Chapter 6

Quantitative Detection of *Aspergillus* spp. by Real-Time Nucleic Acid Sequence-Based Amplification

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Abstract

Rapid and quantitative detection of *Aspergillus* from clinical samples may facilitate an early diagnosis of invasive pulmonary aspergillosis (IPA). As nucleic acid-based detection is a viable option, we demonstrate that *Aspergillus* burdens can be rapidly and accurately detected by a novel real-time nucleic acid assay other than qPCR by using the combination of nucleic acid sequence-based amplification (NASBA) and the molecular beacon (MB) technology. Here, we detail a real-time NASBA assay to determine quantitative *Aspergillus* burdens in lungs and bronchoalveolar lavage (BAL) fluids of rats with experimental IPA.

Key words: Real time, Nucleic acid sequence-based amplification, Molecular beacon, *Aspergillus* burden, Quantitative

1. Introduction

Invasive pulmonary aspergillosis (IPA) is a major cause of morbidity and mortality in immunocompromised patients. Therapeutic success depends on early diagnosis and appropriate initiation of antifungal therapy (1). Nevertheless, early diagnosis of IPA continues to be problematic for this population due to the lack of rapid, sensitive, and specific diagnostic methods. The combination of galactomannan (GM) detection and standard clinical, radiological, and histological examinations has been an important advance in the detection of IPA. However, GM testing has false positives and decreased sensitivity in patients under antifungal prophylaxis (2–4), which highlights a

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critical need for more sensitive, specific, and reliable diagnostic techniques. Molecular diagnostics have emerged as a promising method for the diagnosis of a variety of infectious diseases, including fungal infections (5). Real-time quantitative PCR (qPCR) has been extensively studied and explored as a tool for detecting and identifying *Aspergillus* and other pathogenic fungi in clinical samples (6–10), although the PCR technique still hasn't been approved for defining IA because of lack of standardization and the absence of a commercially available system (11, 12).

Nucleic acid sequence-based amplification (NASBA) is an isothermal amplification process that specifically amplifies RNA even in the presence of genomic DNA (13). The amplification of NASBA is highly robust yielding $>10^{12}$ amplicons in 30 min (14). The amplified single-stranded RNA can be detected easily in real time by molecular beacon (MB) probes, which are self-reporting, hairpin structured, single-stranded nucleic acid probes that brightly fluoresce when bound to their targets (15). As an alternative to PCR for molecular detection, real-time NASBA has been widely used for detecting RNA viruses such as enterovirus and HIV (16–18), certain microbial pathogens including *Legionella* species, *Vibrio cholerae* (19, 20), and pathogens from food and environmental samples (21, 22). To date, only studies of small scope have been reported using NASBA to detect *Aspergillus* species (23, 24), although a good diagnostic value was demonstrated by these studies. To extend the applicability of real-time NASBA assay to the quantification of the fungal load in order to monitor the progress of *Aspergillus* infection, we established a pan-*Aspergillus* real-time NASBA assay and validated with an experimented IPA model (25). We chose to develop a pan-*Aspergillus* assay to gain complete capture of *Aspergillus* species, even though *A. fumigatus* remains the most abundant invasive species.

Quantification of nucleic acids by real-time NASBA is achieved by correlating an increase in probe fluorescence to the amount of starting template. The point at which the fluorescence signal rises above the cutoff value (20% higher than the endpoint signal from no template control) is called the time to positivity (TTP) (Fig. 1a). The dynamic linear quantification range of different probes varies and has to be determined by a standard curve method (Fig. 1b), which shows a linear regression relationship between the log of the amount of starting template and the corresponding TTP value. To achieve reliable *Aspergillus* burden estimation, we have outlined several essential steps which include: (1) total nucleic acid extraction from lung tissue and bronchoalveolar lavage (BAL) fluids, (2) external standard

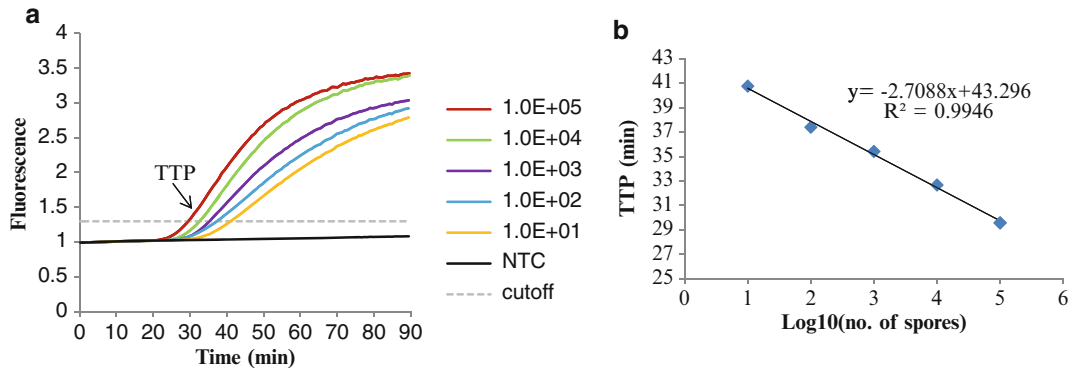

Fig. 1. (**a**) Amplification curves of the real-time pan-*Aspergillus* NASBA assay are produced using total nucleic acids extracted from serially diluted *A. fumigatus* conidia with 10^5 to 10^1 spores. The TTP value increases as the amount of template initially present in the reaction decreases. (**b**) The standard curve is constructed by plotting the log of starting amount against the corresponding TTP value. *Aspergillus* burden estimation is viable by simply applying TTP values from each sample to this linear regression equation.

series preparation, (3) molecular beacon purification and characterization, (4) real-time NASBA assay, and (5) standard curve and burden quantification.

2. Materials

Prepare all solutions using nuclease-free water and DNase, RNase-free molecular grade reagents. All waste materials should be disposed of in accordance with local waste disposal regulations.

2.1. Aspergillus fumigatus Standards

1. Potato dextrose agar (PDA) slant: suspend 39 g of PDA medium in 1 L of purified water. Heat and stir frequently to completely dissolve the medium. Autoclave at 121°C for 15 min. Pour the medium into a 25 ml Flat Base Polystyrene Screw Cap Tube and keep the tube in a slanting position until the agar solidifies.
2. Sterile 0.1% Tween 20 solution.
3. Disposable hemocytometer and an optical microscope with a 200× objective lens.

2.2. Total Nucleic Acid Extraction

1. RNA*later* solution (Ambion, Austin, TX).
2. NucliSENS lysis buffer (bioMérieux, Durham, NC) (see Note 1).
3. Proteinase K solution.
4. Lysing matrix D (MP, Biomedicals, Inc., Solon, OH) (see Note 2).

5. FastPrep®-24 instrument (MP, Biomedicals, Inc., Solon, OH).
6. EasyMAG® wash buffer 1, 2, 3 and EasyMAG® magnetic silica solution (bioMérieux, Durham, NC).

2.3. Molecular Beacon Purification and Melting Curve Analysis

1. Molecular beacon probes were labeled at the 5′-end with fluorophore 5-carboxyfluorescein (FAM) and quencher dabcyl (4-(4-dimethylaminophenyl) diazenylbenzoic acid) at the 3′-end.
2. Beckman Coulter System Gold® high-pressure liquid chromatography (HPLC) systems (Beckman Coulter, Fullerton, CA).
3. Buffer A: 1% triethylamine (TEA), adjust pH to 6.5 by Glacial acetic acid. Filtrate through a NALGENE or equivalent bottle filter (0.45 μm nylon filter).
4. Buffer B: 1% TEA, 75% acetonitrile, adjust pH to 6.5 by Glacial acetic acid. Filtrate through a NALGENE bottle filter (0.45 μm nylon filter) with 3–4 extra sterilized filter paper.
5. 3 M Sodium acetate (NaOAc).
6. 100% ice-cold ethanol.
7. Tris–EDTA (TE) buffer, pH 8.0.
8. Melting curve buffer: 1× PCR buffer with 4 mM $MgCl_2$.
9. Target oligonucleotide (100 μM): short oligonucleotide that is perfectly complementary to the molecular beacon probe sequence.
10. Stratagene Mx4000 or Mx3005P real-time instrument (Agilent Technologies, Santa Clara, CA).

2.4. Real-Time NASBA

1. NucliSENS EasyQ® Basic kit v.2 (bioMèrieux).
2. Primers detecting *Aspergillus* spp. targets 28S ribosomal RNA. The P1 primer is 5′-AATTCTAATAC GACTCACTATA GGGGA GAATCCACATCCAGGTGC-3′, and P2 primer is 5′-CAGCAGTTGGACATGGGTTA-3′ (see Note 3). Prepare a 20 μM working solution of each primer and store at −20°C until use.
3. A molecular beacon is designed for this pan-*Aspergillus* assay. The sequence is 5′-FAM-CGCGAGTGCGCCGTGTGCCGAA ATCGCG-Dabcyl-3′. The working solution is 10 μM and stored at −20°C until use.
4. 0.2 ml thin-wall PCR 8-tube strip (free of RNase, DNase, DNA, and PCR inhibitors).
5. NucliSENS easyQ® system (bioMérieux).

3. Methods

3.1. TNA Extraction from Lung Tissue and BAL Fluids of Rats with IPA

1. Thaw frozen lung homogenates (1 g lung tissue per sample) in RNA*later* or BAL samples (1 ml BAL + 2 ml RNA*later*) at room temperature and centrifuge at 16,000 × *g* for 10 min. Discard supernatant and resuspend the pellet in 1 ml of NucliSENS lysis buffer with proteinase K (100 µg/ml).
2. Transfer the entire solution into the lysing matrix D tube and vigorously vortex in a FastPrep instrument at speed level 6 for 45 s twice (see Note 4). Incubate at 55°C for 1 h (see Note 5).
3. Centrifuge cell lysates at 16,000 × *g* for 10 min. In the meantime, add 2 ml of NucliSENS lysis buffer into each well of the NucliSENS easyMAG® sample cartridge and dilute the magnetic silica twofold. Transfer the supernatant into the easyMAG® sample cartridge and mix with lysis buffer. Mix 140 µl of the diluted magnetic silica solution with each sample.
4. On easyMAG® system, input sample record and choose the extraction protocol "specific B" using the easyMAG® software. The final elution volume option increases from 40 to 110 µl at a 5 µl step. Choose the volume which best fits your experiment.
5. Store the TNA samples at –80°C until use.

3.2. External Standards Preparation and TNA Extraction

1. Grow *Aspergillus fumigatus* wild-type strain R21 on a PDA slant at 37°C for 3 days.
2. Add 2 ml of the 0.1% Tween20 into the slant. Collect and transfer the conidial suspension in a clean 2 ml tube. Vortex until the conidia is evenly distributed in the suspension.
3. Prepare tenfold serial dilutions of the above conidia suspension in 0.1% Tween20. Load 100- and 1,000-fold dilution on the hemocytometer to count the conidia (see Note 6).
4. Based on the cell counts acquired in step 3 prepare 1 ml of 5×10^6 CFU/ml conidia suspension in sterile 0.1% Tween20. Prepare further tenfold serial dilutions down to 5 CFU/ml.
5. Mix a 200 µl aliquot of each dilution with 800 µl of easyMAG lysis buffer (with 100 µg/ml of Proteinase K) and vortex on a FastPrep® instrument as described for lung tissue and BAL samples. The remaining steps of the extraction are the same.

3.3. Molecular Beacon Purification and Melting Curve Analysis

1. Resuspend the crude beacon in 500 µl of Buffer A. Incubate at room temperature for 5 min.
2. Spin product in 0.22 µm filter on a microcentrifuge at maximum speed for 3 min.

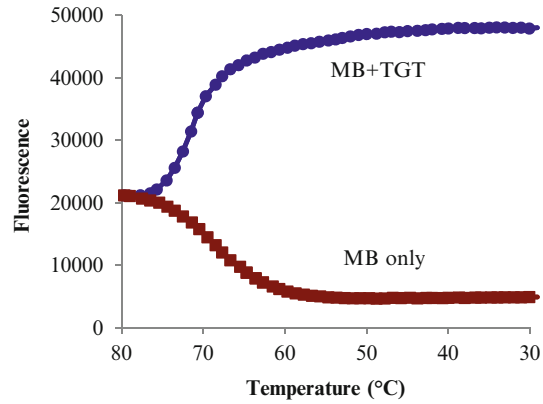

Fig. 2. Thermal denaturation profile shows a wide annealing range of the molecular beacon 28SPA.

3. Collect the filtrate and purify the crude molecular beacon by HPLC through a C-18 reverse-phase column, using a linear elution gradient of 20–80% buffer B that forms over 25 min at a flow rate of 1 ml/min. Monitor the absorption at 260 nm for nucleic acid and at 495 nm for FAM, and collect the fractions with peaks at both wavelengths, as described: http://www.molecular-beacons.com/MB_SC_protocol.html.
4. Precipitate the collected fractions with 2.5 volumes of cold 100% ethanol and 1/10 volume of 3 M NaOAc at –20°C for at least 2 h or overnight (see Note 7).
5. Spin in a microcentrifuge at maximum speed for 20 min at 4°C.
6. Aspirate the supernatant and dry the pellet.
7. Resuspend the pellet in 50 µl of TE buffer.
8. Measure the concentration of the purified molecular beacon. Prepare a 10 µM working solution and store at –20°C.
9. Prepare two 50 µl reactions that contain 200 nM of the molecular beacon in 1× melting curve buffer. Add a fivefold molar excess of the target oligonucleotide to one tube and add only buffer to another tube.
10. On a Stratagene Mx4000 or Mx3005P real-time instrument, run the molecular beacon melting curve program to determine the fluorescence of each solution as a function of temperature. Decrease the temperature of these tubes from 80 to 25°C in 1°C steps, with each hold lasting 30 s, while monitoring the fluorescence during each hold (Fig. 2).

3.4. Real-Time NASBA Assay

1. Prepare enzyme by adding 45 µl of enzyme diluent to enzyme accusphere (red cap), incubate at room temperature for 20 min before use (see Note 8). Each sphere is intended for 8

reactions, for additional reactions pool spheres and add the appropriate amount of diluent. Unused enzyme may be stored at −80°C for up to 2 weeks with 1 freeze–thaw cycle.

2. To create a primer/beacon mixture, add equal parts of primer P1, primer P2, molecular beacon, and Nuclease-free water, e.g.: mix 2 µl of each for a total of 8 µl for 8 reactions.
3. Prepare reagent mixture as follows (for 8 reactions): In a clean tube, add 64 µl of reagent diluent plus 9.6 µl of water and 14.4 µl of KCl (final concentration is 90 mM). Add 8 µl of primer/beacon mix. Add reagent accusphere (blue cap) to tube and vortex immediately (see Note 9).
4. Aliquot 10 µl of reagent mixture to each well in 8 tube strip.
5. Add 5 µl of TNA sample to each well in 8 tube strip.
6. Incubate strips in easyQ incubator for 2 min at 65°C and 2 min at 41°C (program 1).
7. While strips are incubating, add 5 µl enzyme into 8 strip caps.
8. After incubation, secure caps on tubes. Spin briefly in microcentrifuge, flick tube to mix thoroughly, and spin again. Move strips to easyQ analyzer (see Note 10).
9. Open "NucliSens® EasyQ Sample Login," enter sample ID and plate setup, and save the file in the default folder. Open "NucliSens® EasyQ Analysis Software," go to File, and open the file saved above. Select Edit, change the run time to 90 min (see Note 11). Click the Runs button located on the left margin, and place strips onto the NucliSens® EasyQ plate. Confirm that the temperature is 41°C, then click Start (see Note 12).
10. When run is complete (00:00 time is left), click "Out" to move tray to out position. Remove tube strips from the NucliSens® EasyQ Analyzer and discard properly. Go to "NucliSens® EasyQ Data Analyzer" to analyze the data.

3.5. Data Analysis and Burden Quantification

1. Open "NucliSens® EasyQ Data Analyzer." Click the "Start Import of Measurement Data" button, select corresponding file and open.
2. View general tab, calculated data tab, or raw graphic data tabs to view results.
3. Set the threshold as 20% higher than the endpoint signal from the extraction negative control and determine the TTP for each sample on the calculated data tab, then export the data into a new excel spreadsheet.
4. Using the log number of conidia in the external standard and the paired TTP data, draw a standard curve and evaluate the linearity by calculating the R square and the linear equation and determine the best linear quantification range (see Fig. 1b).

5. Calculate the conidia equivalents (CE) for each TNA sample extracted from lungs and BAL fluids by placing the TTP values on the standard curve (see Note 13).
6. Estimate the fungal burden in lung tissue and BAL fluids after accounting for the dilution factor.

4. Notes

1. NucliSENS lysis buffer can be stored at an ambient temperature from 2 to 30°C before opening but should be stored at 2–8°C after opening. Make sure to equilibrate the buffer at room temperature for at least 30 min before use if it is stored at lower temperature.
2. We choose lysing matrix D in this protocol because other matrix tubes provided by MP contain smaller beads, which tend to clog the narrow ends of the disposable tips used on the easyMAG® instrument and may result in an extraction failure.
3. The Pan-*Aspergillus* real-time NASBA assay was designed for detecting *Aspergillus* spp. and showed a high degree of specificity >95%. However, due to the fact that *Penicillium chrysogenum* is 99% homologous to *Aspergillus* spp. in the amplifying region, the cross-reaction from this species would be expected. Other species like the AIDS pathogen *P. marneffei* (26) have the potential to cross-react. This assay did not cross-react with other fungal and bacterial species tested in our lab.
4. Place samples on ice between each FastPrep cycle to rapidly cool down the temperature inside of the tube. When finishing the FastPrep step open cap to degas before the incubation. Because the foam will be produced during the FastPrep, cooling and degassing will help to reduce the volume.
5. The incubation time can be increased if any visible tissue chunk still exists after 1 h incubation. Additional time of digestion and the shaking platform help thorough digestion.
6. Depending on the density of the original conidia suspension, greater dilutions may be needed for accurate counting.
7. At least 2 h incubation time at −20°C is required. Less time for precipitation will reduce the final yield of the purified beacon.
8. Do not vortex the enzyme. Enzyme must be used within 1 h of reconstitution.
9. If more than 8 reactions are desired, pool reagent diluent, water, KCl, and primer/beacon mix. Add appropriate number of reagent accuspheres to tube and vortex immediately. If less

than 8 reactions are desired, add 64 µl reagent diluent directly to reagent accusphere and vortex immediately. Once sphere is in solution, reagent may be aliquoted and stored at –70°C for up to 2 weeks with 1 freeze–thaw. Proceed with addition of water, KCl, and primer/beacon mix. Do not freeze after addition of water, KCl, and primer/beacon mix.

10. These steps should be performed quickly to keep temperature at 41°C.
11. Make sure that the sample ID, plate setup, and running profile are ready before proceeding with NASBA assay setup. Do not leave them until step 8 is finished, otherwise it won't be possible to get the instrument ready without miscalculating the TTP.
12. Do not close the window of "NucliSens® EasyQ Analysis Software" before NASBA run is completed (data will be lost).
13. The CE calculation can only be made within the linear quantification range of the standard curve. Be careful when the TTP value falls outside of the range. The externalization is not recommended. Because the amplification efficiency, which is reflected by the slope of the equation, is different from what is determined for the linear quantification range when the amount of template is too low or too high, the application of the equation on outliers will bias the real fungal burden. Thus, leave the CE estimation as such (<lower linear quantification limit or > higher linear quantification limit) when you have outliers.

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