Title: METHOD FOR SIMULTANEOUS DETECTION OF BACTERIA AND FUNGI IN A BIOLOGICAL PREPARATION BY PCR, PRIMERS AS WELL AS BACTERIA AND FUNGI DETECTION KIT

Abstract: A method for detection of bacteria and fungi in a sample of biological material, wherein the DNA contained in the sample of biological material is subjected to amplification in multiplex real-time PCR, with the use of primers specific for bacteria in the first stage and primers specific for fungi, and in the second stage, the resulting DNA is amplified using primers and probes differentiating fungi into a group of mold fungi and yeast fungi and bacteria into Gram-positive and Gram-negative bacteria. The invention also includes novel oligonucleotide primers for the detection of bacteria and fungi by PCR and a kit for simultaneous detection of bacteria and fungi.
Method for simultaneous detection of bacteria and fungi in a biological preparation by PCR, primers as well as bacteria and fungi detection kit

The object of the invention is a method for simultaneous detection of bacteria and fungi in a sample of biological material by PCR, primers for detection of bacteria and fungi, and a kit for detecting these microorganisms in a sample of biological material. The invention provides a way to accomplish simultaneous DNA detection of Gram-negative bacteria, Gram-positive bacteria, yeast fungi and mold fungi in a sample of biological material, such as patient's saliva or blood.

Infections caused by bacteria and fungi have always been a major medical problem. The most dangerous of these are systemic infections, i.e., sepsis. Despite progress in their treatment achieved primarily through the use of antibiotherapy and the introduction into medical practice of technologies for prolonged life support of patients in critical condition, we are still failing to keep many patients alive. Paradoxically, with the development of medical knowledge and the introduction of newer and newer therapeutic procedures into treatment, the incidence of sepsis is increasing. Lever et al. report that, each year in the U.S., 750,000 people come down with sepsis and it is the cause of more than 215,000 deaths. In the European Union, 146 thousand patients die annually due to severe sepsis. In the UK alone mortality from it ranges from 30 to 50/100,000 a year, which puts sepsis in the forefront of the ten leading causes of death. In developed countries, sepsis develops in 2-4/1,000 liveborn neonates and it is the main cause of their death. In Poland, there is a lack of accurate epidemiological data, but Zielinski et al. provide information that 967 deaths occurred in 2005 due to sepsis, including 43 deaths of children. The growing mortality due to sepsis is the result of increasing resistance to antibiotics, the use of invasive treatment methods and an aging population. Sepsis is the biggest threat to immunocompromised people, especially when they are hospitalized over long periods of time, primarily in intensive care units. It affects particularly patients with neoplastic diseases, immunocompromised patients, patients with burns, the elderly, and children.

The most important and most difficult problem in the treatment of bloodstream infections, determining the effectiveness of treatment and, consequently, the costs and duration of hospitalization, is efficacious diagnosis of factors causing the systemic inflammatory response in the course of sepsis. Identification of the etiological agent (microorganism: fungus or bacterium) allows the employment of effective targeted antibiotherapy. The material subjected to diagnostic testing is preferably blood taken from a patient manifesting
clinical symptoms of sepsis, such as tachycardia, bradycardia, increased or decreased body
temperature, drop in blood pressure, etc.

Blood poses the biggest challenges among all biological materials as regards a material
for microbiological testing. The greatest difficulty is the fact that the microorganisms
responsible for infection can be found in blood in very small quantities, or there is only their
periodic release into blood.

In spite of this, the current diagnostic standard are blood cultures performed on special
media, ideally in automated culture systems (e.g., BACTEC - BectonDickinson). The
advantages of such methods are their simplicity and relatively low costs of testing. Their
weakness is that they are time-consuming, taking up to 5 days (until the test results are
issued), and have low sensitivity, which causes only 15-20% of the culture to obtain
microbial growth. Consequently, in a great majority of cases, the doctor may only apply
empirical antibiotherapy due to the lack of achieving growth of microorganisms responsible
for the infection. The situation is further exacerbated by the fact of subjecting patients to
antibiotherapy before any blood samples are drawn for culture - patients are often treated
with antibiotics prior to manifestation of symptoms of sepsis. Blood cultures are very
troublesome in such a case, due to the fact that it contains antibiotics inhibiting the growth of
microorganisms. In order to increase the chance of detecting microbiological agents in blood,

Another molecular target that allows efficient, accurate and quick diagnosis of
bloodstream infections are microbial nucleic acids which are etiological agents of infection.
Both DNA, as well as RNA, of each organism contains sequences unique to it, constituting a
specific "fingerprint". With the knowledge of these sequences, it is possible to apply
molecular biological methods, such as PCR or hybridization, for determining the presence of
microorganisms in the blood. Sensitivity of molecular methods considerably exceeds the
sensitivity of the culture method. Additionally, the prior use of antibiotic therapy does not
influence the test result due to the fact that there is no need for growth of bacteria or fungi in
culturing medium, but only detection of their DNA or RNA sequences.

Numerous patent applications and specifications, as for example EP2547782, EP2087134,
EP1978111 or EP2009118, disclose the use of PCR methods for the detection of specific
microorganisms based on the developed primers.
Application EP2547782 discloses detection of microorganisms from the *Staphylococcus* group in the multiplex PCR system based on the developed probes and primers, but without the application of the nested PCR system.

The present invention is a method for the detection of bacteria and fungi in a sample of biological material, wherein the DNA contained in the sample of biological material is subjected to amplification in multiplex real-time PCR. The amplification reaction is carried out in two stages with the use of primers specific for bacteria and primers specific for fungi in the first stage, and then the product of the first amplification is used as template in the second stage, i.e., amplification using primers and probes differentiating fungi into a group of mold fungi and yeast-like fungi and bacteria into Gram-positive and Gram-negative bacteria.

Primers specific for the 16S rRNA sequence of bacteria are used as primers specific for bacteria, preferably oligonucleotides with the following sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GGCAGACGGGTAGTAA</td>
</tr>
<tr>
<td>NEST_BAC_R</td>
<td>CGCATTTCACCGCTA</td>
</tr>
</tbody>
</table>

Primers specific for the 18S rRNA sequence of fungi are used as primers specific for fungi, preferably oligonucleotides with the following sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATTGACGGAAGGGCACC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TTCTCGTTGAAGAGCAA</td>
</tr>
</tbody>
</table>

In keeping with the method of the invention, in the second stage of amplification, detection and identification of bacteria are performed with the use of primers with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN/GP_F</td>
<td>GACTCCTACGGGAGGC</td>
</tr>
<tr>
<td>GN/GP_R</td>
<td>GCGGCTGCTGGCAC</td>
</tr>
</tbody>
</table>

and probes with sequences:
while for amplification to detect and identify fungi, primers are used with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP_Probe</td>
<td>Hex- CTGAyssAGCAACGCCGCG -TAMRA</td>
</tr>
<tr>
<td>GN_Probe</td>
<td>Cy5 –CCTGAysCAGCmATGCCGCG- BHQ-2</td>
</tr>
</tbody>
</table>

and probes with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candid_probe</td>
<td>FAM-</td>
</tr>
<tr>
<td>Asperg_probe</td>
<td>TexasRed-</td>
</tr>
</tbody>
</table>

Preferably, in the method of the invention, detection of bacteria and fungi is carried out in a sample of biological material isolated from a patient, preferably from the blood of a patient with symptoms of sepsis.

The invention also includes oligonucleotides with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GGCGGACGGGTTGAGTAA</td>
</tr>
<tr>
<td>NEST_BAC_R</td>
<td>CGCATTTCACCGCTA</td>
</tr>
</tbody>
</table>

for use as primers in a PCR reaction to detect bacteria.

The invention additionally includes oligonucleotides with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATTGACGGAAGGGCACC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TTCCTCGTTGAAGAGCAA</td>
</tr>
</tbody>
</table>

for use as primers in a PCR reaction to detect fungi.
The invention also provides a kit for detection of bacteria and fungi in a sample of biological material by nested-multiplex real-time PCR containing the following oligonucleotides:

for the detection of bacteria, primers specific for 16S rRNA of bacteria:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GGCGGACGCGGTGAGTAA</td>
</tr>
<tr>
<td>NEST_BAC_R</td>
<td>CGCATTTCAAAGTGA</td>
</tr>
</tbody>
</table>

and probes specific for 16S rRNA of bacteria with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP_Probe</td>
<td>FAM-CTGATGCAAGCCAC- TAMRA</td>
</tr>
<tr>
<td>GN_Probe</td>
<td>Cy5-CCTGATGCAAGCCAC-BHQ2</td>
</tr>
</tbody>
</table>

as well as

for the detection of fungi, primers specific for 18S rRNA of fungi:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATTGACGGAAGGCACC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TTCTCAGTGAAGGACAA</td>
</tr>
</tbody>
</table>

and:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUN_F</td>
<td>TTAGTGGAGTGATTGTCTGCT</td>
</tr>
<tr>
<td>FUN_R</td>
<td>TCTAAGGTCATCACAGACCTG</td>
</tr>
</tbody>
</table>

and probes specific for 18S rRNA of fungi with sequences:

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candid_probe</td>
<td>FAM-</td>
</tr>
<tr>
<td>Asperg_probe</td>
<td>TexasRed-</td>
</tr>
</tbody>
</table>
The claimed detection method is based on the multiplex real-time PCR reaction, with a possible simultaneous amplification of at least two DNA sequences. Moreover, the method of the invention realizes the nested-multiplex PCR method, i.e., a two-stage amplification reaction, which significantly increases the sensitivity of detection.

The claimed method provides reliable detection of all species of fungi and bacteria (with differentiation into Gram-positive bacteria, Gram-negative bacteria, yeast fungi and mold fungi) in DNA samples isolated from the blood of patients manifesting symptoms of sepsis. It is possible to employ this method for detecting only bacteria or only fungi but, at the same time, its advantage is the possibility to use it for simultaneous detection of both fungi and bacteria, resulting in lower costs of testing.

Detection of PCR products of the first amplification is not required, as the final result of the diagnostic test is visible in the second amplification. If amplification I fails to obtain multiplication of DNA by using the designed primers, then during the second stage of amplification, another negative result will also be obtained (no microorganisms in the sample of biological material). This does not preclude carrying out product detection upon finishing amplification I with the use of DNA gel electrophoresis, optionally employing spectrophotometric methods.

Detection and identification of PCR products of the second stage of amplification take place already during the process of multiplication of DNA. The used probes, GP_probe, GN_probe, Candid_probe, Asperg_probe, specifically bind to the resulting products of amplification of DNA sequences typical of Gram-positive and Gram-negative bacteria, yeast fungi, and mold fungi and emit fluorescent light that is recorded by the detector during the amplification. Each of the four probes is equipped with a fluorescent marker of a strictly defined, typical for a given probe, light emission wavelength, which allows differentiation of the four particular groups of microorganisms.

The invention encompasses new specific universal primers for bacteria and new universal primers for fungi, the application of which for amplification of genetic material from samples by PCR allows incorporating the entire panel of bacterial and fungal microorganisms (with differentiation into Gram-negative bacteria, Gram-positive bacteria, yeast fungi, and mold fungi), but without typing of specific species. Such information is very useful for the physician in selecting the appropriate treatment before obtaining the result of identification specifying the species of bacteria or fungi from the microbiology lab.
Currently used systems do not possess the indicated universality. The employed systems (e.g. SeptiFast - Roche) make it possible to detect between ten and twenty specific species of microorganisms or (as SeptiTest - Molzym) theoretically permit the detection of any possible species, but sequencing of the PCR product is required, which increases the cost and extends the time of waiting for the result.

The method of the invention utilizing multiplex real-time PCR techniques allows simultaneous detection of bacteria and fungi in real time without the need to wait for the results of DNA electrophoresis, as is the case with standard PCR. Additionally, the use of the nested system allows the increase of sensitivity of the detection method by two orders of magnitude in comparison to one-stage PCR. The application of sequencing of the PCR product is also not necessary in order to identify a particular species of microorganism.

The method of the invention allows rapid detection of all species of fungi (differentiating between yeast fungi and mold fungi) and all species of bacteria (differentiating between Gram-negative and Gram-positive bacteria), without identifying specific species. The detection method enables one to quickly confirm the presence of infection with high sensitivity, overcoming the drawbacks of commercially available methods that require more time and a full spectrum of experiments aimed at a limited number of most common species.

In the claimed method, typing a specific microbial species is also possible upon sequencing of the PCR product obtained in amplification I or II, however, it is not required for initial diagnosis.

In preferred embodiments, the invention is represented in the drawing, in which:

Fig. 1 presents sequences of fungal 18S rRNA with marking of the developed primers, NEST_FUN_F, NEST_FUN_R (gray box), and primers known from the literature, FUN_F, FUN_R (transparent box); the sequences are on one DNA strand, hence the final sequence marked in the gray box is reversed and complementary to the synthesized R equivalent;

Fig. 2 presents sequences of bacterial 16S rRNA with marking of the developed primers, NEST_BAC_F, NEST_BAC_R R (gray box), and primers known from the literature, GN/GP_F, GN/GP_R (transparent box); the sequences are on one DNA strand, hence the final sequence marked in the gray box is reversed and complementary to the synthesized R equivalent;
Fig. 3 shows a comparison of the proportion of positive results obtained from the method of the invention, of 102 blood samples originating from patients with clinical symptoms of sepsis: systemically and broken down into four groups of microorganisms; while

Fig. 4 presents a comparison of the proportion of positive results in the study of 102 blood samples originating from patients with clinical symptoms of sepsis using the culture method in the BACTEC system and in accordance with the method of the invention.

Experimental section

The methodology of microbial DNA amplification was carried out on a DNA template isolated from human blood.

Nested amplification was carried out in two separate amplification stages marked with Roman numerals - I and II. In stage I, newly developed primers were used specific for *Procaryota* (bacteria) and *Eucaryota* (fungi), specific for sequences of 16S rRNA (bacteria) and 18S rRNA (fungi) units. Thereafter, the product of the first (I) PCR amplification was utilized as a template in the second (II) amplification, where primers and probes known from the literature found their application in differentiating fungi into a group of mold fungi and yeast-like fungi and bacteria into Gram-positive and Gram-negative bacteria. Application of nested PCR allows to increase the sensitivity of the method.

In the method of the invention, TaqMan primers and probes known in the literature were applied, and at the same time, multiplex system was developed in the project, which enabled them to be combined in a single reaction.

Primers for amplification I were designed and tested *in silico* with the use of BLAST/NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) base, as shown in Fig. 1. In order to determine the sensitivity of the method, isolation of DNA from blood samples was carried out (originating from healthy volunteers), which were artificially infected with model microorganisms: Gram-negative bacteria - *Escherichia coli* ATCC 25922 (American Type Culture Collection); Gram-positive bacteria - *Staphylococcus aureus* ATCC 33497; yeast-like fungi - *Candida albicans* ATCC 10231, mold fungi - *Aspergillus fumigatus* ATCC 14110, so as to create a gradient of their number in blood. The isolated DNA was used to perform the designed nested-multiplex PCR amplification. The results of the method sensitivity assay are given in Table 1. Table 1 also comprises comparative data for amplification excluding the nested system, which uses the designed primers, however, the sensitivity of the method is then decreased.
Table 1. The sensitivity of detection of bacteria and fungi in blood using the real-time PCR method in two variations: nested-multiplex PCR and multiplex PCR with the designed primers.

<table>
<thead>
<tr>
<th>Groups of microorganisms / species</th>
<th>Multiplex real-time PCR [CFU/ml] method sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NESTED multiplex PCR</td>
</tr>
<tr>
<td>Mold fungi (<em>A. fumigatus</em>)</td>
<td>$4,0 \times 10^1$</td>
</tr>
<tr>
<td>Yeast fungi (<em>C. albicans</em>)</td>
<td>$2,0 \times 10^1$</td>
</tr>
<tr>
<td>Gram (-) bacteria (<em>E. coli</em>)</td>
<td>$6,5 \times 10^1$</td>
</tr>
<tr>
<td>Gram (+) bacteria (<em>S. aureus</em>)</td>
<td>$6,0 \times 10^1$</td>
</tr>
</tbody>
</table>

Sequences of the applied oligonucleotides (probes and primers) are compiled in Table 2.

Table 2. Sequences of primers and probes used in the study.

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
<th>Target sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F *</td>
<td>GGCGGACGGGTGAGTAA</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>NEST_BAC_R *</td>
<td>CGCATTTCCACGCTA</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>NEST_FUN_F *</td>
<td>AATTGACGGAAGGGAACC</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>NEST_FUN_R *</td>
<td>TTCCTCGTTGAAGGCAAA</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>FUN_F</td>
<td>TTGGTGGAGTGATTTTGCTGCT</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>FUN_R</td>
<td>TCTAAGGGCATCACGACCTG</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Candid_probe</td>
<td>FAM-TTAACCTACTAAAATAGTGCTGAGC-BHQ1</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>Asperg_probe</td>
<td>TexasRed-TCGGCCCTTAAATAGCCCGTGCGTC-G-Eclipse</td>
<td>18S rRNA</td>
</tr>
</tbody>
</table>
New sequences of primers, designed for the purposes of the invention

Composition of multiplex PCR reaction mixtures and nested-multiplex PCR are given in Table 3, where additionally the reagents used and amplification thermal profiles are provided.

Table 3. Composition of reaction mixtures, the reagents involved and PCR thermal profiles.

<table>
<thead>
<tr>
<th>NESTED multiplex PCR</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>amplification I [final volume 25 µl]</td>
<td>amplification II [final volume 10 µl]</td>
</tr>
<tr>
<td>1. Water 6,7 µl</td>
<td>1. Water 2,08 µl</td>
</tr>
<tr>
<td>2. B buffer 2,5 µl 2,5 µl</td>
<td>2. B buffer 1,0 µl 1,0 µl</td>
</tr>
<tr>
<td>3. NEST_BAC_F 0,125 µl</td>
<td>3. GN/GP_F 0,2 µl 0,2 µl</td>
</tr>
<tr>
<td>4. NEST_BAC_R 0,125 µl</td>
<td>4. GN/GP_R 0,2 µl 0,2 µl</td>
</tr>
<tr>
<td>5. NEST_FUN_F 0,125 µl</td>
<td>5. GN_probe 0,05 µl 0,05 µl</td>
</tr>
<tr>
<td>6. NEST_FUN_R 0,125 µl</td>
<td>6. GN_probe 0,05 µl 0,05 µl</td>
</tr>
<tr>
<td>7. dNTP’s 2,5 µl</td>
<td>7. FUN_F 0,2 µl 0,2 µl</td>
</tr>
<tr>
<td>8. MgCl₂ 2,5 µl</td>
<td>8. FUN_R 0,2 µl 0,2 µl</td>
</tr>
<tr>
<td>9. Perpetual Tag 0,3 µl</td>
<td>9. Asperg_prob 0,05 µl 0,05 µl</td>
</tr>
<tr>
<td></td>
<td>10. Candid_prob 0,05 µl 0,05 µl</td>
</tr>
<tr>
<td></td>
<td>11. dNTP’s 1,0 µl 1,0 µl</td>
</tr>
<tr>
<td></td>
<td>12. MgCl₂ 1,8 µl 1,8 µl</td>
</tr>
<tr>
<td></td>
<td>13. Perpetual Tag 0,12 µl 0,12 µl</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14. DNA 3,0 µl 3,0 µl</td>
</tr>
</tbody>
</table>
Example 1

Nested-multiplex real-time PCR for simultaneous detection of bacteria and fungi.

A study was conducted using the developed nested-multiplex real-time PCR method on 102 DNA samples isolated from the blood of patients manifesting clinical symptoms of sepsis in order to detect Gram-positive bacteria, Gram-negative bacteria, yeast fungi, and mold fungi. The first amplification of the collected DNA was performed in the final volume of 25 µl in the presence of the newly designed primers: NEST_BAC_F, NEST_BAC_R, NEST_FUN_F, NEST_FUN_R with sequences listed in Table 1, using Perpetual Taq Polymerase, carrying out 30 cycles with temperature and time parameters presented in Table 4. Afterwards, 3 µl of the mixture from the first stage of amplification containing amplified DNA of the detected microorganism was subjected to the second amplification in the final volume of 10µl of the mixture described in Table 4, performing 40 thermal cycles.
Table 4. Composition of reaction mixtures, the reagents involved and PCR reaction thermal profiles

<table>
<thead>
<tr>
<th></th>
<th>amplification I [final volume 25 µl]</th>
<th>amplification II [final volume 10 µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Water</td>
<td>6.7 µl</td>
<td>1. Water</td>
</tr>
<tr>
<td>2. B buffer</td>
<td>2.5 µl</td>
<td>2. B buffer</td>
</tr>
<tr>
<td>3. NEST_BAC_F</td>
<td>0.125 µl</td>
<td>3. GN/GP_F</td>
</tr>
<tr>
<td>4. NEST_BAC_R</td>
<td>0.125 µl</td>
<td>4. GN/GP_R</td>
</tr>
<tr>
<td>5. NEST_FUN_F</td>
<td>0.125 µl</td>
<td>5. GP_probe</td>
</tr>
<tr>
<td>6. NEST_FUN_R</td>
<td>0.125 µl</td>
<td>6. GN_probe</td>
</tr>
<tr>
<td>7. dNTP’s</td>
<td>2.5 µl</td>
<td>7. FUN_F</td>
</tr>
<tr>
<td>8. MgCl₂</td>
<td>2.5 µl</td>
<td>8. FUN_R</td>
</tr>
<tr>
<td>9. Perpetual Taq</td>
<td>0.3 µl</td>
<td>9. Asperg_prob</td>
</tr>
<tr>
<td></td>
<td>Polymerase</td>
<td>10. Candid_prob</td>
</tr>
<tr>
<td>10. DNA</td>
<td>10 µl</td>
<td>11. dNTP’s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12. MgCl₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13. Perpetual Taq Polymerase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14. DNA (product of amplification I)</td>
</tr>
</tbody>
</table>

95°C – 5 min
95°C – 20 sec
46°C – 20 sec
72°C – 30 sec
30 x

95°C – 5 min
95°C – 15 sec
65°C – 1 min
40 x

Detection and identification of the PCR products of the second amplification was carried out in the course of the process of DNA multiplication. The probes used: GP_probe, GN_probe, Candid_probe, Asperg_probe, upon attaching specifically to the resulting products of amplification of DNA sequences typical of Gram-positive bacteria, Gram-negative bacteria, yeast fungi and mold fungi emitted fluorescent light recorded by the detector in the course of amplification, allowing identification of the amplified product.

The obtained results were compared with the available results from cultures of the same 102 blood samples acquired from the traditional method of diagnosis for sepsis, based on a culture using BACTEC (BectonDickinson) automated system. In all samples, the results
generated by culture were confirmed, and additionally, positive results were obtained for the presence of bacteria and fungi in a portion of negative samples in the culture. This validates high sensitivity of the new method. Detailed results are presented in Figures 3 and 4.

Example 2.

Nested-multiplex real-time PCR for the detection of Gram-positive and Gram-negative bacteria

The detection was performed analogously to the method employed in Example 1 using NEST_BAC_F and NEST_BAC_R primers for amplification of bacterial DNA, in the conditions described in Table 5.

Table 5. The composition of reaction mixtures, the reagents involved and PCR thermal profiles for the detection of Gram-positive and Gram-negative bacteria.

<table>
<thead>
<tr>
<th>NESTED multiplex PCR</th>
<th>amplification I</th>
<th>[final volume 25 µl]</th>
<th>amplification II</th>
<th>[final volume 10 µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Water</td>
<td>6.95 µl</td>
<td>1) Water</td>
<td>2.58 µl</td>
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</tr>
<tr>
<td>2) B buffer</td>
<td>2.5 µl</td>
<td>2) B buffer</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>3) NEST_BAC_F</td>
<td>0.125 µl</td>
<td>3) GN/GP_F</td>
<td>0.2 µl</td>
<td></td>
</tr>
<tr>
<td>4) NEST_BAC_R</td>
<td>0.125 µl</td>
<td>4) GN/GP_R</td>
<td>0.2 µl</td>
<td></td>
</tr>
<tr>
<td>5) dNTP’s</td>
<td>2.5 µl</td>
<td>5) GP_probe</td>
<td>0.05 µl</td>
<td></td>
</tr>
<tr>
<td>6) MgCl₂</td>
<td>2.5 µl</td>
<td>6) GN_probe</td>
<td>0.05 µl</td>
<td></td>
</tr>
<tr>
<td>7) Polymerase</td>
<td>0.3 µl</td>
<td>7) dNTP’s</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>8) Perpetual Taq</td>
<td></td>
<td>8) MgCl₂</td>
<td>1.8 µl</td>
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<tr>
<td>9) DNA</td>
<td>10 µl</td>
<td>9) Polymerase</td>
<td>0.12 µl</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>11) DNA</td>
<td>3.0 µl</td>
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</table>

Example 3.

Nested-multiplex real-time PCR for the detection of yeast fungi and mold fungi.
The detection was performed analogously to the method employed in Example 1 using NEST_FUN_F and NEST_FUN_R primers in amplification I for the detection of fungi, in the conditions described in Table 6, followed by amplification II in the mixture described in the table, carrying out thermal cycles as defined there.

Table 6. The composition of reaction mixtures, the reagents involved and PCR thermal profiles for the detection of yeast fungi and mold fungi

<table>
<thead>
<tr>
<th>NESTED multiplex PCR</th>
<th>amplification I [final volume 25 µl]</th>
<th>amplification II [final volume 10 µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Water</td>
<td>6.95 µl</td>
<td>1) Water</td>
</tr>
<tr>
<td>2) B buffer</td>
<td>2.5 µl</td>
<td>2) B buffer</td>
</tr>
<tr>
<td>3) NEST_FUN_F</td>
<td>0.125 µl</td>
<td>3) FUN_F</td>
</tr>
<tr>
<td>4) NEST_FUN_R</td>
<td>0.125 µl</td>
<td>4) FUN_R</td>
</tr>
<tr>
<td>5) dNTP’s</td>
<td>2.5 µl</td>
<td>5) Asperg_prob</td>
</tr>
<tr>
<td>6) MgCl₂</td>
<td>2.5 µl</td>
<td>6) Candid Probe</td>
</tr>
<tr>
<td>7) Polymerase</td>
<td>0.3 µl</td>
<td>7) dNTP’s</td>
</tr>
<tr>
<td>8) Perpetual Taq</td>
<td>10 µl</td>
<td>8) MgCl₂</td>
</tr>
<tr>
<td>9) DNA</td>
<td>10 µl</td>
<td>9) Polymerase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10) Perpetual Taq</td>
</tr>
</tbody>
</table>

- DNA (product of amplification I) 3.0 µl

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
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</thead>
<tbody>
<tr>
<td>95°C – 5 min</td>
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<tr>
<td>95°C – 20 sec</td>
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<tr>
<td>46°C – 20 sec</td>
<td>30 x</td>
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<td>72°C – 30 sec</td>
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</tr>
<tr>
<td>95°C – 15 sec</td>
<td>40 x</td>
</tr>
<tr>
<td>65°C – 1 min</td>
<td></td>
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</tbody>
</table>
Claims

1. A method for detection of bacteria and fungi in a sample of biological material in which DNA contained in the sample of biological material is subjected to amplification in multiplex real-time PCR, wherein the amplification reaction is carried out in two stages with the use of primers specific for bacteria and primers specific for fungi in the first stage, and then the product of the first amplification is used as a template in the second stage - amplification using primers and probes differentiating fungi into a group of mold fungi and yeast fungi and bacteria into Gram-positive and Gram-negative bacteria.

2. The method according to claim 1, wherein primers specific for the sequence of bacterial 16S rRNA are used as primers specific for bacteria.

3. The method according to claim 2, wherein as primers specific for bacterial 16S rRNA, oligonucleotides are used with the following sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GCGGACGGGTAGTAA</td>
</tr>
<tr>
<td>NEST_BAC_R</td>
<td>CGCATTCACCGCTA</td>
</tr>
</tbody>
</table>

4. The method according to claim 1, wherein as primers specific for fungi, primers specific for the sequence of fungal 18S rRNA are used.

5. The method according to claims 3 or 4, wherein as primers specific for fungal 18S rRNA, oligonucleotides are used with the following sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATGCCAGGAGGCCAC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TCCCTCGTGAAGAGCA</td>
</tr>
</tbody>
</table>

6. The method according to claims 1-5, wherein in the second stage of amplification, primers with the following sequences are used for detection and identification of bacteria:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN/GP_F</td>
<td>GACTCCTACGGAGGC</td>
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<tr>
<td>GN/GP_R</td>
<td>GCGGCTGCTGGCAC</td>
</tr>
</tbody>
</table>

and probes with sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5’ - 3’</th>
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</thead>
</table>
7. The method according to claim 1-5, wherein in the second stage of amplification, primers with the following sequences are used for detection and identification of fungi:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUN_F</td>
<td>TTGGTGAGTGATTTTGCTGCT</td>
</tr>
<tr>
<td>FUN_R</td>
<td>TCTAAGGCGATCACAGACCTG</td>
</tr>
</tbody>
</table>

and probes with sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candid Probe</td>
<td>FAM-TTAAACCTACTAAATAGTGCTGCTAGC-BHQ1</td>
</tr>
<tr>
<td>Asperg Probe</td>
<td>TexasRed-TCGGCCCTTTAAATAGCCCGGTCCGC-Eclipse</td>
</tr>
</tbody>
</table>

8. The method according to any of claims 1 or 3 or 5 or 6 or 7, wherein the detection of bacteria and fungi is carried out in a sample of biological material isolated from a patient, preferably from the blood of a patient with symptoms of sepsis.

9. Oligonucleotides with the sequence:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GCCGGACGGGTAGTAAN</td>
</tr>
<tr>
<td>NEST_BAC_R</td>
<td>CGCATTTCCACCGCTA</td>
</tr>
</tbody>
</table>

for use as PCR primers for the detection of bacteria.

10. Oligonucleotides with the sequence:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATTGACGGAGGGCACCC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TTCCTCGTTGAAGAGCAA</td>
</tr>
</tbody>
</table>

for use as PCR primers for the detection of fungi.

11. A kit for detection of bacteria and/or fungi in a sample of biological material using the method described in claim 1, containing the following oligonucleotides:

for detection of bacteria, primers specific for bacterial 16S rRNA:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_BAC_F</td>
<td>GCCGGACGGGTAGTAAN</td>
</tr>
</tbody>
</table>
NEST_BAC_R | CGCATTTCACCGCTA

and

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN/GP_F</td>
<td>GACTCCTACGGGAGGC</td>
</tr>
<tr>
<td>GN/GP_R</td>
<td>GCGGCTGCTGGCAC</td>
</tr>
</tbody>
</table>

and probes specific for bacterial 16S rRNA with sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP_Probe</td>
<td>Hex-CTGAYssAGCAACGCGCGC-G-TAMRA</td>
</tr>
<tr>
<td>GN_Probe</td>
<td>Cy5-CCTGAYssCAGCmATGCGCGC-BHQ-2</td>
</tr>
</tbody>
</table>

and/or

for the detection of fungi primers specific for fungal 18S rRNA:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEST_FUN_F</td>
<td>AATTGACGGAAGGCACC</td>
</tr>
<tr>
<td>NEST_FUN_R</td>
<td>TTCCTCGTTGAAAGCAC</td>
</tr>
</tbody>
</table>

and:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUN_F</td>
<td>TTGGTGGAGTGATTTGTCTGCT</td>
</tr>
<tr>
<td>FUN_R</td>
<td>TCTAAGGCGCATACAGACCTG</td>
</tr>
</tbody>
</table>

and probes specific for fungal 18S rRNA with sequences:

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candid_probe</td>
<td>FAM-TTAACCTACTAAATAGTGCTGCTAGC-BHQ1</td>
</tr>
<tr>
<td>Asperg_probe</td>
<td>TexasRed-TCGGCCCTTAAATAGCCCCGGTCCG -Eclipse</td>
</tr>
</tbody>
</table>
Fig. 1
Sequences of fungal 18S rRNA with the designed primers NEST_FUN_F, NEST_FUN_R marked (gray box) and primers FUN_F, FUN_R known from the literature (transparent box)
Fig. 2
Sequences of bacterial 16S rRNA with the designed primers NEST_BAC_F, NEST_BAC_R marked (gray box) and primers GN/GP_F, GN/GP_R known from the literature (transparent box)

CLUSTAL W (1.83) multiple sequence alignment

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KC150149
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KC150200
CTTTCCTAGCTGAGGAGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG
JQ613981
CTTTCITATAGCTGAGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG
KC155289
CTTTATAGCTGAGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG
KC150142
CACTCAAGGGAGACAAGAGGCTAGCTGAGGCTTGGTCTGTAAGTAGGCTACG

* ******************** *

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JQ613981
GAACGGCTGCTTAACAGTCCGCAAGCGGTGTCGACTAACGCTGCTGTAAGTAGGCTACGAGGTATACG
KC155289
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JQ613981
CAATGGCGCGACCAGGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG
KC155289
CAATGGCGCGACCAGGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG
KC150142
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* ******************** *

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JQ613981
AGTACCTTTACCGGGAGGAGAA---GGTGGATAGTGGGTAACCTTGACCAAGGATACC
KC155289
AGTACCTTTACCGGGAGGAGAA---GGTGGATAGTGGGTAACCTTGACCAAGGATACC
KC150142
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GGACACCAAGGGAGACAAGAGGCTAGCTGAGGCTTGGTCTGTAAGTAGGCTACGAGGTATACG

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KC155289
GTAACTGGGATTACAGGGTGGGTAACCTTGACCAAGGATACC
KC150142
GTAACTGGGATTACAGGGTGGGTAACCTTGACCAAGGATACC

* ******************** *
```
Fig. 3

Percentage of infection of blood samples examined by Nested-multiplex real-time PCR for the presence of microorganisms in total and different types of: G+ bacteria, G- bacteria, Candida fungi and Aspergillus fungi.

Fig. 4

Comparison of sensitivity of detection of bacteria and fungi by culture and Nested-multiplex real-time PCR.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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Further documents are listed in the continuation of Box C. See patent family annex.

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    "A" document defining the general state of the art which is not considered to be of particular relevance
    "E" earlier application or patent but published on or after the international filing date
    "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
    "O" document referring to an oral disclosure, use, exhibition or other means
    "P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"A" document member of the same patent family

Date of the actual completion of the international search
11 September 2014

Date of mailing of the international search report
07/10/2014

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer
Hennard, Christophe
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<td>YANAGIHARA KATSUNORI ET AL: &quot;Evaluation of pathogen detection from clinical samples by real-time polymerase chain reaction using a sepsis pathogen DNA detection kit&quot;, CRITICAL CARE, BIOMED CENTRAL LTD, LONDON, GB, vol. 14, no. 4, 24 August 2010 (2010-08-24), page R159, XP021085524, ISSN: 1364-8535, DOI: 10.1186/CC9234 page 3, tabl e 1, col umn 2; page 8, concl usion</td>
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Form PCT/ISA/210 (continuation of second sheet) (April 2005)
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