SPOOR & FISHER

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FULL NAMES OF APPLICANT

71	UNIVERSITY OF THE FREE STATE

FULL NAMES OF INVENTORS

72	SEBOLAI, OLIHILE MOSES OGUNDEJI, ADEPEMI OLAWUNMI ALBERTYN, JACOBUS POHL, CAROLINA HENRIETTA KOCK, JOHAN LODEWYK FRANSCISCUS

TITLE OF INVENTION

54	METHOD FOR THE IDENTIFICATION OF CLINICAL CRYPTOCOCCUS NEOFORMANS AND CRYPTOCOCCUS GATTI ISOLATES

METHOD FOR THE IDENTIFICATION OF CLINICAL CRYPTOCOCCUS NEOFORMANS AND CRYPTOCOCCUS GATTI ISOLATES

BACKGROUND OF THE INVENTION

The present invention relates to a method of detecting the presence or absence, and if present the identity of a *Cryptococcus* pathogen in a biological sample from a subject. The invention also provides for a kit for the detection and identification of a *Cryptococcus pathogen* in a biological sample.

Cryptococcosis is a life-threatening fungal disease that may present in either immunocompetent or immunocompromised persons (Day, 2004). Persons with an impaired cell-mediated immunity are more vulnerable to the disease. The advent of HIV has led to an increase in the number of cryptococcosis cases, and in Sub Saharan Africa, it is estimated that

cryptococcosis annually accounts for over 500 000 deaths of HIV-infected individuals (Park *et al.,* 2009).

Although cryptococcosis can be managed, its clinical diagnosis may often be inaccurate. A diagnostic error could leave survivors with neurological problems or have fatal consequences (Saha *et al.,* 2008). Moreover, inaccurate diagnosis undermines efforts to understand the extent and scope of this infectious disease, and by necessary implication, could have a negative impact on plans and management strategies by health authorities.

Over the years, Indian ink preparations have been used to diagnose cryptococcosis (Saha *et al.,* 2008, 2009). However, Indian ink has poor sensitivity, and there have been reported cases of acapsular strains inducing pathogenesis (del Poeta, 2004). Although serological tests tend to be more specific, they are not always consistent in diagnosing cryptococcosis due to the generation of false positive or negative results (Saha *et al.,* 2009). More importantly, traditional serological techniques cannot resolve the identity of the responsible etiological agent. This information is crucial in understanding the epidemiology, pathogenesis, clinical presentation and drug resistance of clinical isolates (Ito-Kuwa *et al.,* 2007). In addition, the identity of the etiological agent can also provide information relating to the agent's oxylipin production pattern and, possibly, associated pathogenesis (Sebolai *et al.,* 2007, 2012).

The availability of an extensive molecular database has allowed for easy identification and referencing of yeasts (Scozetti *et al.,* 2002). Traditionally, *Cryptococcus* (*Cr.*) *neoformans* has been classified into three varieties viz. *Cr. neoformans* var. *neoformans* (serotype D), *Cr. neoformans* var. *grubii* (serotype A), *Cr. neoformans* var. *gattii* (serotype B and C) as well as a hybrid (AD) (Chen *et al.,* 2010). Recently, it has been proposed that the species complex should be reclassified into two distinct species viz. *Cr. neoformans* (serotypes A, AD and D) and *Cr. gattii* (serotype B and C) (Kwon Chung & Varma, 2006). Based on this proposal, the inventors

sought to examine the usefulness of sequencing the internal transcribed spacers (ITS) including the 5.8S gene, as a reliable method for the identification of etiological agents from clinical strains obtained from patients with cryptococcosis. Importantly, the inventors point out that the uncovering of nucleotide polymorphism in the ITS region, implies strain identification can be achieved without the need to sequence amplicons. In resource-limited settings, a simple molecular method would have a clear competitive advantage over more elegant typing techniques that are too expensive and laborious for routine use (Sidrum *et al.,* 2010). We also sought to confirm cases of *Cr. gattii*, found to be less prevalent in temperate regions such as South Africa, by cultivation on carvanine-glycine-bromothymol blue media.

SUMMARY OF THE INVENTION

The present invention provides a rapid diagnostic method for the detection of cryptococcosis etiological agents in a biological sample in a patient who has been diagnosed with cryptococcosis and a kit for performing the method of the invention.

More specifically, the invention relates to using a PCR based screening method to detect the presence or absence of a *Cryptococcus* pathogen in the sample. If a *Cryptococcus* pathogen is present in the sample, the PCR product may be digested with a restriction endonuclease in order to detect a polymorphism which results in the formation of a unique signature fragmentation pattern. The nucleotide polymorphism or restriction site is only present in the distinct species *Cr. neoformans* and absent in the distinct but closely related species *Cr. gattii.* Even more specifically, this invention relates to a method for detection of the specific variety of the *Cryptococcus* pathogen in a sample i.e. *Cr. neoformans* var. *grubii* or *Cr. neoformans* var. *neoformans*, by screening for the presence of the polymorphism.

According to a first embodiment of the invention, there is provided a method of detecting the presence or absence of a *Cryptococcus* pathogen

in a biological sample. The method comprising the steps of screening the biological sample using a PCR based method in order to determine the presence or absence of the *Cryptococcus* pathogen in the sample. If the *Cryptococcus* pathogen is present in the sample, the method further comprises a step of digesting the PCR product with a restriction endonuclease, wherein digestion of the PCR product is indicative of the identity of the variety or of the identity of the species of *Cryptococcus* in the sample.

In one embodiment of the invention the *Cryptococcus* varieties are selected from *Cryptococcus neoformans* var. *neoformans* (serotype D), *Cryptococcus neoformans* var. *grubii* (serotype A), a hybrid of the former varieties (serotype AD) or *Cryptococcus neoformans* var. *gattii* (serotype B or C).

In an alternative embodiment of the invention the *Cryptococcus* varieties are classified into two distinct species *Cryptococcus neoformans* (serotypes A, D or AD) and *Cryptococcus gattii* (sertotype B or C).

The restriction endonuclease used in the method of the invention is *Ssp*I. As a result of a polymorphism present in the *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *grubii* varieties, digestion with *Ssp*I results in the PCR product being cleaved into two fragments. Alternatively, the polymorphism is present in the *Cryptococcus neoformans* species.

Screening according to the method of the invention is carried out using PCR to amplify a portion of an internal transcribed spacer (ITS) including the 5.8S gene. PCR primers used in the method of the invention may have the sequences set out in SEQ ID NO: 1 (5'- TCC TCC GCT TAT TGA TAT GC -3') and SEQ ID NO: 2 (5'- GGA AGT AAA AGT CGT AAC AAG G -3') or a sequence which has 95% identity to either of these the sequences and is capable of amplifying a fragment of an ITS including the 5.8S gene.

The biological sample may be a blood sample, a cerebrospinal fluid sample, a positive culture sample, or a lung biopsy sample. Preferably, the sample is a positive culture sample or a cerebrospinal fluid samples.

In particular, the method may include the steps of:

(a) extracting total DNA from a biological sample of a subject;
(b) amplifying a portion of the ITS and 5.8S gene to generate a PCR product; and
(c) determining the presence or absence of the PCR product,

wherein the presence of a PCR amplification product is indicative of the presence of *Cryptococcus* infection in the subject.

The method may further include a step of:

(d) digesting the PCR product with an *Ssp*I restriction endonuclease

wherein digestion of the PCR product to form 2 fragments is indicative of the presence of *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *grubii* varieties, alternatively indicative of *Cryptococcus neoformans* in the biological sample. Further, wherein non-digestion of the PCR products is indicative of a *Cryptococcus neoformans* var. *gatti* variety, alternatively it is indicative of the *Cryptococcus gatti* species in the biological sample.

According to a further embodiment of the invention, there is provided a kit for detecting the presence of a *Cryptococcus* infection in a biological sample obtained from a subject, the kit comprising:

(a) primers for amplification of a portion of ITS and the 5.8S gene;

(b) a *Ssp*I restriction endonuclease; and

(c) a detection means for detecting the amplification and/or restriction enzyme digestion products.

The kit may additionally comprise instructions for performing the method of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Non-limiting embodiments of the invention will now be described by way of example only and with reference to the following figures:

Figure 1: Electropherogram showing PCR products. Desired DNA fragments of between 600 and 700 bases were obtained for all strains tested depicting the approximate size of the ITS region. M = DNA ladder, O'GeneRuler™. DNA Ladder = 1 kb).

Figure 2: Multiple sequence alignment (MSA). A MSA was performed on DNA sequences obtained from all 70 strains studied. Part of the alignment is shown here. The variable bases, highlighted in grey colour-coded boxes, revealed the intra-specific variation that allowed for the delineation of the studied strains into the three traditional varieties of *Cryptococcus neoformans.* These variable bases were also consistent in their delineation when applied against a number of sequences limited to *Cr. neoformans* species complex from the GeneBank. Spaces (-) were introduced in order to improve alignment. The MSA was generated with ClustalX.

Figure 3 Multiple sequence alignment (MSA). A MSA was performed on DNA sequences obtained from all 70 strains studied. Part of the alignment is shown here. A closer examination of the sequence data limited to the distinct species, *Cryptococcus* (*Cr.*) *neoformans* (as constituted by *Cr. neoformans* var. *grubii* and *neoformans*) revealed a restriction site that is absent in *Cr. gattii* (i.e. *Cr. neoformans* var. *gattii*). The restriction site was present when also aligning sequences limited to the distinct species, *Cr. neoformans* from the GeneBank for referencing.

Spaces (-) were introduced in order to improve alignment. The MSA was generated with ClustalX.

Figure 4: A pictogram showing growth results of tested clinical isolates on carnavanine-glycine-bromothymol blue agar after five days of cultivation at 30 °C. Isolates were differentiated according to media colouration after 5 days. All *Cr. neoformans* isolates (varietal forms: *neoformans* and *grubii*) produced no colour change (symbolises a negative reaction) while all *Cr. gattii* isolates (varietal form: *gattii*) produced a blue media colouration (symbolises a positive reaction).

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the invention are shown.

The invention as described should not to be limited to the specific embodiments disclosed and modifications and other embodiments are intended to be included within the scope of the invention. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

As used throughout this specification and in the claims which follow, the singular forms "a", "an" and "the" include the plural form, unless the context clearly indicates otherwise.

The terminology and phraseology used herein is for the purpose of description and should not be regarded as limiting. The use of the terms "comprising", "containing", "having" and "including" and variations thereof used herein, are meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

In the present invention clinical isolates of *Cryptococcus* varieties were identified using a PCR based method as a tool for the early diagnosis of cryptococcosis. The PCR based method of the invention will assist in

eliminating misdiagnosis of cryptococcosis and will thus contribute to efforts of fully understanding the extent and scope of this infectious disease. Compared to less sensitive conventional methods, PCR is an excellent tool as it can detect low fungal loads and can be used on small sample sizes. In clinical practise, it is often difficult to obtain large sample sizes particularly from paediatric patients (Jarvis *et al.,* 2011).

The present invention further provides for a method of delineating the tested strains into three varieties viz. *Cr. neoformans* var. *neoformans*, *Cr. neoformans* var. *grubii* and *Cr. neoformans* var. *gattii.* through the use of sequence variation within the ITS region.

The invention also provides for a rapid diagnostic method for identifying *Cr. neoformans* as a result of a unique restriction site in the ITS region of this variety.

The use of the methods of the present invention provides a rapid diagnostic method for the routine diagnosis of cryptococcosis in resource-limited settings. This is as a result of the method being simple to use as compared to: (1) more elegant typing methods, and (2) performing sequencing analysis. It is also possible to confirm cases of *Cr. gattii* using CGB media. Nonetheless, CGB is not ideal as a point-of-care test due to the long waiting period before reading the results. Nonetheless, it is a reliable method for confirmatory purposes.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Clinical Strains

Seventy unidentified cryptococcosis positive cultures were examined in this study. These cultures (originating as pure individual strains) were obtained from Universitas Academic Hospital, Bloemfontein,

South Africa. These strains were maintained on yeast-malt-extract (YM) agar (3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, 5 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ glucose, 16 $g.L^{-1}$ agar; Merck, South Africa).

DNA Extraction

DNA was extracted using a thermal shock method. In brief, 18 h cells, sub-cultured on YM agar at 30 °C, were suspended in 25 µL of triple distilled water and lysed with a pipette tip followed by boiling for 10 min at 96 °C.

Sequencing

DNA samples were amplified by PCR in a reaction mixture (50 µL) containing 1.5 mM magnesium chloride, 200 µM deoxynucleoside triphosphates, 100 pmoL of each primer, 0.5 U Taq DNA polymerase (New England BioLabs®). Amplification was performed in a thermal cycler (Applied Biosystems 2720) using the following steps: 94 °C for 2 min followed by 30 cycles of 30 sec denaturation at 94 °C and 30 sec annealing at 52 °C with 1 min extension followed by final extension for 5 min at 72 °C. The primer sequences used for amplification were ITS4 (SEQ ID NO:1 - 5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS5 (SEQ ID NO:2 - 5'- GGA AGT AAA AGT CGT AAC AAG G -3') (White *et al.,* 1990). The PCR products were visualised on a 0.8 % agarose gel. PCR products were purified and concentrated using the DNA Clean and Concentrator™ kit (Zymo Research) and the sequencing reaction was performed with the ABI Prism™ Big Dye terminator™ V3.1 cycle sequencing ready reaction kit (Applied Biosystems®) and data collected on an ABI 3130XL genetic analyzer (Applied Biosystems®). Data was analysed using GeneiousPRO 5.6.2. The sequences were compared to yeast sequences in the NCBI database by using the basic local alignment search tool (BLAST).

Resolving Identity of the Isolated Strains

The gel electrophoresis patterns of ITS amplification products, obtained from 12 of the 70 strains representing the seventy clinical isolates,

are shown in Figure 1. Using the primers ITS4 and ITS5 to amplify the ITS region, we obtained DNA fragments of between 600 and 700 bases for all strains tested depicting the approximate size of the ITS region. Sequencing of these amplicons followed by editing resulted in an ITS 1-5.8S-ITS 2 sequence of 478 bases. These sequences were multi-aligned and analysed in order to determine intra-specific variation within the various *Cryptococcus* varieties (Figure 2). The analysis revealed variable bases between the strains; which allowed for their delineation into three distinct varieties viz. *Cr. neoformans* var. *neoformans* (variable bases: T [position: 28], A [position: 117]), *Cr. neoformans var. grubii* (variable base: A [position: 230]) as well as *Cr. neoformans* var. *gattii* (variable base: C [position: 19]) (Table 1). These variable bases were also consistent in their delineation when applied against a number of sequences limited to *Cr. neoformans* species complex available from GeneBank.

Table 1: Strain identification based on analysis of ITS sequence analysis and CGB media results.

Strain delineation						
[+]Strain	**ITS intraspecific variation**		**ITS polymorphism**		**[++]Growth on CGB media**	
	Variable base	**Position**	**Signature**	**Endonuclease**	**Colour**	**Interpretation**
Cr. neo var. *neo*	T; A	28; 117	AATATT	*Ssp*I	Yellow	Negative
Cr. neo var. *gru*	A	230	AATATT	*Ssp*I	Yellow	Negative
Cr. neo var. *gat*	C	19	-	-	Blue	Positive

[+] Cr = *Cryptococcus*; *neo* = *neoformans*; var = variety; *gru* = *grubii*; *gat* = *gattii*. [++] Negative = *Cr. neofomans*; Positive = *Cr. gattii*.

The inventors have resolved the tested strains into distinct species viz. *Cr. neoformans* (*Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii*) and *Cr. gattii* (*Cr. neoformans* var. *gattii*) as proposed by Kwon-Chung & Varma (2006). We further uncovered a restriction site for *Ssp*I present only in the distinct species *Cr. neoformans* (as constituted by *Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii* strains), which is absent in the distinct species *Cr. gattii* (as constituted by *Cr. neoformans* var. *gattii* strains) (Figure 3). Amplification of this region and its subsequent

digestion allows for the identification of these two distinct species without sequencing, thus enhancing the speed at which identities can be resolved whilst eliminating sequencing costs.

EXAMPLE 2

Preparation of carvanine-glycine-bromothymol blue (CGB) media and cultivation

The media was prepared as previously described (Kwon Chung *et al.,* 1982). Strains were cultivated in duplicate at 30 °C for 5 days, before reading the results. The media was fitted with carvananine (growth inhibitor) and bromothymol blue; which turns the media blue due to a change in pH as positive strains cleave glycine to produce ammonia. As such, a negative result was interpreted as an agar plate that remained yellow after 5 days of cultivation.

Confirmation of studied strain identities

Traditionally, *Cr. gattii* has been thought to be limited to tropical and subtropical regions of the world (Day, 2004). In this part of the study, the inventors sought to confirm cases of *Cr. gattii* as identified from the sequencing results. All 64 *Cr. neoformans* (*Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii* strains) CGB plates remained yellow; while all 6 *Cr. gattii* (*Cr. neoformans* var. *gattii* strains) plates were blue after five days (Fig. 4 and Table 1). Identification of *Cr. gattii* as demonstrated in our study does not seem to be an isolated incidence, as this pathogen has also been isolated in other temperate regions viz. North America, thereby supporting the idea of a more worldwide ecological distribution (Datta *et al.,* 2009). Speculatively, this may in part be due to the exportation of eucalyptus trees, suggested to be the primary source of *Cr. gattii* (Kidd *et al.,* 2007).

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CLAIMS

1. A method of detecting the presence or absence, and if present the identity of a *Cryptococcus* pathogen in a biological sample from a subject, the method comprising the steps of:

(i) screening the biological sample using a PCR based method; and

(ii) digesting the PCR product with a restriction endonuclease,

wherein the presence or absence of a PCR product is indicative of the presence or absence of a *Cryptococcus* pathogen in the sample, and further wherein digestion of the PCR product is indicative of the identity of the *Cryptococcus* pathogen in the sample.

2. The method of claim 1, wherein the *Cryptococcus* pathogen is selected from the group consisting of *Cryptococcus neoformans* var. *neoformans* (serotype D), *Cryptococcus neoformans* var. *grubii* (serotype A), a hybrid Cryptococcus of serotype A and serotype D (serotype AD) or *Cryptococcus neoformans* var. *gattii* (serotype B or C).

3. The method of claim 1 or 2, wherein the *Cryptococcus* pathogens are classified into two distinct species *Cryptococcus neoformans* (serotypes A, D or AD) and *Cryptococcus gattii* (serotypes B or C).

4. The method of any one of claims 1 to 3, wherein the screening step comprises amplifying a portion of an internal transcribed spacer (ITS) including the 5.8S gene with oligonucleotide primers of SEQ ID NO: 1 (5'-TCC TCC GCT TAT TGA TAT GC -3') and SEQ ID NO: 2 (5'- GGA AGT AAA AGT CGT AAC AAG G -3').

5. The method of claim 4, wherein the oligonucleotide primers comprise sequences having at least 95% sequence identity to SEQ ID

NO:1 or SEQ ID NO:2 and wherein the oligonucleotide primers are capable of amplifying a fragment of an ITS including the 5.8S gene.

6. The method of any one of claims 1 to 5, wherein the restriction endonuclease is *Ssp*I.

7. The method of any one of claims 1 to 6, wherein the biological sample is selected from the group consisting of a blood sample, a cerebrospinal fluid sample, a positive culture sample, or a lung biopsy sample.

8. The method of claim 7, wherein the biological sample is a positive culture sample or a cerebrospinal fluid sample.

9. The method of any one of claims 1 to 8, wherein the subject is a human.

10. A kit for detecting the presence or absence, and if present the identity of a *Cryptococcus* pathogen in a biological sample obtained from a subject, the kit comprising:

(i) oligonucleotide primers for amplification of a portion of ITS and the 5.8S gene;
(ii) a *Ssp*I restriction endonuclease; and
(iii) a detection means for detecting the presence or absence of a PCR amplification product and/or restriction enzyme digestion products.

11. The kit according to claim 9, wherein the oligonucleotide primers comprise SEQ ID NO:1 and SEQ ID NO:2.

12. The kit according to claim 9 or 10, additionally comprising instructions for performing the method of the invention.

DATED THIS 6TH DAY OF NOVEMBER 2014

SPOOR & FISHER
APPLICANT'S PATENT ATTORNEY

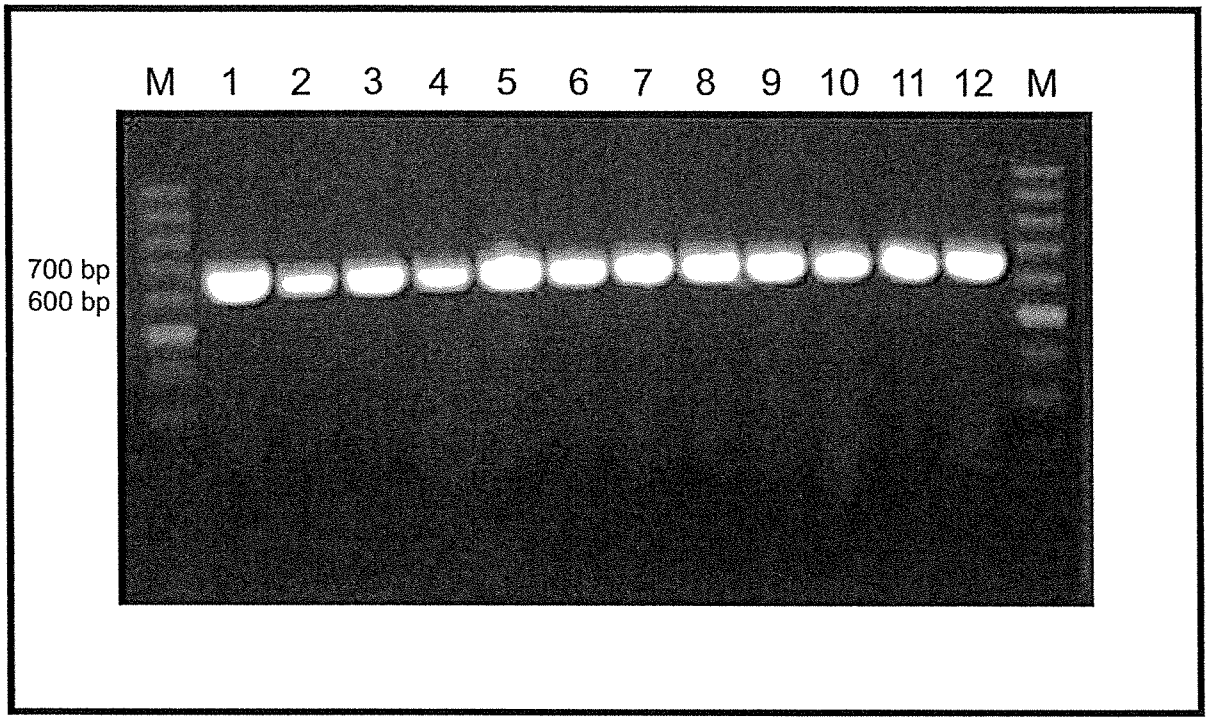

FIGURE 1

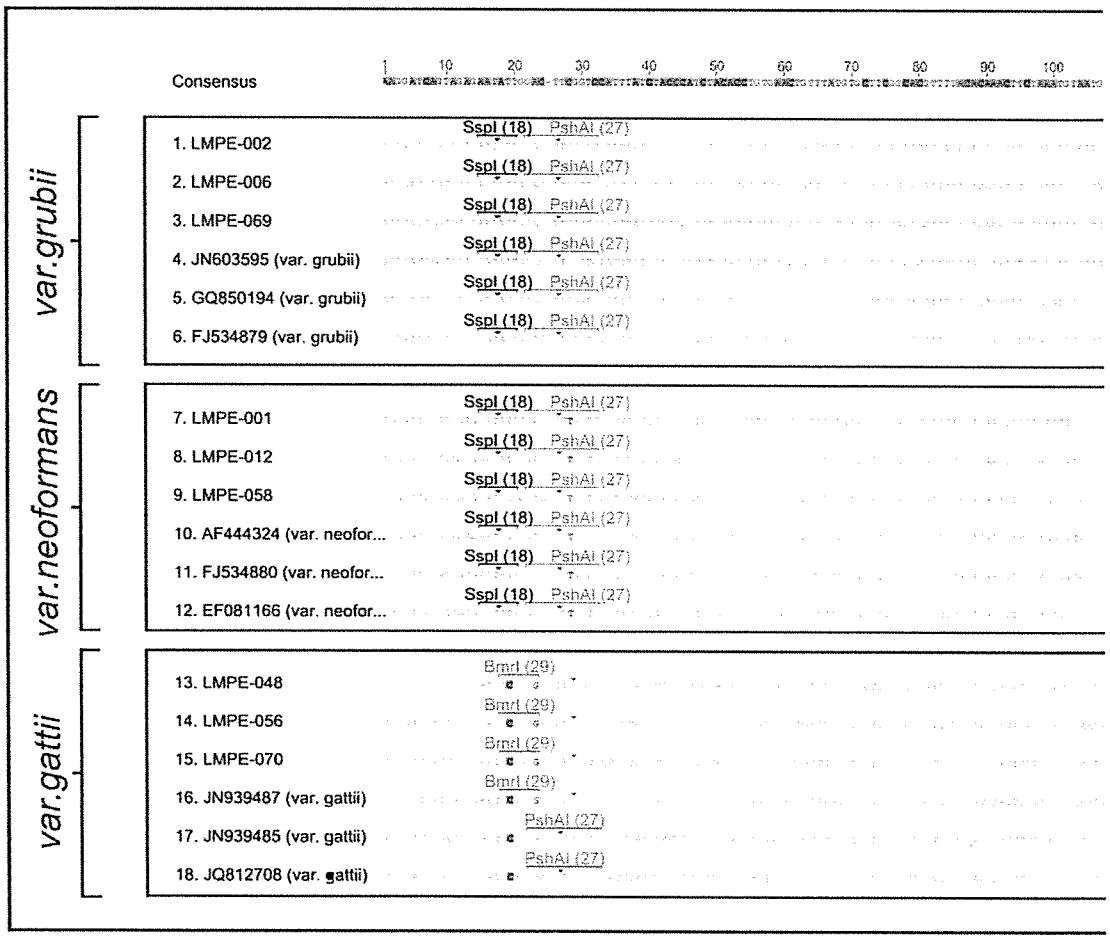
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FIGURE 2

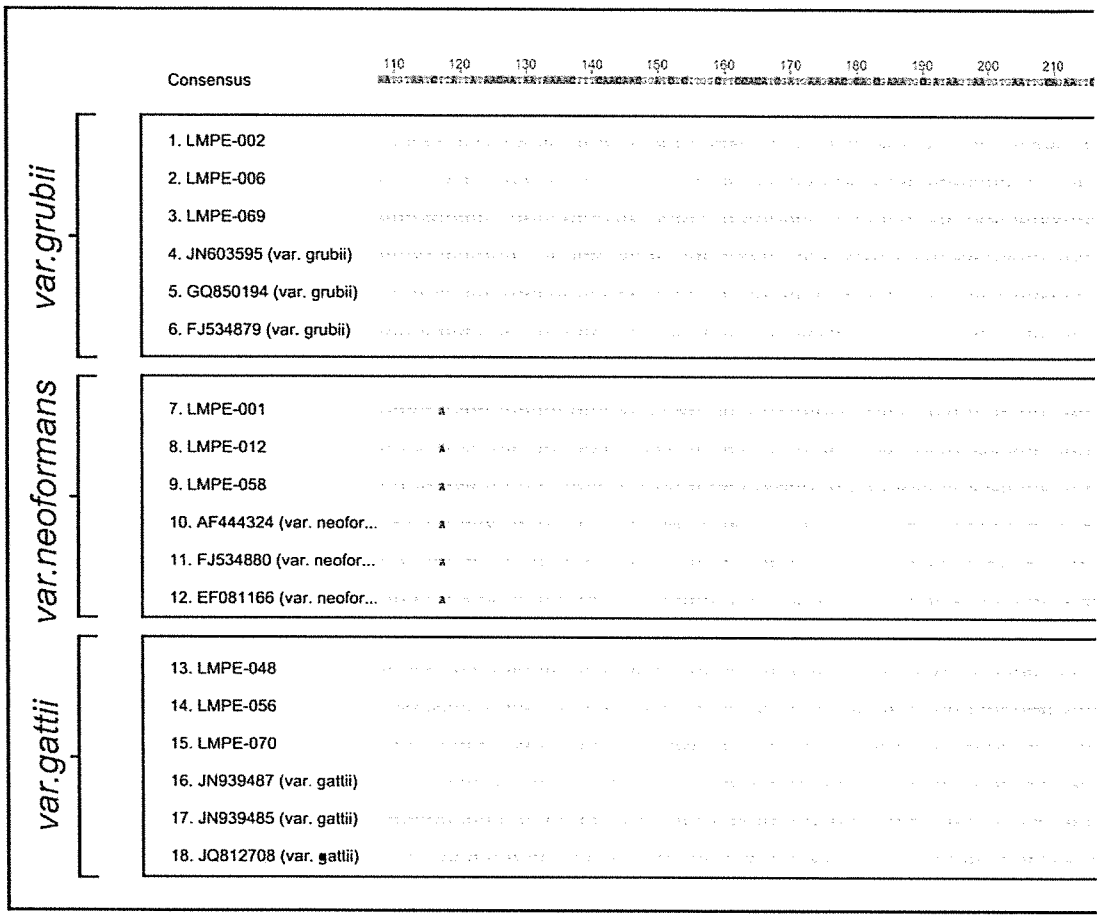
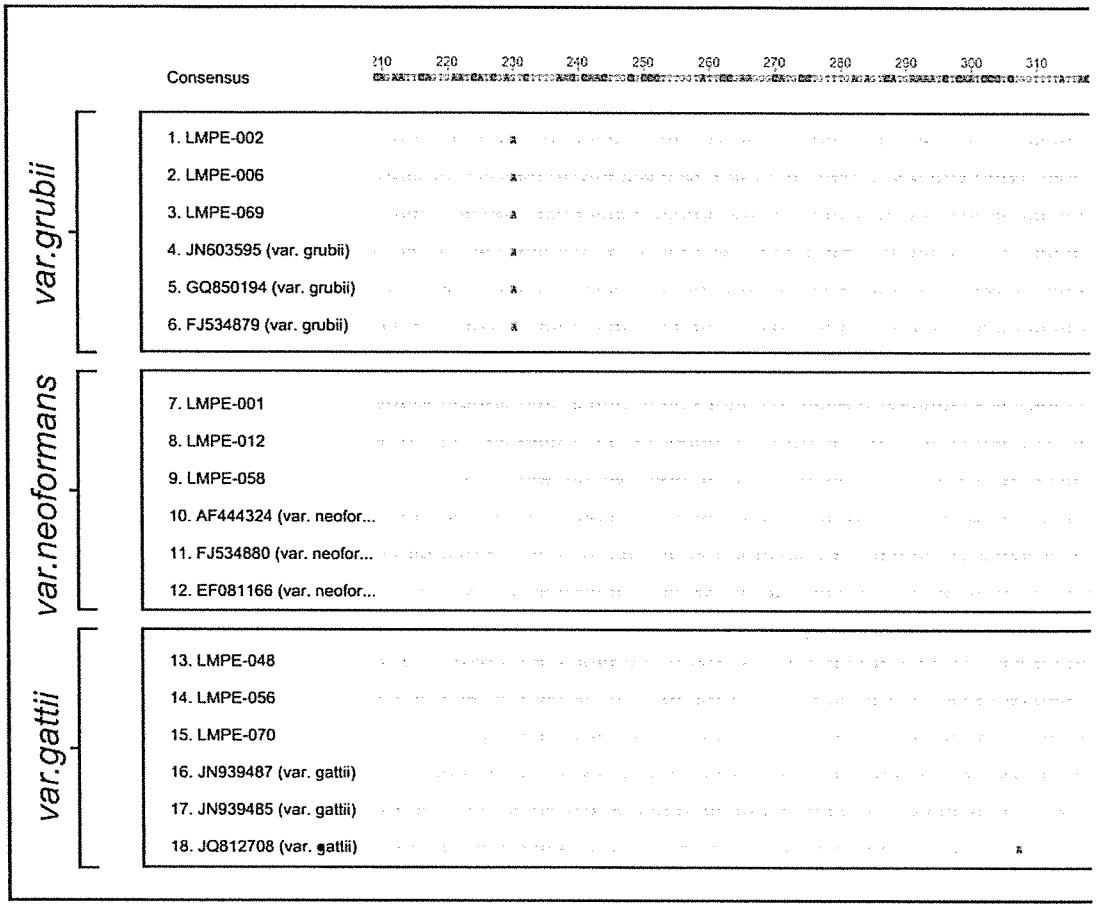


FIGURE 2 (CONT)



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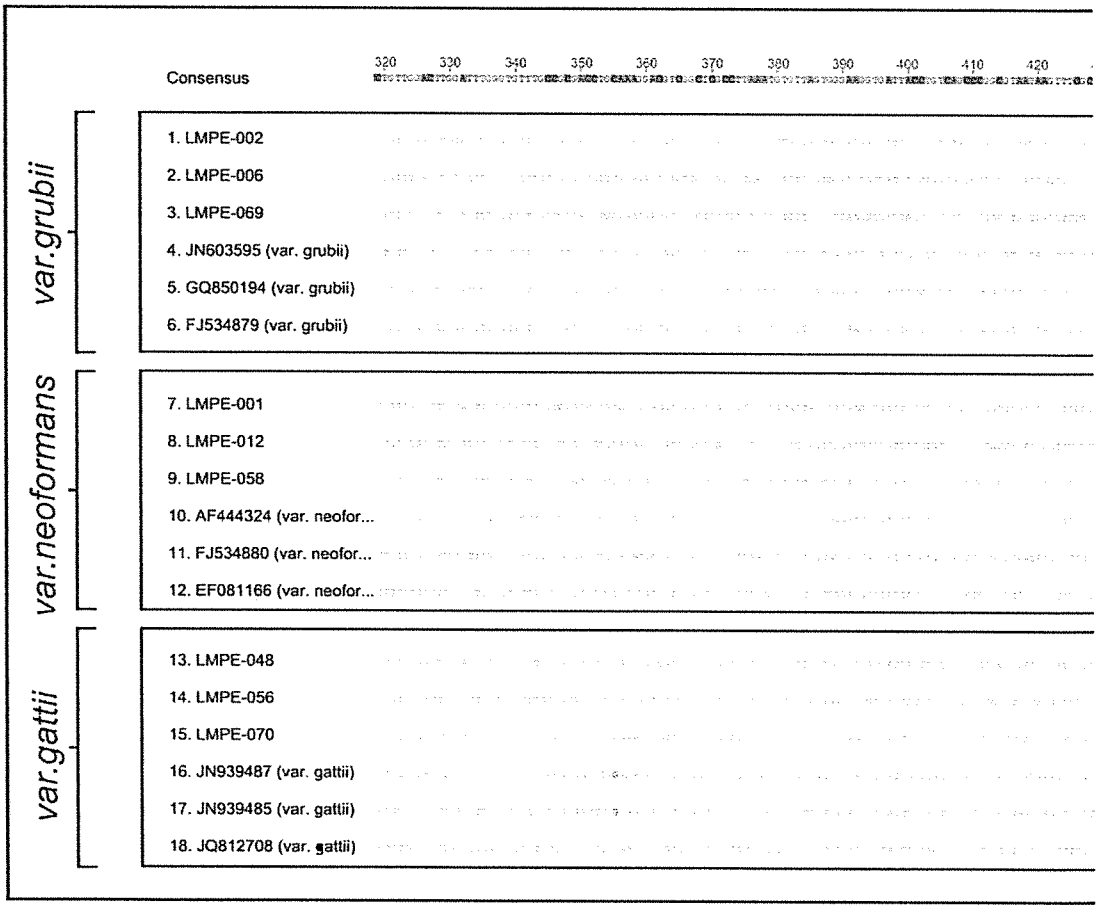

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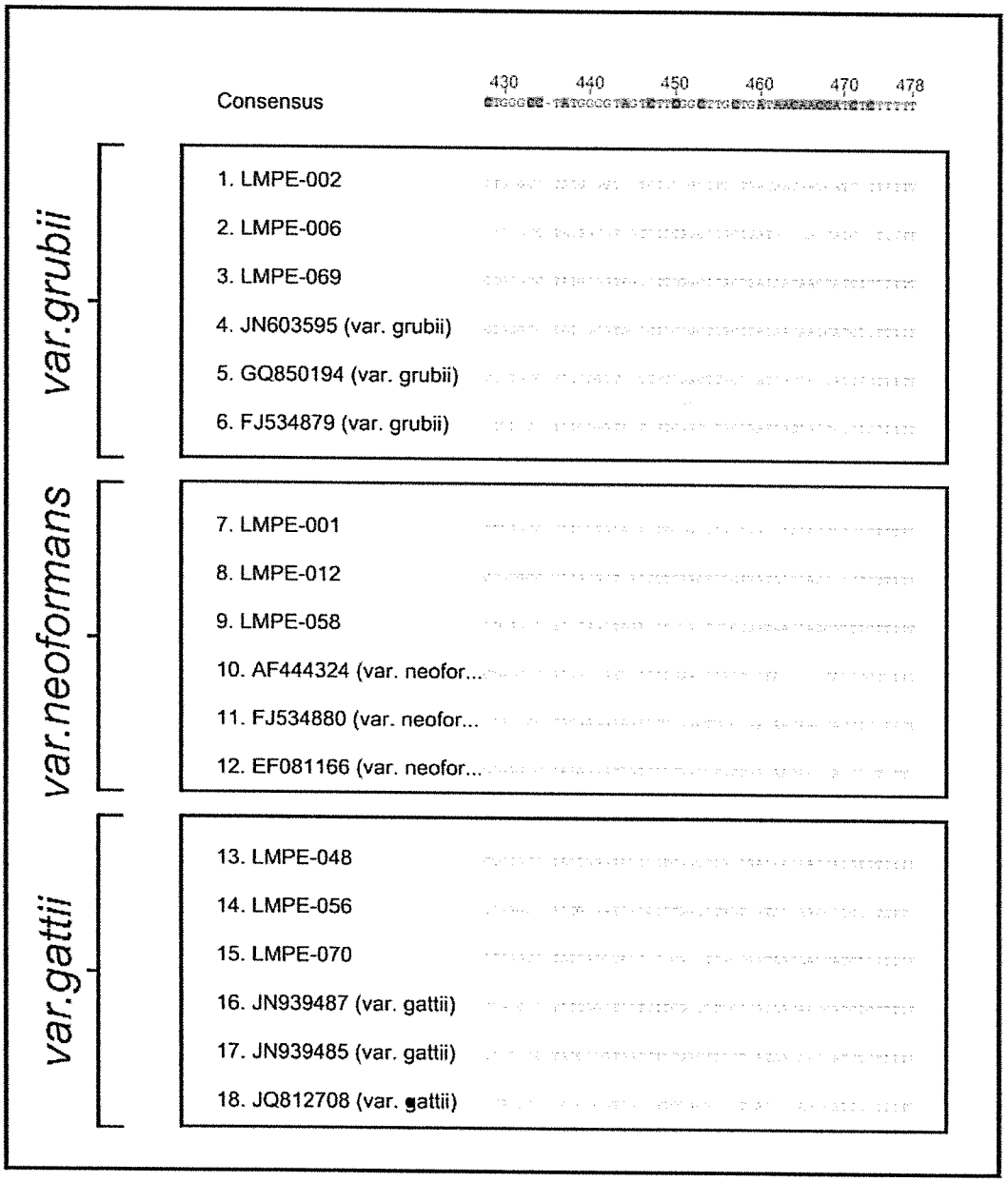

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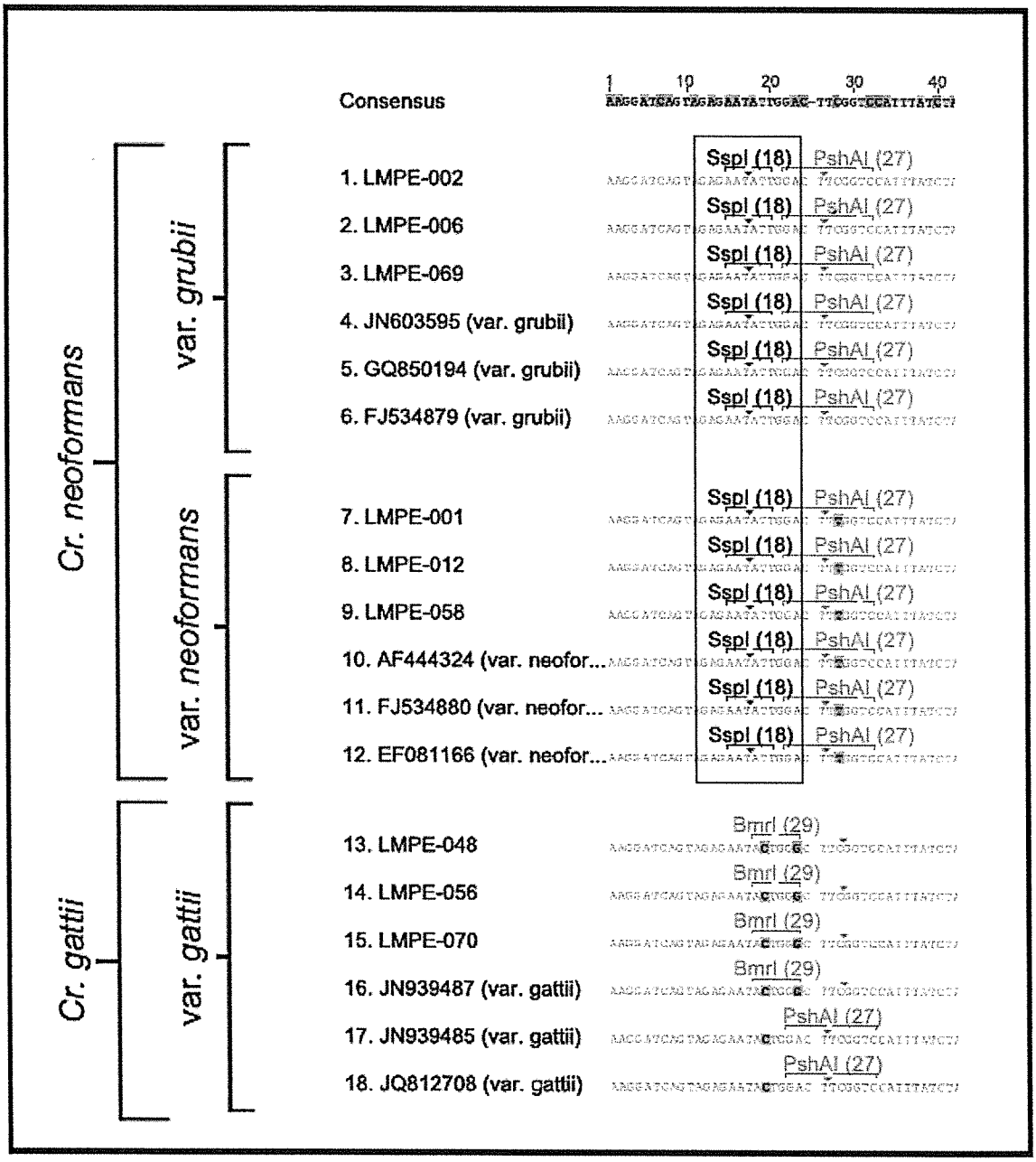
Cr. neoformans
var. grubii
var. neoformans
Cr. gattii
var. gattii
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2. LMPE-006
3. LMPE-069
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FIGURE 3

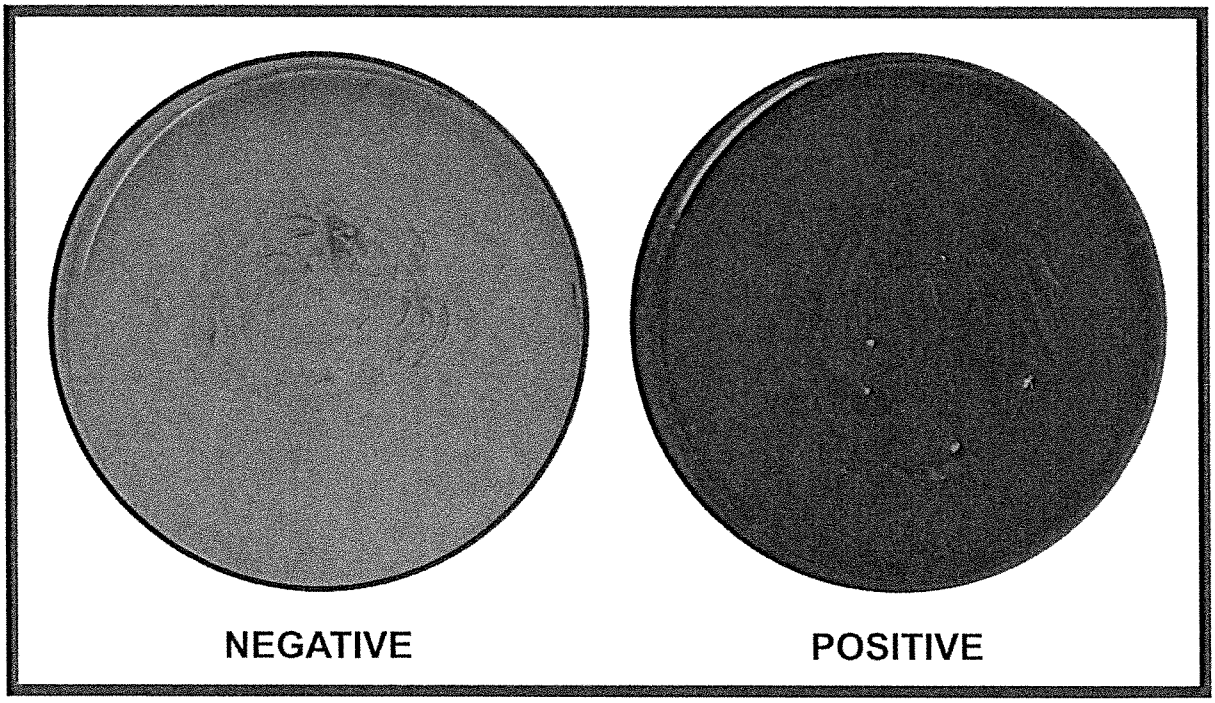

FIGURE 4