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

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(52) **U.S. Cl.**

CPC ***C12Q 1/6895*** (2013.01); *C12Q 2600/16* (2013.01)

(57) **ABSTRACT**

The invention discloses an in vitro method for the identification of *Candida tropicalis*, the sequences associated to said identification, as well as diagnosis kits for identifying *Candida tropicalis*.

FIG. 1

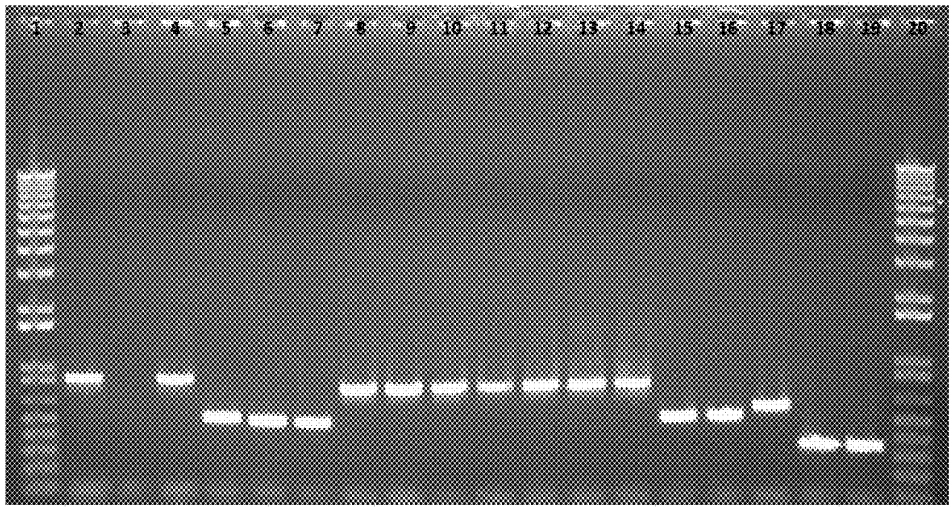


FIG. 2A

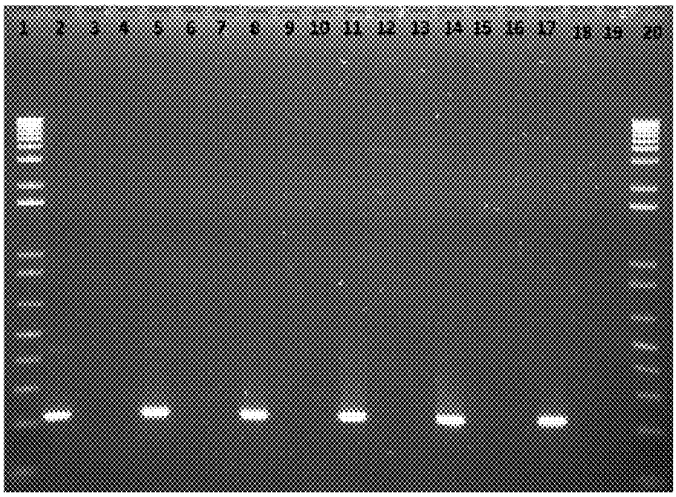


FIG. 2B

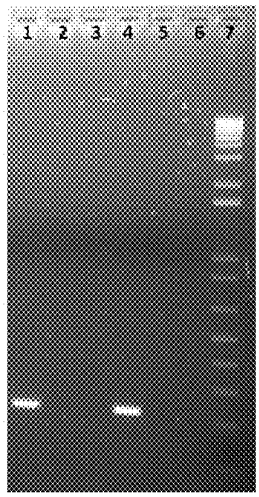


FIG. 3A

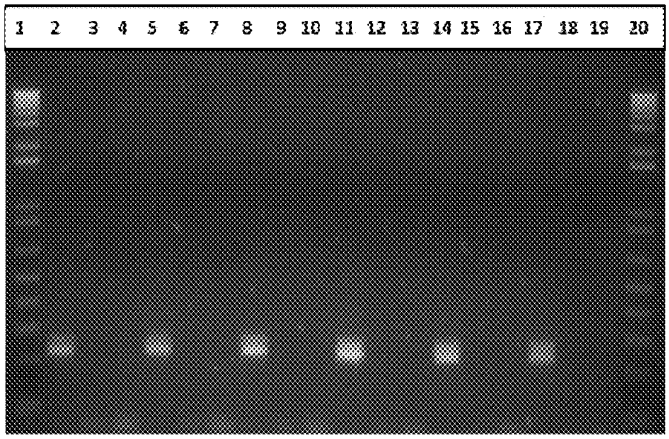


FIG. 3B

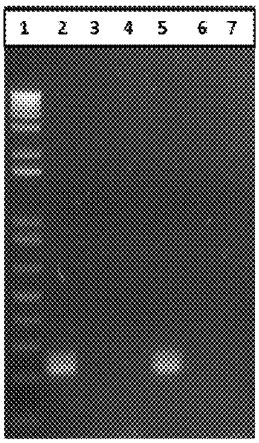


FIG. 4

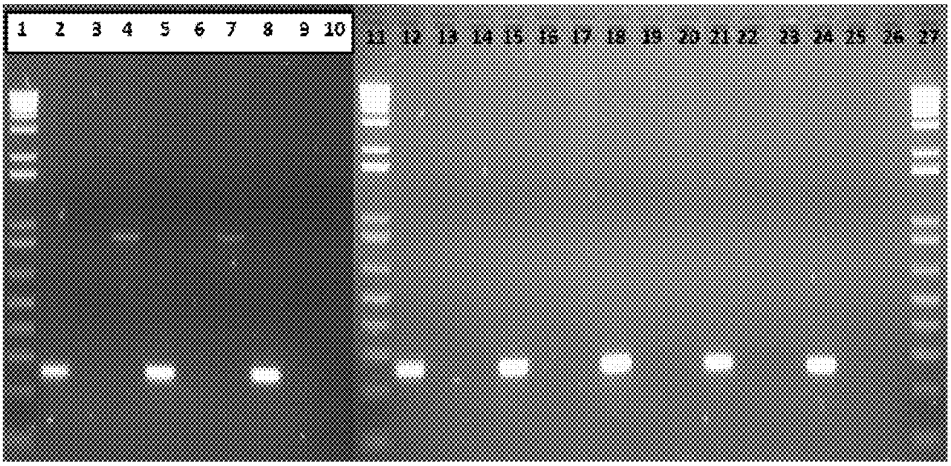


FIG. 5A FIG. 5B FIG. 5C FIG. 5D

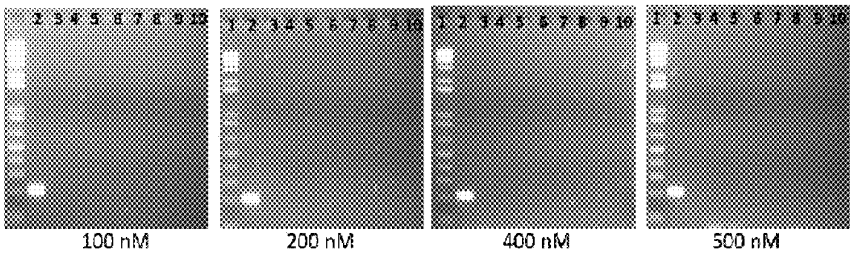


FIG. 5E FIG. 5F FIG. 5G FIG. 5H

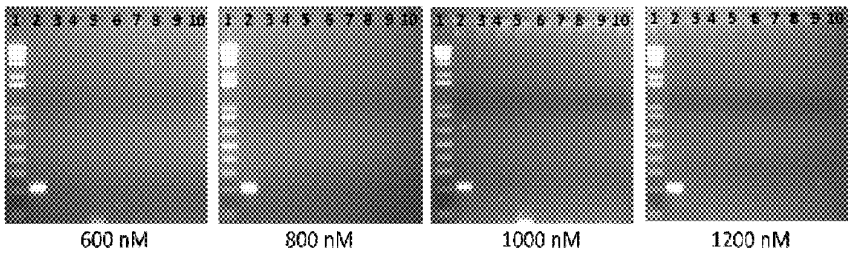


FIG. 6A FIG. 6B FIG. 6C FIG. 6D

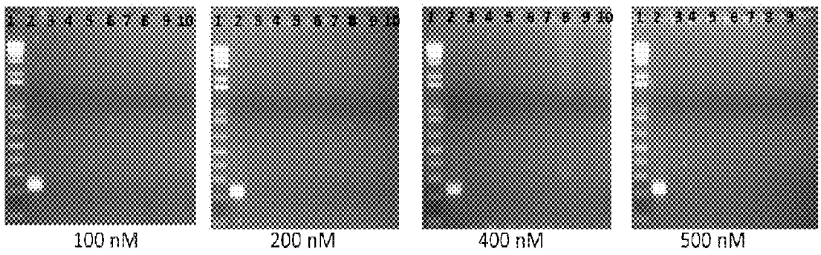


FIG. 6E FIG. 6F FIG. 6G FIG. 6H

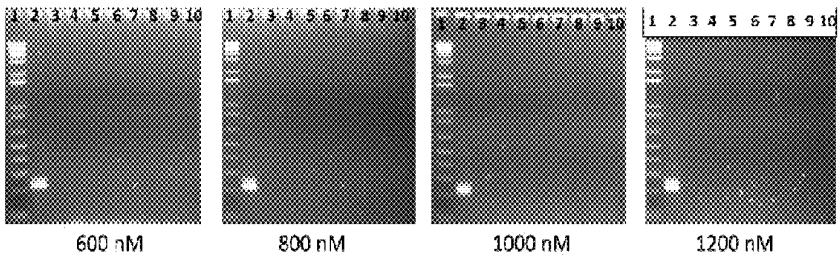
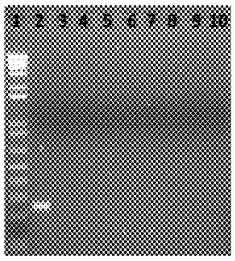
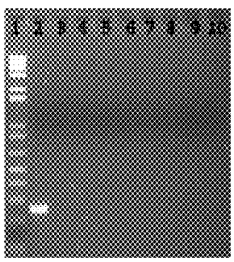


FIG. 7A



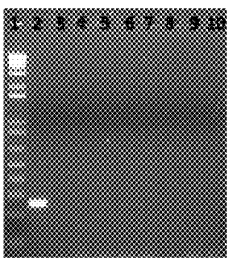
100 nM

FIG. 7B



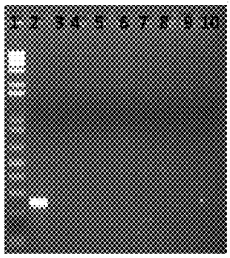
200 nM

FIG. 7C



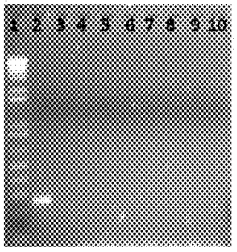
400 nM

FIG. 7D



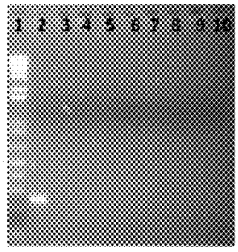
500 nM

FIG. 7E



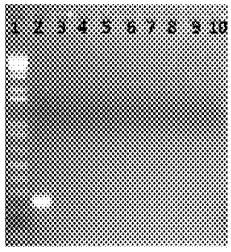
600 nM

FIG. 7F



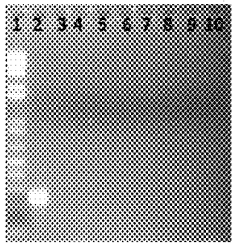
800 nM

FIG. 7G



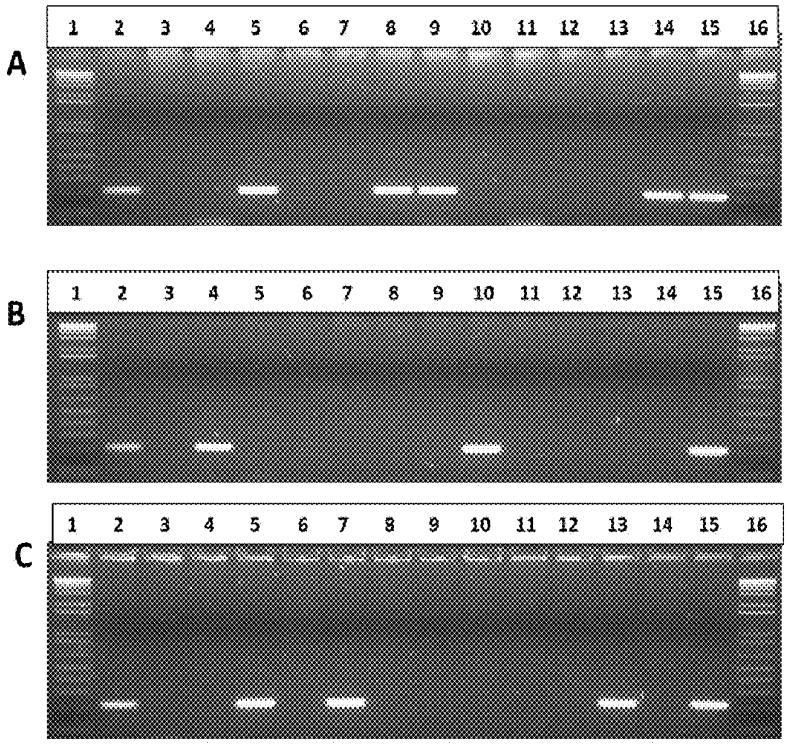
1000 nM

FIG. 7H

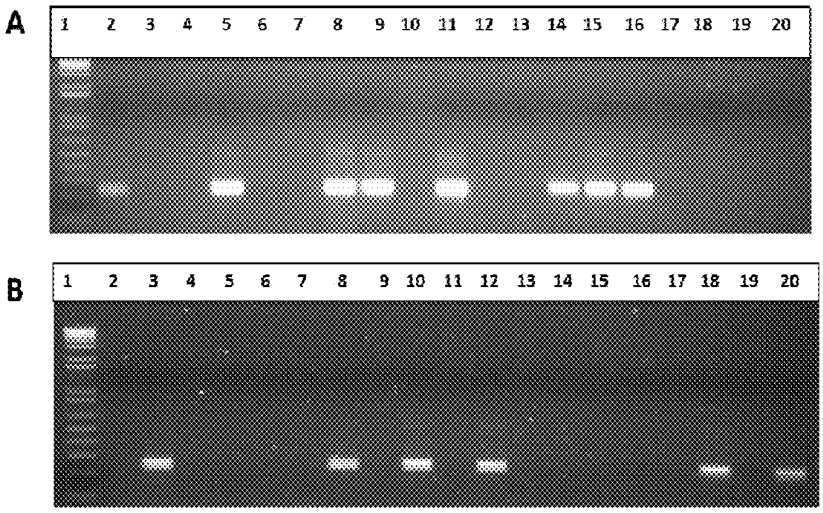


1200 nM

FIGURES 8A-8C



FIGURES 9A- 9B



FIGURES 10A- 10B

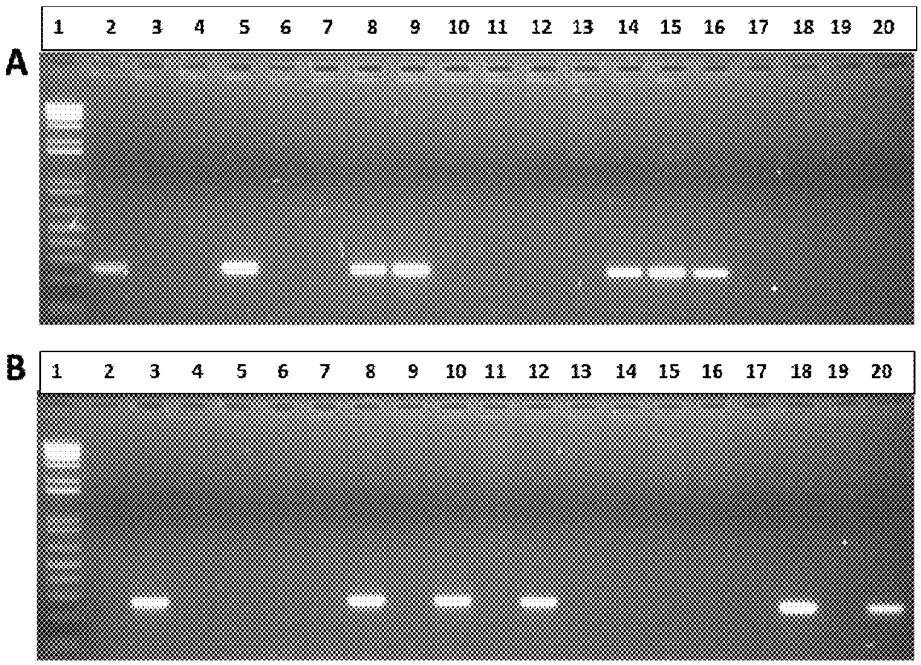
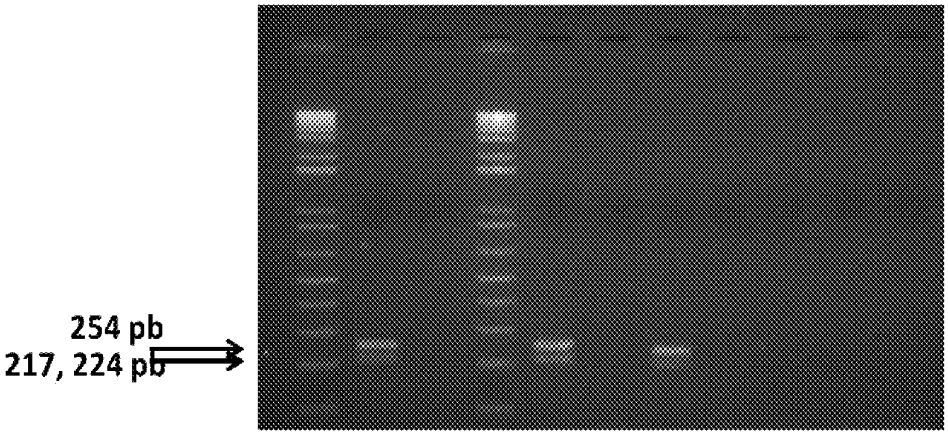
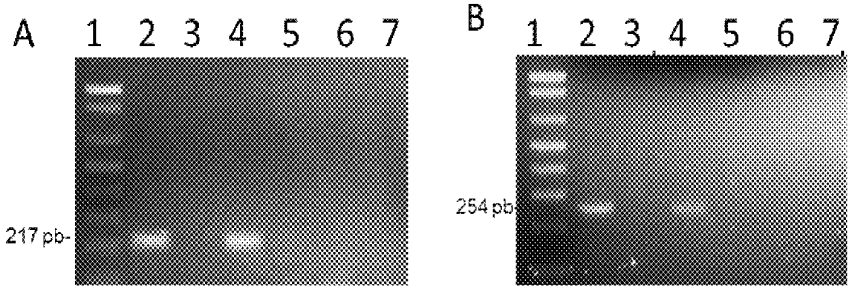


FIG. 11



FIGUREs 12A-12B



CANDIDA TROPICALIS 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.

[0007] 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. 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. Therefore, there is a need of an specific diagnosis of *Candida tropicalis*, since current methods cannot differentiate between other *Candida* species, with certain rate of cross-reacting (HSEIN CHANG CHANG, et al, JOURNAL OF CLINICAL MICROBIOLOGY, October 2001, p. 3466-3471; which discloses that ITS PCR detection of *C. tropicalis* produces exactly the same size of amplicon than *C. albicans*, and also is difficult to differentiate it before *C. parapsilosis*, thus further assays should be carried out).

[0008] It has been reported that several *Candida* species have chromosome rearrangements 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.

[0009] In light of the above, the present invention discloses an in vitro method for detecting and identifying *Candida tropicalis*, with at least one specific oligonucleotide, but also with an in-block multiplex set of specific oligonucleotides, which allows identification of *Candida tropicalis* in clinical samples of different population subgroups.

SUMMARY OF THE INVENTION

[0010] The present invention claims and discloses oligonucleotides for the specific identification of *Candida tropicalis*, consisting of a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 12 or a complement thereof.

[0011] In a further embodiment, it is further disclosed an in vitro method for the specific identification of *C. tropicalis*, 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 an specific embodiment the amplification of DNA fragments is carried out with at least one pair of oligonucleotides or at least two pair of oligonucleotides.

[0012] In an additional embodiment, a kit for the specific identification of *Candida tropicalis*, comprising at least one oligonucleotide as above mentioned is also disclosed; wherein in an specific embodiment, said kit comprises at least one oligonucleotide pair or at least two pair of oligonucleotides.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] 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 ⅕ 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. guillermondii; Lane* 18 shows *C. krusei* 1; Lane 19 shows *C. krusei* 2 and Lane 20 shows the: molecular weight marker.

[0014] FIGS. **2**A-**2**B. show a 2% agarose gels showing temperature gradient for *C. tropicalis* detection (Ct18- clinical isolated strain) using the oligonucleotides pair Ct1. The unspecific strip for the positive control disappears as the oligonucleotides annealing temperature increases, for this oligonucleotides pair, the optimal temperature selected is 64.4° 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. tropicalis* has a length of 174 bp.

[0015] In FIG. **2**A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-4 show Annealing temperature 62° C.: Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative control with-

out DNA; Lane 4 shows *C. dubliniensis*. Lanes 5-7 show annealing temperature 62.6° C.: Lane 7 shows the positive control *C. tropicalis*; Lane 8 shows the negative control without DNA; Lane 9 shows *C. dubliniensis*. Lanes 8-10 show annealing temperature 63.4° C.: Lane 8 shows the positive control *C. tropicalis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. dubliniensis*. Lanes 11-13 show annealing temperature 64.4° C.: Lane 11 shows the positive control *C. tropicalis*; Lane 12 shows the negative control without DNA; Lane 13 shows *C. dubliniensis*. Lanes 14-16 show annealing temperature 65.8° C.: Lane 14 shows the positive control *C. tropicalis*; Lane 15 shows the: negative control without DNA; Lane 16 shows *C. dubliniensis*. Lanes 17-19 show annealing temperature 66.9° C.: Lane 17 shows the positive control *C. tropicalis*; Lane 18 shows the negative control without DNA; Lane 19 shows *C. dubliniensis*. Lane 20 shows the molecular weight marker.

[0016] In FIG. **2**B Lanes 1-3 show annealing temperature 67.6° C.: Lane 1 shows the positive control *C. tropicalis*; Lane 2 shows the negative control without DNA; Lane 3 shows *C. dubliniensis*. Lanes 4-6 show annealing temperature 68° C.: Lane 4 shows the positive control *C. tropicalis*; Lane 5 shows the negative control without DNA; Lane 6 shows *C. dubliniensis*. Lane 7 shows the molecular weight marker.

[0017] FIGS. **3**A-**3**B shows a 2% agarose gel showing temperature gradient for *C. tropicalis* detection (Ct18 clinical isolated strain) using the oligonucleotides pair Ct3. For this oligonucleotides pair, the optimal temperature selected is 60.1° C. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls.

[0018] In FIG. **3**A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lanes 2-4 show Annealing temperature 55° C.: Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. dubliniensis*. Lanes 5-7 show annealing temperature 56.2° C.: Lane 7 shows the positive control *C. tropicalis*; Lane 8 shows the negative control without DNA; Lane 9 shows *C. dubliniensis*. Lanes 8-10 show annealing temperature 58° C.: Lane 8 shows the positive control *C. tropicalis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. dubliniensis*. Lanes 11-13 show annealing temperature 60.1° C.: Lane 11 shows the positive control *C. tropicalis*; Lane 12 shows the negative control without DNA; Lane 13 shows *C. dubliniensis*. Lanes 14-16 show annealing temperature 63.1° C.: Lane 14 shows the positive control *C. tropicalis*; Lane 15 shows the negative control without DNA; Lane 16 shows *C. dubliniensis*. Lanes 17-19 show annealing temperature 65.5° C.: Lane 17 shows the positive control *C. tropicalis*; Lane 18 shows the negative control without DNA; Lane 19 shows *C. dubliniensis*. Lane 20 shows the molecular weight marker.

[0019] In FIG. **3** B Lanes 1-3 show annealing temperature 67.1° C.: Lane 1 shows the positive control *C. tropicalis*; Lane 2 shows the negative control without DNA; Lane 3 shows *C. dubliniensis*. Lanes 4-6 show annealing temperature 68° C.: Lane 4 shows the positive control *C. tropicalis*. Lane 5 shows the negative control without DNA; Lane 6 shows *C. dubliniensis*. Lane 7 shows the molecular weight marker.

[0020] FIG. **4** shows a 2% agarose gel showing temperature gradient for *C. tropicalis* detection (Ct18- clinical isolated strain) using the oligonucleotides pair Ct5. The unspecific strip for *C. dubliniensis* disappears as the oligonucleotides alignment temperature increases, for this oligonucleotides pair, the optimal temperature selected is 65.5° C. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls. In FIG. **4**, Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene). Lanes 2-4 show annealing temperature 55° C. 2: positive control *C. tropicalis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. dubliniensis*. Lanes 5-7, show annealing temperature 56.2° C. Lane 5 shows the positive control *C. tropicalis*; Lane 6 shows the negative control without DNA; Lane 7 shows *C. dubliniensis*. Lanes 8-10, show annealing temperature 58° C. Lane 8 shows the positive control *C. tropicalis*; Lane 9 shows the negative control without DNA; Lane 10 shows *C. dubliniensis*. Lane 11 shows the molecular weight marker. Lanes 12-14 show annealing temperature 60.1° C. Lane 12 shows the positive control *C. tropicalis*; Lane 13 shows negative control without DNA; Lane 14 shows *C. dubliniensis*. Lanes 15-17 show annealing temperature 63.1° C. Lane 15 shows the positive control *C. tropicalis*; Lane 16 show the negative control without DNA; Lane 17 shows *C. dubliniensis*. Lanes 18-20 show annealing temperature 65.5° C. Lane 18 shows the positive control *C. tropicalis*; Lane 19 shows the negative control without DNA; Lane 20 shows *C. dubliniensis*. Lanes 21-23 show annealing temperature 67.2° C. Lane 21 shows the positive control *C. albicans*; Lane 22 shows the negative control without DNA; Lane 23 shows *C. dubliniensis*. Lanes 24-26 show annealing temperature 68° C. Lane 24 shows the positive control *C. albicans*; Lane 25 shows the negative control without DNA; Lane 26 shows *C. dubliniensis*. Lane 27 shows the molecular weight marker.

[0021] FIG. **5** A-H. show a 2% agarose gel showing oligonucleotide concentration analysis for *C. tropicalis* detection (clinical Ct18- isolated) using the oligonucleotides pair Ct1. For this oligonucleotides pair, the optimal concentration selected is 100 nM. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls.

[0022] FIG. **5** A shows the oligonucleotides pair having a concentration of 100 nM;

[0023] FIG. **5**B shows the oligonucleotides pair having a concentration of 200 nM;

[0024] FIG. **5**C shows the oligonucleotides pair having a concentration of 400 nM;

[0025] FIG. **5**D shows the oligonucleotides pair having a concentration of 500 nM;

[0026] FIG. **5**E shows the oligonucleotides pair having a concentration of 600 nM;

[0027] FIG. **5**F shows the oligonucleotides pair having a concentration of 800 nM;

[0028] FIG. **5**G shows the oligonucleotides pair having a concentration of 1000 nM;

[0029] FIG. **5**H shows the oligonucleotides pair having a concentration of 1200 nM. For each gel, the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. parapsilosis*; Lane 7 shows *C. dubliniensis*; Lane 8 shows *C. bracarensis*; Lane 9 shows *C. guillermondii*; Lane 10 shows *C. krusei.*

[0030] FIGS. **6**A-**6**H show a 2% agarose gel showing oligonucleotide concentration analysis for *C. tropicalis* detection (clinical isolate Ct18-) using the oligonucleotides pair Ct3. For this oligonucleotides pair, the optimal concentration

selected is 100 nM. For electrophoresis, the samples were run at a concentration 4 times higher than the one used for the controls.

[0031] FIG. **6** A shows the oligonucleotides pair having a concentration of 100 nM;

[0032] FIG. **6**B shows the oligonucleotides pair having a concentration of 200 nM;

[0033] FIG. **6**C shows the oligonucleotides pair having a concentration of 400 nM;

[0034] FIG. **6**D shows the oligonucleotides pair having a concentration of 500 nM;

[0035] FIG. **6**E shows the oligonucleotides pair having a concentration of 600 nM;

[0036] FIG. **6**F shows the oligonucleotides pair having a concentration of 800 nM;

[0037] FIG. **6**G shows the oligonucleotides pair having a concentration of 1000 nM;

[0038] FIG. **6**H shows the oligonucleotides pair having a concentration of 1200 nM. For each gel, the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. parapsilosis*; Lane 7 shows *C. dubliniensis*; Lane 8 shows *C. bracarensis*; Lane 9 shows *C. guillermondii*; Lane 10 shows *C. krusei.*

[0039] FIGS. **7**A-**7**H show a 2% agarose gel showing oligonucleotide concentration analysis for *C. tropicalis* detection (clinical isolate Ct18) using the oligonucleotides pair Ct5. For this oligonucleotides pair, the optimal concentration selected is 200 nM.

[0040] FIG. **6** A shows the oligonucleotides pair having a concentration of 100 nM;

[0041] FIG. **6**B shows the oligonucleotides pair having a concentration of 200 nM;

[0042] FIG. **6**C shows the oligonucleotides pair having a concentration of 400 nM;

[0043] FIG. **6**D shows the oligonucleotides pair having a concentration of 500 nM;

[0044] FIG. **6**E shows the oligonucleotides pair having a concentration of 600 nM;

[0045] FIG. **6**F shows the oligonucleotides pair having a concentration of 800 nM;

[0046] FIG. **6**G shows the oligonucleotides pair having a concentration of 1000 nM;

[0047] FIG. **6**H shows the oligonucleotides pair having a concentration of 1200 nM. For each gel, the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative control without DNA; Lane 4 shows *C. glabrata*; Lane 5 shows *C. albicans*; Lane 6 shows *C. parapsilosis*; Lane 7 shows *C. dubliniensis*; Lane 8 shows *C. bracarensis*; Lane 9 shows *C. guillermondii*; Lane 10 shows *C. krusei.*

[0048] FIGS. **8**A-**8**C. show a 2% agarose gels showing the analysis of the 36 clinical isolated samples for *C. tropicalis* detection (Ct18 clinical isolate sample) using the oligonucleotides pair Ct1. There were 12 samples detected as positive; the isolated sample AN8 wasn't positive for Ct1 neither for Ct5, but it was positive with Ct3. In all FIGS. **8** A-**8**C, lane 1 and 16 show the molecular weight marker (1 Kb DNA Ladder Invitrogene); lane 2 shows the positive control *C. tropicalis*; lane 3 shows the negative controls, without DNA. Remaining lanes 4 to 15 show clinical samples.

[0049] FIGS. **9**A-**9**B show a 2% agarose gel showing the analysis of the 36 clinical isolated samples for *C. tropicalis* detection (Ct18 clinical isolate sample) using the oligonucleotides pair Ct3. There were 13 samples detected as positive; the isolated sample AN8 (lane 11) was not positive for Ct1 neither for Ct5, but it was positive with Ct3.

[0050] In FIG. **9** A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative controls, without DNA. Remaining lanes 4 to 20 show clinical samples.

[0051] In FIG. **9**B: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Remaining lanes 2 to 20 show clinical samples.

[0052] FIGS. **10**A-**10**B show a 2% agarose gel showing the analysis of the 36 clinical isolated samples for *C. tropicalis* detection (Ct18 clinical isolated sample) using the oligonucleotides pair Ct5. There were 12 samples detected as positive; the isolated sample AN8 (lane 11) wasn't positive for Ct1 neither for Ct5, but it was positive with Ct3.

[0053] In FIG. **10** A: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Lane 2 shows the positive control *C. tropicalis*; Lane 3 shows the negative controls, without DNA. Remaining lanes 4 to 20 show clinical samples.

[0054] In FIG. **10** B: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene); Remaining lanes 2 to 20 show clinical samples.

[0055] FIG. **11** shows a 2% agarose gel showing a multiplex test for *C. tropicalis*. Ct1, Ct3 and Ct5 oligonucleotide pairs were tested in several conditions. Predicted amplification sizes 217, 224 and 254 base pairs were detected in samples containing only *C. tropicalis*. Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene). 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. parapsilosis, C. dubliniensis, S. cerevisiae,* 100 ng each. Lane 4 shows the molecular weight marker. Lane 5 shows *C. tropicalis*. Lane 6 shows the negative control without DNA. Lane 7 shows *C. tropicalis*. Lane 8 shows *C. albicans*. Lane 9 shows *C. glabrata*. Lane 10 shows *C. parapsilosis*. Lane 11 shows *C. dubliniensis.*

[0056] FIGS. **12** A-B show a 2% agarose gel showing specificity test. FIG. **12**A A with Ct1 and FIG. **12** B with Ct5 oligonucleotide pairs. For both figures the lane order is: Lane 1 shows the molecular weight marker (1 Kb DNA Ladder Invitrogene). Lane 2 shows the positive control *C. tropicalis*. Lane 3 shows the negative control without DNA. Lane 4 shows *C. tropicalis* 100 ng plus 50 ng *C. albicans, C. parapsilosis, C. glabrata, C. dubliniensis, C. bracarensis, C. guilliermondii, C. krusei, C. metapsilosis, C. orthopsilosis, S. cerevisiae* each. Lane 5 shows *C. tropicalis* 10 ng plus 50 ng *C. albicans, C. parapsilosis, C. glabrata, C. dubliniensis, C. bracarensis, C. guilliermondii, C. krusei, C. metapsilosis, C. orthopsilosis, S. cerevisiae* each. Lane 6 shows *C. tropicalis* 1 ng plus 50 ng *C. albicans, C. parapsilosis, 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. parapsilosis, C. glabrata, C. dubliniensis, C. bracarensis, C. guilliermondii, C. krusei, C. metapsilosis, C. orthopsilosis, S. cerevisiae* each.

DETAILED DESCRIPTION OF THE INVENTION

[0057] The present invention discloses an in vitro method for detecting and identifying *Candida tropicalis*, with at least one set of specific oligonucleotides, but also with an in-block multiplex set of specific oligonucleotides, which allows identification of *Candida tropicalis* in clinical samples of different population subgroups with 100% of specificity and sensitivity.

[0058] Several oligonucleotides have been designed in order to specifically detect different chromosomal sites of *Candida tropicalis*. 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 tropicalis* 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. Said oligonucleotides are part of a composition which further comprises a suitable acceptable carrier.

[0059] "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.

[0060] 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.

[0061] "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.

[0062] The present invention discloses several oligonucleotides for the specific identification of *C. tropicalis*, 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. tropicalis*, 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.

[0063] Also, the oligonucleotides for the specific identification of *Candida tropicalis*, consist of a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 22 or complements thereof.

[0064] Said oligonucleotides are sufficiently complementary to the target sequences of *C. tropicalis*. 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.

[0065] This invention also discloses an in vitro method for the specific identification of *C. tropicalis*, 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 22 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.

[0066] 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.

[0067] Furthermore, a kit for the specific identification of *Candida tropicalis*, 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 tropicalis* such as those disclosed on SEQ ID NOS: 1 to 22 or complements thereof. In the multiplex embodiment, the kit comprises at least one oligonucleotide pair or more preferably, at least two oligonucleotide pairs.

[0068] The use of said oligonucleotides specifically designed for the specific identification of *Candida tropicalis*, is disclosed as well.

[0069] As an additional embodiment, the present invention discloses at least one probe useful for the specific identification of *Candida tropicalis*. 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.

[0070] 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:

[0071] Traditional method of identification of *Candida* in urine, urine samples are analyzed in an automated urine analyzer coupled Urisys type UF-IOOi. The analysis was performed by flow cytometry with an argon laser. The UF-IOOi 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 Iμl 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. 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. tropicalis*, is 6 days, with a sensitivity of about 85%.

[0072] In the method for identifying *C. tropicalis*, of the present invention, the urine samples are analyzed in an automated urine analyzer coupled Urisys type OF-I00i. The analysis was performed by flow cytometry with an argon laser.

[0073] The UF-I00i 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. tropicalis*, together as an in-block multiplex or separately. The total test time is 6 hours.

[0074] 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_2. The release of CO_2 is detected by the computer and automatically marked as blood cultures positive for yeast. 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. tropicalis*. The total time for identification is a minimum of 9 days.

[0075] Method to detect *C. tropicalis*, 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_2. The release of CO_2 is detected by the computer and automatically marked as 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. The genomic DNA obtained from PCR annealing is used where any of the oligonucleotides generated from SEQ ID Nos. 1 to 22, in optimal reaction conditions. The PCR products were separated by agarose gel electrophoresis and the products are analyzed for the correct identification of *C. tropicalis*. The total test time is 3 days.

[0076] 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.

[0077] 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.

[0078] 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

[0079] *Candida tropicalis* 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 *Candida tropicalis*.

Oligo-nucleotide pair No.	Seq ID. No.	Forward (Fw) or Reverse (Rv)	Bp number	5' a 3' Sequence	Amplicon lenght (bp)	Contig Name
Ct1	Seq. ID. No. 1	Fw	22	CTG TCA TGG TTT ATG TTC CAC C	217	XM_002546113.1
	Seq. ID. No. 2	Rv	20	GAA TCA GTA CCA CCT GGC TC		

TABLE 1 -continued

Examples of oligonucleotides for the identification of *Candida tropicalis*.

Oligo-nucleotide pair No.	Seq ID. No.	Forward (Fw) or Reverse (Rv)	Bp number	5' a 3' Sequence	Amplicon lenght (bp)	Contig Name
Ct2	Seq. ID. No. 3	Fw	18	CCC AAG AAT GGA CAA GAG	211	XM_00254231
	Seq. ID. No. 4	Rv	18	CTT CAG CAA GTA AGC CAG		
Ct3	Seq. ID. No. 5	Fw	18	CAC TGT GAC GAC CAT AGA	224	RG_06258
	Seq. ID. No. 6	Rv	18	GCG CCA TAT ATC TGT GTG		
Ct4	Seq. ID. No. 7	Fw	18	CGT ATT TCG TGT CGC ATC	310	RG_06258
	Seq. ID. No. 8	Rv	18	CTT TGC TGT GTT TGG CAG		
Ct5	Seq. ID. No. 9	Fw	18	CAT GTG TAC ACA TGC GAC	254	RG_06258
	Seq. ID. No. 10	Rv	18	CTT TGC TGT GTT TGG CAG		
Ct6	Seq. ID. No. 11	Fw	18	CAA CCA TGT CGC TGT TAC	279	RG_06258
	Seq. ID. No. 12	Rv	18	CTT TGC TGT GTT TGG CAG		
Ct7	Seq. ID. No. 13	Fw	18	CAG TTG CAC TCT GTT TGG	178	XM_0025455188.1
	Seq. ID. No. 14	Rv	18	GTT CCC AAA CTT ACA CCG		
Ct8	Seq. ID. No. 15	Fw	18	CTC ACT TCG TTA TGG AGC	359	XM_0025455188.1
	Seq. ID. No. 16	Rv	20	CAC CTT TGA TAG GTC TCT CG		
Ct9	Seq. ID No. 17	Fw	18	CTC ACT TCG TTA TGG AGC	153	XM_0025455188.1
	Seq. ID. No. 18	Rv	18	GTT GTC CAA CTG CTC AAG		
Ct10	Seq. ID. No. 19	Fw	18	CTC ACT TCG TTA TGG AGC	601	XM_0025455188.1
	Seq. ID. No. 20	Rv	18	GAT TGG CAC ACC ATA ACG		
Ct11	Seq. ID. No. 21	Fw	18	CTC ACT TCG TTA TGG AGC	662	XM_0025455188.1
	Seq. ID. No. 22	Rv	18	CCA CCG GTA CCA AAT ACA		

[0080] The locations of the corresponding contigs are accordance with GenBank database (http://www.ncbi.nlm.nih.gov).

[0081] Said oligonucleotide pairs were tested for optimizing the amplification conditions. Thus, oligonucleotide pairs Ct1 to Ct 11 have annealing temperatures from about 54° C. to 61° C. These oligonucleotide pairs were tested on genomic DNA for amplification testing carrying out PCR reactions. The oligonucleotides are contained in a composition further comprising a suitable acceptable carrier, such as, but not limited, water, buffer, etc. 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 10 X	1X	3.0 μL
$MgCl_2$ 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

[0082] 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

[0083] 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.

[0084] During electrophoresis, the samples belonging to other *Candida* species different to *Candida tropicalis*, 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

[0085] 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.

[0086] 3 oligonucleotide pairs are shown in order to reflect the sensitivity and selectivity of the 22 oligonucleotides and probes for identifying *C. tropicalis*. These examples are illustrative but not limitative for the scope of the invention.

[0087] Optimal PCR Reaction Conditions:

[0088] Firstly, annealing conditions were tested with a temperature threshold. Results are shown in Table 4.

[0089] 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		Temperature Threshold (° C.)								Best selected
No.	pair	Min							Max	Temperature (° C.)
1	Ct1	62	62.6	63.4	64.4	65.8	66.9	67.6	68	64.4
2	Ct3	55	56.2	58	60.1	63.1	65.5	67.1	68	60.1
3	Ct5	55	56.2	58	60.1	63.1	65.5	67.1	68	65.5

[0090] FIGS. **2** to **4** 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.

[0091] Oligonucleotide Concentration

[0092] Once the optimal annealing temperature has been selected for each oligonucleotide pair, the optimal oligonucleotide concentration was determined for PCR reactions.

[0093] The concentrations tested were: 100 nM, 200 nM, 400 nM, 500 nM, 600 nM, 800 nM, 1000 nM y 1200 nM.

[0094] The minimal oligonucleotide concentration wherein a clear band was detected in the positive control, was selected. Table 5 shows the best concentrations. FIGS. **5** to **7** 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 *C. tropicalis.*

No	Oligonucleotide pair	Best selected concentration
1	Ct1	100 nM
2	Ct3	100 nM
3	Ct5	200 nM

[0095] 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. tropicalis*, genomic DNA can be detected in an amount of at least 0.5 ng.

Example 3

Candida Detection on Isolated Clinical Samples

[0096] The above exemplified oligonucleotide pairs were tested to detect *Candida tropicalis* on clinical isolated samples from hospitalized patients.

[0097] FIGS. **8** to **10** show the results of said tests. All the oligonucleotide pairs detect only the specific *Candida* specie for which they were designed. In most of the cases all the oligonucleotide pairs detect the same positive samples with one exception: Ct3 pair from *C. tropicalis* detect an additional sample (lane 10) than the other 2 pairs for the same specie (Ct1 and Ct5). All the agarose gels are at a concentration of 2% and were run at 110-130 V.

[0098] Comparing PCR results with Vitek identification methods reveals that PCR test has a sensibility of 98% and a specificity of 100% in contrast to VITEK tests with an 85% and 33% respectively. Vitek method could not identify one *C. tropicalis* clinical sample (the result was *C. albicans*), however the PCR was positive. To confirm the result, said clinical sample was reanalyzed by API ID32 C (BioMerieux®), which reconfirmed that said sample was effectively *C. tropicalis*. Vitek test identified three clinical isolates as *C. tropicalis*, however, by PCR tests of the invention identified them as *C. parapsilosis* and two *C. albicans*. These results were also confirmed by API ID32C test.

Example 4

Multiplex Assay

[0099] Since it is possible to have rearrangements within the genome of *C. tropicalis*, as shown with clinical sample 7 (see FIG. **10**A lane 10) 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.

[0100] FIG. **11** shows the use of oligonucleotides pairs Ct1, Ct3 and Ct5 simultaneously in samples containing *C. tropicalis* alone or in mixture with *C. glabrata, C. albicans, C. parapsilosis, 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. tropicalis*, and not in the control lanes (lane 3, 6, 8 to 11). Therefore, a multiplex kit for detecting *C. tropicalis* has been designed with a 100% of sensibility and specificity.

Example 5

Specificity Assay

[0101] FIGS. **12** A and **12**B show that the oligonucleotides tested are specific for *C. tropicalis* and do not cross-link with other microbial species. For example, *C. tropicalis* mixed with another 10 microbial species such as *C. albicans, C. parapsilosis, C. glabrata, C. dubliniensis, C. bracarensis, C. guilliermondii, C. krusei, C. metapsilosis, C. orthopsilosis, S. cerevisiae* (50 ng each for a total of 500 ng). *C. tropicalis* 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 (217 bp for Ct1, 224 bp for Ct3 and 254 for Ct5) and its resequencing test. Negative control without *C. tropicalis* DNA did not show any amplification band. This confirms that the assay is 100% specific for *C. tropicalis.*

[0102] Finally, from the totality of clinical samples tested, 12 were classified as *C. tropicalis* with a 100% sensitivity and specificity, compared with Vitek tests.

SEQUENCE LISTING

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<400> SEQUENCE: 1

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<400> SEQUENCE: 2

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gaatcagtac cacctggctc 20

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What is claimed is:

1. An oligonucleotide for a specific identification of *Candida tropicalis* comprising a nucleic acid having at least 90% sequence homology to one of SEQ ID NOS: 1 to 22 or a complement thereof.

2. An in vitro method for a specific identification of *C. tropicalis* 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 a specific identification of *Candida tropicalis*, 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**.

* * * * *