Candida* species. Also, further analysis is required, since the ITS or rDNA sequences are of similar size and should be re-sequenced before a final result is provided.

**[0007]** (Chang Chang H, et al., 2001 J. Clin. Microbiol., 39:3466-3471, which discloses that *C. parapsilosis* is difficult to differentiate upon *C. tropicalis*). Examples of these kind of inventions are disclosed in EP2315853B1, US2008305487A1, JP2012120535A, US20100311041A1, CA2136206A1, which are incorporated only as reference and should not be considered as prior art for the instant invention. *C. parapsilosis* has also been detected with PCR of an FKS1 gene fragment (Garcia-Effron, et al. J. Clin Microbiol. 2011, Vol. 49 3257-3261). This method however requires a subsequent step of EcoRI digestion. An additional method is Secondary alcohol dehydrogenase-restriction fragment length polymorphism as described by Tavanti et al. (Tavanti et al., 2005, J. Clin Microbiol. 43:284-292)

**[0008]** Therefore, there is a need of an specific and rapid diagnosis of *Candida parapsilosis*, since current methods

need further analysis (such as resequencing or enzyme digestion) or cannot differentiate between other *Candida* species.

**[0009]** It has been reported that several *Candida* species have chromosome re-arrangements that may cause loss of genetic material. (Butler, G., et al, Nature 459(7247):657-662 (2009)). This can be associated with variations in molecular diagnosis, since the target sequence may vary or lost.

**[0010]** In light of the above, the present invention discloses an in vitro method for detecting and identifying *Candida parapsilosis*, with at least one specific oligonucleotide, but also with an in-block multiplex set of specific oligonucleotides, which allows identification of *Candida parapsilosis* in clinical samples of different population subgroups.

SUMMARY OF THE INVENTION

**[0011]** The present invention claims and discloses oligonucleotides for the specific identification of *Candida parapsilosis*, consisting of a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 4 or a complement thereof.

**[0012]** In a further embodiment, it is further disclosed an in vitro method for the specific identification of *C. parapsilosis*, comprising the steps of: a) amplifying DNA fragments from a biological sample with at least one oligonucleotide as above defined; and b) identify the amplified DNA fragments; wherein in a specific embodiment the amplification of DNA fragments is carried out with at least one pair of oligonucleotides or at least two pair of oligonucleotides.

**[0013]** In an additional embodiment, a kit for the specific identification of *Candida parapsilosis*, comprising at least one oligonucleotide as above mentioned is also disclosed; wherein in a specific embodiment, said kit comprises at least one oligonucleotide pair or at least two pair of oligonucleotides, wherein said oligonucleotides are in a composition with a suitable acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

**[0014]** FIG. 1 shows a 2% agarose gel showing ribosomal DNA amplification of multiple *Candida* species by using the universal oligonucleotides ITS1 and ITS4. *C. glabrata* was used as positive control (BG14). For the electrophoresis, it was employed 1/5 of the total volume (2 µL) of the PCR amplification product of all of the samples and products. Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. glabrata*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. tropicalis*; Lane 7 shows *C. parapsilosis*; Lane 8 shows *C. bracarensis* 1; Lane 9 shows *C. bracarensis* 2; Lane 10 shows *C. bracarensis* 3; Lane 11 shows *C. bracarensis* 4; Lane 12 shows *C. bracarensis* 5; lane 13 shows *C. bracarensis* 6; Lane 14 shows *C. bracarensis* 7; Lane 15 shows *C. dubliniensis* 1; Lane 16 shows *C. dubliniensis* 2; Lane 17 shows *C. guillermoidii*; Lane 18 shows *C. krusei* 1; Lane 19 shows *C. krusei* 2 and Lane 20 shows the molecular weight marker.

**[0015]** FIGS. 2A-2C. show a 2% agarose gels showing temperature gradient for *C. parapsilosis* detection (Cp20-clinical isolated strain) using the oligonucleotides pair Cp1. The unspecific strip for *C. dubliniensis* and *C. krusei* disappears as the oligonucleotides annealing temperature increases, for this oligonucleotides pair, the optimal temperature selected is 62.0° C. For electrophoresis, the samples were

run at a concentration 4 times higher than the one used for the controls. The amplification band for *C. parapsilosis* has a length of 311 bp.

**[0016]** In FIG. 2A lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-8 show Annealing temperature 60° C.: 2: positive control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. albicans*. Lane 5 shows *C. glabrata*. Lane 6 shows *C. dubliniensis*. Lane 7 shows *C. guilliermondii*, Lane 8 shows *C. krusei*. Lanes 9-15 show annealing temperature 60.8° C.: Lane 9 shows the positive control *C. parapsilosis*; Lane 10 shows the negative control without DNA; Lane 11 shows *C. albicans*. Lane 12 shows *C. glabrata*. Lane 13 shows *C. dubliniensis*. Lane 14 shows *C. guilliermondii*, Lane 15 shows *C. krusei*. Lanes 16-19 show annealing temperature 61.9° C.: Lane 16 shows the positive control *C. parapsilosis*; Lane 17 shows the negative control without DNA; Lane 18 shows *C. albicans*. Lane 19 show *C. glabrata*. Lane 20 shows the molecular weight marker.

**[0017]** In FIG. 2B: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-4 show Annealing temperature 61.9° C.: Lane 2 shows *C. dubliniensis*. Lane 3 shows *C. guilliermondii*, Lane 4 shows *C. krusei*. Lanes 5-11 show Annealing temperature 63.2° C.: Lane 5 shows the positive control *C. parapsilosis*; Lane 6 shows the negative control without DNA; Lane 7 shows *C. albicans*. Lane 8 shows *C. glabrata*. Lane 9 shows *C. dubliniensis*. Lane 10 shows *C. guilliermondii*, Lane 11 shows *C. krusei*. Lanes 12-18 show Annealing temperature 65° C.: Lane 12 shows the positive control *C. parapsilosis*; Lane 13 shows the negative control without DNA; Lane 14 shows *C. albicans*. Lane 15 shows *C. glabrata*. Lane 16 shows *C. dubliniensis*. Lane 17 shows *C. guilliermondii*, Lane 18 shows *C. krusei*. Lanes 19-20 show Annealing temperature 66.5° C. Lane 19 shows the Positive control *C. parapsilosis*; Lane 20 shows the negative control without DNA.

**[0018]** In FIG. 2C: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-6 show Annealing temperature 66.5° C.: Lane 2 shows *C. albicans*. Lane 3 shows *C. glabrata*. Lane 4 shows *C. dubliniensis*. Lane 5 shows *C. guilliermondii*, Lane 6 shows *C. krusei*. Lanes 7-13 show Annealing temperature 67.5° C.: Lane 7 shows the positive control *C. parapsilosis*; Lane 8 shows the negative control without DNA; Lane 9 shows *C. albicans*. Lane 10 shows *C. glabrata*. Lane 11 shows *C. dubliniensis*. Lane 12 shows *C. guilliermondii*, Lane 13 shows *C. krusei*. Lanes 14-20 show Annealing temperature 68° C.: Lane 14 shows the positive control *C. parapsilosis*; Lane 15 shows the negative control without DNA; Lane 16 shows *C. albicans*. Lane 17 shows *C. glabrata*. Lane 18 shows *C. dubliniensis*. Lane 19 shows *C. guilliermondii*, Lane 20 shows *C. krusei*.

**[0019]** FIGS. 3A-3E. show a 2% agarose gel showing temperature gradient for *C. parapsilosis* detection (Cp20-clinical isolated strain) using the oligonucleotides pair Cp2. The unspecific strip for *C. glabrata*, *C. tropicalis*, *C. bracarensis* and *C. dubliniensis* disappears as the oligonucleotides alignment temperature increases, for this oligonucleotides pair, the optimal temperature selected is 68.4° C. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls. The amplification band for *C. parapsilosis* has a length of 174 bp.

**[0020]** In FIG. 3A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-7 show Annealing temperature 56° C.: Lane 2 shows the positive

control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*. Lane 5 shows *C. tropicalis*. Lane 6 shows *C. bracarensis*. Lane 7 shows *C. dubliniensis*. Lanes 8-13 show annealing temperature 57.1° C.: Lane 8 shows the positive control *C. parapsilosis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. glabrata*. Lane 11 shows *C. tropicalis*. Lane 12 shows *C. bracarensis*. Lane 13 shows *C. dubliniensis*. Lanes 14-19 show annealing temperature 58.8° C.: Lane 14 shows the positive control *C. parapsilosis*; Lane 15: negative shows the control without DNA; Lane 16 shows *C. glabrata*. Lane 17 shows *C. tropicalis*. Lane 18 shows *C. bracarensis*. Lane 19 shows *C. dubliniensis*. Lane 20 shows the molecular weight marker.

**[0021]** In FIG. 3B: Lane 1 shows the molecular weight marker. Lanes 2-7 show Annealing temperature 60.8° C.: Lane 2 shows the positive control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*. Lane 5 shows *C. tropicalis*. Lane 6 shows *C. bracarensis*. Lane 7 shows *C. dubliniensis*. Lanes 8-13 show annealing temperature 63.5° C.: Lane 8 shows the positive control *C. parapsilosis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. glabrata*. Lane 11 shows *C. tropicalis*. Lane 12 shows *C. bracarensis*. Lane 13 shows *C. dubliniensis*. Lanes 14-19 show annealing temperature 65.7° C.: Lane 14 shows the positive control *C. parapsilosis*; Lane 15 shows the negative control without DNA; Lane 16 shows *C. glabrata*. Lane 17 shows *C. tropicalis*. Lane 18 shows *C. bracarensis*. Lane 19 shows *C. dubliniensis*. Lane 20 shows the molecular weight marker.

**[0022]** In FIG. 3C: Lane 1 shows the molecular weight marker. Lanes 2-7 show Annealing temperature 67.2° C.: Lane 2 shows the positive control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*. Lane 5 shows *C. tropicalis*. Lane 6 shows *C. bracarensis*. Lane 7 shows *C. dubliniensis*. Lanes 8-13 show annealing temperature 68° C.: Lane 8 shows the positive control *C. parapsilosis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. glabrata*. Lane 11 shows *C. tropicalis*. Lane 12 shows *C. bracarensis*. Lane 13 shows *C. dubliniensis*. Lane 14 shows the molecular weight marker.

**[0023]** In FIG. 3D: Lane 1 shows the molecular weight marker. Lanes 2-4 show annealing temperature 68.4° C. Lane 2 shows the positive control *C. parapsilosis*, Lane 3 shows the negative control. Lane 4 shows *C. dubliniensis*. Lanes 5-7 show annealing temperature 69° C. Lane 5 shows the positive control *C. parapsilosis*, Lane 6 shows the negative control. Lane 7 shows *C. dubliniensis*. Lanes 8-10 show annealing temperature 69.6° C. Lane 8 shows the positive control *C. parapsilosis*, Lane 9 shows the negative control Lane 10 shows *C. dubliniensis*.

**[0024]** In FIG. 3E: Lane 1 shows the molecular weight marker. Lanes 2-4 show annealing temperature 71.8° C. Lane 2 shows the positive control *C. parapsilosis*, Lane 3 shows the negative control. Lane 4 shows *C. dubliniensis*. Lanes 5-7 show annealing temperature 72° C. Lane 5 shows the positive control *C. parapsilosis*, Lane 6 shows the negative control. Lane 7 shows *C. dubliniensis*.

**[0025]** FIGS. 4A-4H. show a 2% agarose gel showing oligonucleotide concentration analysis for *C. parapsilosis* detection (clinical isolate Cp20) using the oligonucleotides pair Cp1. For this oligonucleotides pair, the optimal concentration selected is 200 nM.



[0026] FIG. 4A shows a concentration of oligonucleotides pair of 100 nM;

[0027] FIG. 4B shows a concentration of oligonucleotides pair of 200 nM;

[0028] FIG. 4C shows a concentration of oligonucleotides pair of 400 nM;

[0029] FIG. 4D shows a concentration of oligonucleotides pair of 500 nM;

[0030] FIG. 4E shows a concentration of oligonucleotides pair of 600 nM;

[0031] FIG. 4F shows a concentration of oligonucleotides pair of 800 nM;

[0032] FIG. 4G shows a concentration of oligonucleotides pair of 1000 nM;

[0033] FIG. 4H shows a concentration of oligonucleotides pair of 1200 nM. For each gel, the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogen); Lane 2 shows the positive control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. tropicalis*; Lane 7 shows *C. dubliniensis*; Lane 8 shows *C. bracarensis*; Lane 9 shows *C. guilliermondii*; Lane 10 shows *C. krusei*.

[0034] FIGS. 5A-5H show a 2% agarose gel showing oligonucleotide concentration analysis for *C. parapsilosis* detection (clinical isolated Cp20) using the oligonucleotides pair Cp2. For this oligonucleotides pair, the optimal concentration selected is 600 nM. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls.

[0035] FIG. 5A shows a concentration of oligonucleotides pair of 100 nM;

[0036] FIG. 5B shows a concentration of oligonucleotides pair of 200 nM;

[0037] FIG. 5C shows a concentration of oligonucleotides pair of 400 nM;

[0038] FIG. 5D shows a concentration of oligonucleotides pair of 500 nM;

[0039] FIG. 5E shows a concentration of oligonucleotides pair of 600 nM;

[0040] FIG. 5F shows a concentration of oligonucleotides pair of 800 nM;

[0041] FIG. 5G shows a concentration of oligonucleotides pair of 1000 nM;

[0042] FIG. 5H shows a concentration of oligonucleotides pair of 1200 nM. For each gel, the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogen); Lane 2 shows the positive control *C. parapsilosis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. tropicalis*; Lane 7 shows *C. dubliniensis*; Lane 8 shows *C. bracarensis*; Lane 9 shows *C. guilliermondii*; Lane 10 shows *C. krusei*.

[0043] FIGS. 6A-6C. show a 2% agarose gels showing the analysis of the 36 clinical isolated samples for *C. parapsilosis* detection (Cp20 clinical isolated sample) using the oligonucleotides pair Cp1. There were 3 samples detected as positive.

[0044] In FIG. 6A-6C, lane 1 and lane 16 show the molecular weight marker (1 Kb DNA Ladder Invitrogen); lane 2 shows the positive control *C. parapsilosis*; lane 3 shows the negative controls, without DNA. Remaining lanes 4 to 15 show clinical samples.

[0045] FIGS. 7A-7B. show a 2% agarose gel showing the analysis of the 36 clinical isolated samples for *C. parapsilosis* detection (Cp20 clinical isolated sample) using the oligonucleotides pair Cp2. There were 3 samples detected as positive.

[0046] In FIG. 7A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogen); Lane 2 shows the positive control *C. parapsilosis*; Lane 3 shows the negative controls, without DNA. Remaining lanes 4 to 20 show clinical samples.

[0047] In FIG. 7B: Lane 1 to 19 show clinical samples. Lane 20 shows the molecular weight marker (1 Kb DNA Ladder Invitrogen);

[0048] FIG. 8. shows a 2% agarose gel showing a multiplex test for *C. parapsilosis*. Cp1 and Cp2 oligonucleotide pairs were tested in several conditions. Predicted amplification sizes 311 and 174 base pairs were detected in samples containing only *C. parapsilosis*. Lane 1 shows the Molecular weight marker (1 Kb DNA Ladder Invitrogen). Lane 2 shows *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *S. cerevisiae*, 100 ng each. Lane 3 shows the negative control containing *C. glabrata*, *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *S. cerevisiae*, 100 ng each. Lane 4 shows the molecular weight marker. Lane 5 shows *C. parapsilosis*. Lane 6 shows the negative control without DNA. Lane 7 shows *C. parapsilosis*. Lane 8 shows *C. albicans*. Lane 9 shows *C. glabrata*. Lane 10 shows *C. tropicalis* Lane 11 shows *C. dubliniensis*.

[0049] FIGS. 9A-9B. show a 2% agarose gel showing specificity test. FIG. 9 A with Cp1 and FIG. 9B with Cp2 oligonucleotide pairs. For both Figures the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogen). Lane 2 shows the positive control *C. parapsilosis*. Lane 3 shows the negative control without DNA. Lane 4 shows *C. parapsilosis* 100 ng plus 50 ng *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. bracarensis*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *S. cerevisiae* each. Lane 5 shows *C. parapsilosis* 10 ng plus 50 ng *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. bracarensis*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *S. cerevisiae* each. Lane 6 shows *C. parapsilosis* 1 ng plus 50 ng *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. bracarensis*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *S. cerevisiae* each. Lane 7 shows 50 ng *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. bracarensis*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *S. cerevisiae* each.

#### DETAILED DESCRIPTION OF THE INVENTION

[0050] The present invention discloses an in vitro method for detecting and identifying *Candida parapsilosis*, with at least one set of specific oligonucleotides, but also with an in-block multiplex set of specific oligonucleotides, which allows identification of *Candida parapsilosis* in clinical samples of different population subgroups with 100% of specificity and sensitivity.

[0051] Several oligonucleotides have been designed in order to specifically detect different chromosomal sites of *Candida parapsilosis*. The amplified sequences are located in several chromosomes and in contigs that have unique regions that allow said specific detection. The different sizes among the amplification products of each pair of oligonucleotides allow that they are rapidly recognized in separate or a single multiplex assay. *Candida parapsilosis* can be specifically

detected by any amplification method, such as PCR, RT-PCR, Q-PCR, multiplex-PCR, nested-PCR, or any other amplification or nucleic acid detection methods such as Southern blot, Dot blot, etc.

**[0052]** “Amplification” should be interpreted as a process for artificial increasing the number of copies of a particular nucleic acid fragments into millions of copies through the replication of the target segment.

**[0053]** By “complementary” is meant a contiguous sequence that is capable of hybridizing to another sequence by hydrogen bonding between a series of complementary bases, which may be complementary at each position in the sequence by standard base pairing (e.g., G:C, A:T or A:U pairing) or may contain one or more positions, including a basic ones, which are not complementary bases by standard hydrogen bonding. Contiguous bases are at least 80%, preferably at least 90%, and more preferably about 100% complementary to a sequence to which an oligomer is intended to specifically hybridize. Sequences that are “sufficiently complementary” allow stable hybridization of a nucleic acid oligomer to its target sequence under the selected hybridization conditions, even if the sequences are not completely complementary.

**[0054]** “Sample preparation” refers to any steps or methods that prepare a sample for subsequent amplification and detection of *Candida* nucleic acids present in the sample. Sample preparation may include any known method of concentrating components from a larger sample volume or from a substantially aqueous mixture, e.g., any biological sample that includes nucleic acids. Sample preparation may include lysis of cellular components and removal of debris, e.g., by filtration or centrifugation, and may include use of nucleic acid oligomers to selectively capture the target nucleic acid from other sample components.

**[0055]** The present invention discloses a composition comprising several oligonucleotides for the specific identification of *C. parapsilosis*, in a suitable acceptable carrier, wherein said oligonucleotides comprises a continuous sequence of about 18 to 22 nucleotides of a target sequence. Said target sequence is located along the chromosomes of said *C. parapsilosis*, in exclusive sites that allows non-cross reactions with any other kind of organism, including other *Candida* species and microbial or eukaryotic nucleic acid that can be contained in a biological sample.

**[0056]** Also, the oligonucleotides for the specific identification of *Candida parapsilosis*, consist of a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 4 or complements thereof.

**[0057]** Said oligonucleotides are sufficiently complementary to the target sequences of *C. parapsilosis*. For the experimental procedures, the amplified sequences were re-sequenced in order to make sure that the amplified product corresponds to the disclosed genomic region.

**[0058]** This invention also discloses an in vitro method for the specific identification of *C. parapsilosis*, comprising the steps of: a) amplifying nucleic acid fragments from a biological sample by an amplification method with at least one of the specifically designed oligonucleotides, such as those disclosed on SEQ ID NOS: 1 to 4 or a complement thereof; and b) identify the amplified nucleic acid fragments. In this method the biological sample is derived from one subject to study. The subject to study is a mammal, wherein as a preferred embodiment, but not limited, is a human. Additionally, in a preferred embodiment, said biological sample is selected

from the group consisting of any sample containing DNA, fluids, tissue, or cell debris, midstream urine, urine culture tube, growing by nephrostomy (right and left kidney), water, hemodialysis, pleural fluid, culture pyogenic, mieloculture, bone marrow, blood lysis (peripheral blood), blood culture (blood), leukocyte concentrate, concentrated red cell, throat, nasal discharge, vaginal discharge, exudate prostate sputum, catheter, biopsies from different tissues such as lymph node, subcutaneous tissue, cornea, lung, pulmonary nodule, pancreas, jaw, skin, skin quantitative (cellulite, breast, scrotum, arm, hand), hair, nails, warm muscle, bone, breast, synovial fluid, scar, thigh, joint capsule, knee, omentum, bronchoalveolar lavage (lingula, upper and lower lobe (left and right), left and right LBA (airways)); post-mortem (liver, lung, spleen), wound swabs (perianal, vaginal, ulcer (foot, hand)), abscess (thigh, kidney, perianal) or peripancreatic.

**[0059]** Furthermore, a kit for the specific identification of *Candida parapsilosis*, with at least one oligonucleotide or as a multiplex identification kit is disclosed. Said kits comprise at least one oligonucleotide specifically designed for the identification of *Candida parapsilosis* such as those disclosed on SEQ ID NOS: 1 to 4 or complements thereof. In the multiplex embodiment, the kit comprises at least one oligonucleotide pair or more preferably, at least two oligonucleotide pairs.

**[0060]** The use of said oligonucleotides specifically designed for the specific identification of *Candida parapsilosis*, is disclosed as well.

**[0061]** As an additional embodiment, the present invention discloses at least one probe useful for the specific identification of *Candida parapsilosis*. Said identification is carried out by an in vitro method comprising coupling nucleic acid fragments from a biological sample with said probes and identifying the hybridized nucleic acid fragments, wherein said steps are carried out by any hybridization method.

**[0062]** In order to test fully the competitive advantage of the methods of the present invention against traditional diagnostic methods, below is a comparison of the times of two tests:

**[0063]** Traditional method of identification of *Candida* in urine, urine samples are analyzed in an automated urine analyzer coupled Urisys type UF-100i. The analysis was performed by flow cytometry with an argon laser. The UF-100i measures the properties of scattered light and fluorescence to count and identify the particles in the urine. The volume of the particles is determined from the impedance signals. Thus, according to the scatterplots, the result indicates which urine samples are likely to contain yeast cells. These samples are marked as YLC urine samples (yeast cells). In urine samples taken YLC marked 1 $\mu$ l and plating medium Sabourand/Dextrose (SDA) and medium Sabourand/Dextrose with cefoperazone (CFP). These plates are incubated at 30° C. for 72 hours. Urine cultures with growth less than 10,000 CFU/ml, as no growth plates, are reported as not developed fungi (negative) urine cultures with equal or greater development to 10,000 CFU/ml pass germ tube test, with incubation for 2 hours at 35° C. In the case of negative germ tube is reported as *Candida* sp. To identify the species from the report of *Candida* sp.

**[0064]** Vitek cards are used that allow the identification by means of assimilation of carbohydrates. These cards are incubated for a period of 24 to 48 hours, at which time the cards are read. The minimum total time to identify *C. parapsilosis*, is 6 days, with a sensitivity of about 85%.

**[0065]** In the method for identifying *C. parapsilosis*, of the present invention, the urine samples are analyzed in an auto-

mated urine analyzer coupled Urisys type UF-100i. The analysis was performed by flow cytometry with an argon laser.

[0066] The UF-100i measures the properties of scattered light and fluorescence to count and identify the particles in the urine. The volume of the particles is determined from the impedance signals. Thus, according to the scatterplots, the result indicates which urine samples are likely to contain yeast cells. These samples are marked as YLC urine samples (yeast cells). The time of this first stage is 2 hours. Next, in urine samples taken YLC marked as 1 ml, centrifuged, the supernatant is discarded, resuspended and boiled the pill. The genomic DNA obtained is used for PCR analysis using primers generated from the SEQ ID Nos. 1 to 22, under optimal conditions reaction. The PCR products were separated by agarose gel electrophoresis and the products are analyzed for the correct identification of *C. parapsilosis*, together as an in-block multiplex or separately. The total test time is 6 hours.

[0067] Traditional method of identification of *Candida* in blood samples: Blood samples are incubated for 72 hours in the automated equipment BACTEC9240. When no growth of microorganisms metabolize these nutrients in the culture medium by releasing CO<sub>2</sub>. The release of CO<sub>2</sub> is detected by the computer and automatically marked as blood cultures positive for yeast.

[0068] Positive blood cultures for yeasts are grown on plates Sabourand/Dextrose (SDA) and Sabourand/Dextrose with cefoperazone (CFP) and incubated at 30° C. for 72 hours. Blood cultures with lower growth of 10,000 CFU/ml as well as those without growth, are reported as not develop fungus (negative), the blood cultures with growth equal to or greater than 10,000 CFU/ml was performed germ tube test for 2 hours at 35° C. In the case of negative germ tube is reported as *Candida* sp. To identify the species from the report of *Candida* sp. Vitek cards are used that allow the identification by means of assimilation of carbohydrates. These cards are incubated for 24 to 48 hours and are read to identify *C. parapsilosis*. The total time for identification is a minimum of 9 days.

blood cultures positive for yeast. Blood samples positive for yeast marked 100 µl taken, centrifuged, the supernatant is discarded, re-suspended and boiled the pill.

[0070] The genomic DNA obtained from PCR annealing is used where any of the oligonucleotides generated from SEQ ID Nos. 1 to 4, in optimal reaction conditions. The PCR products were separated by agarose gel electrophoresis and the products are analyzed for the correct identification of *C. parapsilosis*. The total test time is 3 days.

[0071] An alternate method is to take as the patient's blood sample without being seeded by blood culture. In this case, follow the above procedure and the total test time is 4 hours.

[0072] Thus, the critical step is to obtain sufficient genomic DNA from any of the types of samples described above, and from them, using genomic DNA obtained as the PCR template, and using any one of the oligonucleotides generable or generated in the regions above disclosed, such as, but not limited to the 12 sequences disclosed. The PCR products are obtained and analyzed by any conventional method, such as but not limited to agarose gel electrophoresis, dot-blot hybridizations, Southern blotting, Northern blotting and similar RT-PCR, PCR-ELISA, and others known in the art (for example, but not limited to, Molecular Diagnostic PCR handbook. (2005), Gerrit J. Viljoen, Louis H. and John R. Crowther Nei. Springer Publishers) to correctly identify *C. albicans* in a multiplex assay or single assay. Note that these oligonucleotides may comprise nucleotide unmarked or marked, such as but not limited to, radioactive labeling, brand quiomiluminiscente, luminescent, fluorescent, biotinylated.

[0073] Experimental selected examples, which must be considered only as supporting technical evidence, but without limiting the scope of the invention, are provided herein below.

EXAMPLES

Example 1

Oligonucleotide Design

[0074] *Candida parapsilosis* oligonucleotides and probes were specifically designed from unique sites located on the genome. Non-limiting examples of the specifically designed oligonucleotides are disclosed in Table 1.

TABLE 1

Examples of oligonucleotides for the identification of <i>Candida parapsilosis</i>							
Oligo-nucleotide pair No.	Seq ID No.	Foward (Fw) or Reverse (Rv)	Bp number	5' a 3' Sequence	Amplicon lenght (bp)	Contig Name	
Cp1	Seq. ID. No. 1	Fw	19	GTC AGT TCG GGA GCA ATA G	311	AG_03079	
	Seq. ID. No. 2	Rv	20	GTT GCT TCT CCT TCA CTT GC			
Cp2	Seq. ID. No. 3	Fw	20	GGG TGT ATG AGA CTA CTT GG	174	CPAG_00126	
	Seq. ID. No. 4	Rv	18	CTG AAA GAG GAA CGT CAC			

[0069] Method to detect *C. parapsilosis*, according to the present invention in blood samples: Blood samples are incubated for 72 hours BACTEC9240 automated equipment. When no growth of microorganisms metabolize these nutrients in the culture medium by releasing CO<sub>2</sub>. The release of CO<sub>2</sub> is detected by the computer and automatically marked as

[0075] The locations of the corresponding contigs are accordance with GenBank database (<http://www.ncbi.nlm.nih.gov>).

[0076] Said oligonucleotide pairs were tested for optimizing the amplification conditions. Thus, oligonucleotide pairs Cp1 and Cp2 have annealing temperatures from about 56° C.

to 72° C. These oligonucleotide pairs were tested on genomic DNA for amplification testing carrying out PCR reactions. For example the oligonucleotide pairs were analyzed in a final product volume of 30 µL, as follows:

TABLE 2

PCR general experimental conditions.		
Reagents	Concentration	Volume (µL)
Genomic DNA	Variable	0.5 µL
Buffer 10X	1X	3.0 µL
MgCl <sub>2</sub> 20X	1X	1.5 µL
dNTPs 2 Mm	30 µM	0.45 µL
Primer Forward	500 nM	3.0 µL
Primer Reverse	500 nM	3.0 µL
Amplificase	500 U	0.4 µL
Water		18.15 µL
	Final Volume	30.0 µL

[0077] As a control, the quality of the genomic DNA was evaluated by amplifying rDNA regions with universal oligonucleotides ITS1 and ITS4 (Table 3), using the same concentrations and final volume as above disclosed. The genomic DNA was pure, non-degraded and free of molecules that could interfere with further PCR reactions. (FIG. 1)

TABLE 3

Universal oligonucleotides for amplifying ITS on fungi genes.		
Name	5' a 3' Sequence	Lenght
ITS1	TCCGTAGGTGAACCTGCGG	19
ITS4	TCCTCCGCTTATTGATATGC	20

[0078] The amplified fragments resulting from the PCR reactions of each oligonucleotide pairs were tested on 2% agarose gels during 60 minutes at 100-130 volts.

[0079] During electrophoresis, the samples belonging to other *Candida* species different to *Candida parapsilosis*, were loaded at higher concentrations to those used for positive and negative controls. This was made in order to be sure of the oligonucleotide's sensitivity.

Example 2

Standardization Techniques

[0080] Herein below, standardization results from some selected oligonucleotides are shown. This selection should not be taken as limiting the scope of the invention, but to illustrate the applicability of all the designed oligonucleotides.

[0081] Cp1 and Cp2 oligonucleotide pair were tested in order to reflect the sensitivity and selectivity of the 4 oligonucleotides and probes for identifying *C. parapsilosis*. These examples are illustrative but not limitative for the scope of the invention.

[0082] Optimal PCR Reaction Conditions:

[0083] Firstly, annealing conditions were tested with a temperature threshold. Results are shown in Table 4.

[0084] Annealing temperatures were tested for each oligonucleotide pairs, the maximum and minimum temperatures

wherein the reaction is effective was pointed out in the thermocycling and the intermediate temperatures were calculated.

TABLE 4

Annealing temperatures were tested for each oligonucleotide pairs.									
Oligonucleotide pair	Temperature Threshold (° C.)								Best selected Temperature (° C.)
	Min							Max	
Cp1	60	60.8	61.9	63.2	65	66.5	67.5	68	64.4
Cp2	56	57.1	58.8	60.8	63.5	65.7	67.2	68	68.4
	68	68.4	69	69.6	70.5	71.3	71.8	72	

[0085] FIGS. 2 and 3 show the minimum temperature threshold wherein the oligonucleotides are more specific compared with other species which show unspecific bands in the first analysis. All the agarose gels are at a concentration of 2% and were run at 110-130 V.

[0086] Oligonucleotide Concentration

[0087] Once the optimal annealing temperature has been selected for each oligonucleotide pair, the optimal oligonucleotide concentration was determined for PCR reactions.

[0088] The concentrations tested were: 100 nM, 200 nM, 400 nM, 500 nM, 600 nM, 800 nM, 1000 nM y 1200 nM.

[0089] The minimal oligonucleotide concentration wherein a clear band was detected in the positive control, was selected. Table 5 shows the best concentrations. FIGS. 4 and 5 show the optimization results with exemplifying oligonucleotide pairs. All the agarose gels are at a concentration of 2% and were run at 110-130 V.

TABLE 5

Best oligonucleotide concentration for oligonucleotide pairs designed for <i>C. parapsilosis</i> .		
No	Oligonucleotide pair	Best selected concentration
1	Cp1	200 nM
2	Cp2	600 nM

[0090] Genomic DNA detected. The amount of genomic DNA that can be detected for each oligonucleotide pair was tested from 100 ng to 0.02 ng with a control without DNA. For *C. parapsilosis*, genomic DNA can be detected in an amount of at least 0.1 ng.

Example 3

*Candida* Detection on Isolated Clinical Samples

[0091] The above exemplified oligonucleotide pairs were tested to detect *Candida parapsilosis* on clinical isolated samples from hospitalized patients.

[0092] FIGS. 6 and 7 show the results of said tests. All the oligonucleotide pairs detect only the specific *Candida* specie for which they were designed. In all cases both oligonucleotide pairs detect the same positive samples.

[0093] Comparing PCR results with Vitek identification methods reveals that PCR test has a sensibility of 100% and a specificity of 100% in contrast to VITEK tests with an 85% and 33% respectively. Vitek test identified one clinical isolate

as *C. tropicalis*, however, the PCR test of the invention identified it as *C. parapsilosis*. This result was also confirmed by API ID32C test.

#### Example 4

##### Multiplex Assay

**[0094]** Since it is possible to have rearrangements within the genome of *C. parapsilosis*, a multiplex assay was designed in order to confirm with 100% specificity, the presence of the microorganism in clinical samples. Since the oligonucleotide pairs are located in several chromosomes, the probability of having more than one rearrangement within a clinical sample is low.

**[0095]** FIG. 8 shows the use of oligonucleotides pairs Cp1 and Cp2 simultaneously in samples containing *C. parapsilosis* alone or in mixture with *C. glabrata*, *C. albicans*, *C. tropicalis*, *C. dubliniensis*, *S. cerevisiae*, wherein each microorganism is in an amount of 100 ng. As predicted, the amplified fragments are present only in the lanes containing *C.*

*parapsilosis*, and not in the control lanes (lane 3, 6, 8 to 11). Therefore, a multiplex kit for detecting *C. parapsilosis* has been designed with a 100% of sensibility and specificity.

#### Example 5

##### Specificity Assay

**[0096]** FIGS. 9A and 9B show that the oligonucleotides tested are specific for *C. parapsilosis* and do not cross-link with other microbial species. For example, *C. parapsilosis* mixed with another 10 microbial species such as *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. bracarensis*, *C. guilliermondii*, *C. krusei*, *C. metapsilosis*, *C. orthopsilosis*, *S. cerevisiae* (50 ng each for a total of 50 ng). *C. parapsilosis* DNA was added in different amounts: 100 ng, 10 ng, 1 ng and a control without DNA. As shown, the amplified bands detected correspond to the predicted size (311 bp for Cp1, and 174 bp for Cp2) and its resequencing test. Negative control without *C. parapsilosis* DNA did not show any amplification band. This confirms that the assay is 100% specific for *C. parapsilosis*.

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#### SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 4

<210> SEQ ID NO 1

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: *Candida parapsilosis*

<400> SEQUENCE: 1

gtcagttcgg gagcaatag

19

<210> SEQ ID NO 2

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: *Candida parapsilosis*

<400> SEQUENCE: 2

gttgettctc cttcacttgc

20

<210> SEQ ID NO 3

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: *Candida parapsilosis*

<400> SEQUENCE: 3

gggtgtatga gactacttgg

20

<210> SEQ ID NO 4

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: *Candida parapsilosis*

<400> SEQUENCE: 4

ctgaaagagg aacgtcac

18

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What is claimed is:

1. An oligonucleotide for the specific identification of *Candida parapsilosis* comprising a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 4 or a complement thereof.

2. An in vitro method for the specific identification of *C. parapsilosis*, comprising the steps of:

a) amplifying DNA fragments from a biological sample with at least one oligonucleotide as defined in claim 1; and

b) identify the amplified DNA fragments.

3. The method according to claim 2, wherein the amplification of DNA fragments is carried out with at least one pair of oligonucleotides as defined in claim 1.

4. The method according to claim 2, wherein the amplification of DNA fragments is carried out with at least two pair of oligonucleotides as defined in claim 1.

5. A kit for the specific identification of *Candida parapsilosis*, comprising at least one oligonucleotide as defined in claim 1.

6. The kit according to claim 5, comprising at least one oligonucleotide pair as defined in claim 1.

7. The kit according to claim 6, comprising at least two pair of oligonucleotides as defined in claim 1.

\* \* \* \* \*