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Update on the diagnosis of invasive fungal infection

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ABSTRACT

The number of patients at risk of suffering invasive fungal infection (IFI) is increasing. Because of its high mortality, new rapid and accurate diagnostic tools are needed. Last advances in invasive candidiasis diagnosis comprise Peptide Nucleic Acid Fluorescent In-Situ Hybridization (PNA-FISH), direct MALDI-TOF or multiplex acid nucleic testing. While all of them rely in positive blood cultures, T2Candida® uses PCR coupled with T2Magnetic resonance detection directly in whole blood, allowing detection of 1-3 UFC/mL of *Candida* in about four hours. Beyond galactomannan (GM), novelties in IFI caused by molds include the international standardization of PCR techniques, with several commercial kits available. A combination of GM and PCR appears to be a good diagnostic strategy for invasive aspergillosis. PCR coupled to electrospray ionization/mass spectrometry and detection of volatile organic compounds in exhaled air by gas chromatography/mass spectrometry are other promising approaches to IFI diagnostic that still need to be validated.

Key words: diagnosis, *Candida*, mold.

Actualización en el diagnóstico de la infección fúngica invasora

RESUMEN

El número de pacientes en riesgo de padecer infección fúngica invasora (IFI) está en aumento. Debido a su elevada mortalidad, es necesario disponer de nuevas herramientas diagnósticas más rápidas, sensibles y específicas que las que disponemos en la actualidad. Los últimos avances en el diagnóstico de la candidiasis invasora incluyen Hibridación In Situ de Ácidos Péptidonucleicos (PNA-FISH), MALDI-TOF directo o PCR múltiple. Mientras que todas estas técnicas se realizan sobre frascos de hemocultivo positivos, T2Candida® se basa en una PCR con detección por resonancia magnética T2 directamente en sangre total, y permite la detección de entre 1-3 UFC/mL de *Candida* en aproximadamente 4 horas. Más allá del galactomanano (GM), una de las últimas novedades en el diagnóstico de IFI causada por hongos filamentosos es la estandarización internacional de las técnicas moleculares, con la aparición de varios kits comerciales. Una buena estrategia para el diagnóstico de aspergilosis invasora es la combinación de GM y PCR. La PCR asociada a ionización por electrospray/espectrometría de masas y la detección de compuestos orgánicos volátiles en aire exhalado mediante cromatografía de gases asociada a espectrometría de masas son otras aproximaciones prometedoras al diagnóstico de IFI que aún deben ser validadas.

Palabras clave: diagnóstico, *Candida*, hongo filamentoso.

An increasing number of patients are at risk of suffering opportunistic invasive fungal infection (IFI): solid organ transplant (SOT) recipients, haematological patients undergoing hematopoietic stem cell trans-plantation (HSCT), neoplastic diseases, AIDS, immunosuppressive therapy, major surgery, chronic pulmonary diseases, etc. Among them, invasive candidiasis causes almost 70% of all IFIs around the world, followed by cryptococosis (20%), and aspergillosis (10%)[1]. Other molds such as *Zygomycetes*, *Fusarium* and *Scedosporium* species are emerging in the last few years and represent a cause of concern[2]. The mortality attributed to invasive candidiasis varies from 30-50%, and it can reach almost 100% in some molds[3]. Late initiation of antifungal therapy significantly increases mortality in invasive candidiasis[4], which is why early diagnosis techniques are urgent.

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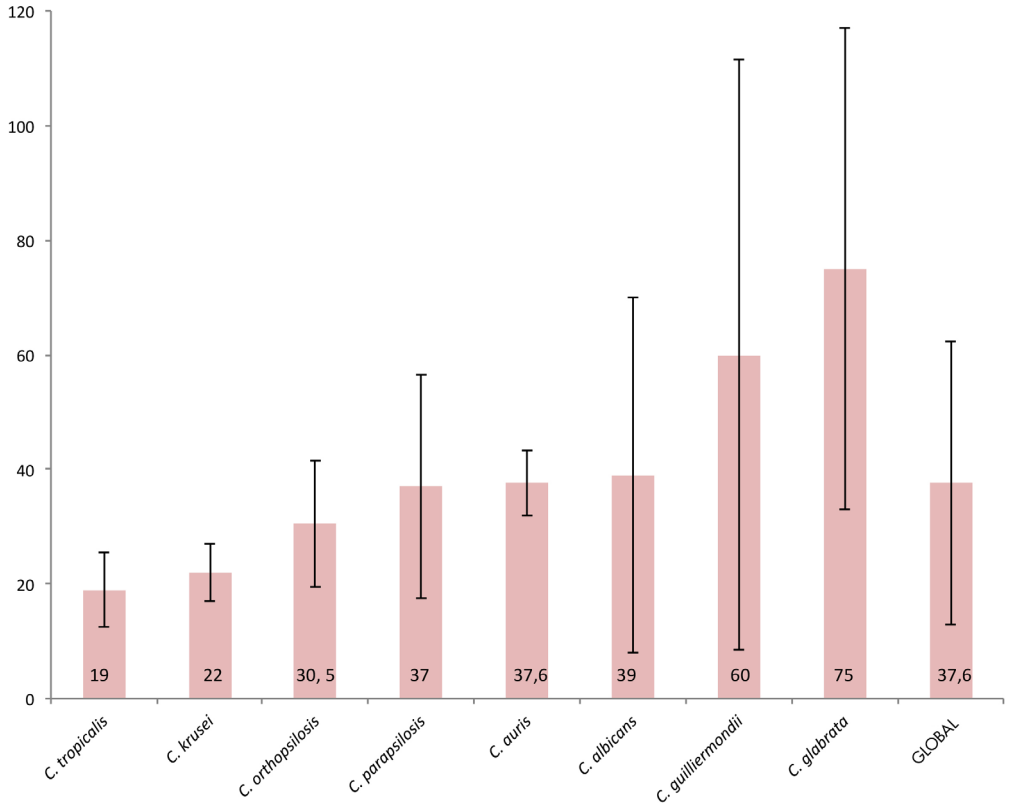

Figure 1 | **Medium time of growth (hours) of different *Candida* species in blood cultures Data from 258 candidemia episodes in intensive care units at La Fe University Hospital**

INVASIVE CANDIASIS

Despite its low sensitivity (50-60%), blood culture remains as the gold standard method to diagnose candidemia. Time to positivization of blood cultures varies between different species of *Candida* from 19-22 hours in *Candida tropicalis* and *Candida krusei* to 60-75 hours in species such as *Candida guillermondii* or *Candida glabrata*, with a mean time of growth of 37.6 hours (data from 258 candidemia episodes at La Fe University Hospital, figure 1). Once the blood culture is positive, it takes about 15 minutes to learn whether the causing agent of the blood stream infection is a yeast with a gram stain. However, final identification with traditional methods such as Auxa*Color*™ 2 (Bio-Rad, Marnes-la-Coquette France), Vitek® 2 YST ID card (bioMérieux, Marcy l'Etoile, France) or CHROMagar™ Candida (CHROMagar, Paris, France) takes around 24-48 hours. Some novel strategies to shorten this diagnostic period include Peptide Nucleic Acid Fluorescent In-situ Hybrydization (PNA-FISH), direct MALDI-TOF from the blood culture bottle, multiplex acid nucleic testing like Filmarray, or non-blood culture based methods such as T2Candida (figure 2):

Yeast Traffic Light PNA FISH™ (AdvanDX; Woburn, MA) is a next-generation, three-probe Peptid Nucleic Acid Fluorescence In-Situ Hybridization system which is FDA cleared for the rapid identification of *C. albicans/C. parapsilosis* (green fluorescence), *C. glabrata/C. krusei* (red fluorescence) *and C. tropicalis* (yellow fluorescence). It is performed from positive blood cultures in 90 minutes. The colour also provides a preliminary indication of fluconazole susceptibility, with green suggesting likely susceptibility, red indicating likely resistance, and yellow suggesting concern for inducible resistance. Other *Candida* spp., non-*Candida* yeast, and bacteria show no fluorescence[5]. The utilization of PNA FISH has demonstrated a reduction in the median time required for identification in *C. albicans* to 9.5 h compared to the standard culture median time of 44 h. The most pronounced effect of the PNA FISH test is on the reduction of echinocandin usage in patients with candidemia due to *C. albicans*. In these patients fluconazole substituted caspofungin after notification of the PNA FISH results, with a significant decrease in antifungals costs of $1,978 per patient[6].

MALDI-TOF technology is available in more hospitals each day. The biggest advantage of this technique is its promptness. It takes no more than 5 minutes to identify a microorganism from isolated colonies. To accelerate even more this process microbiologists have developed a protocol to identify yeasts with MALDI-TOF directly from positive blood culture bottles in 30 minutes without performing a subculture. The proto-

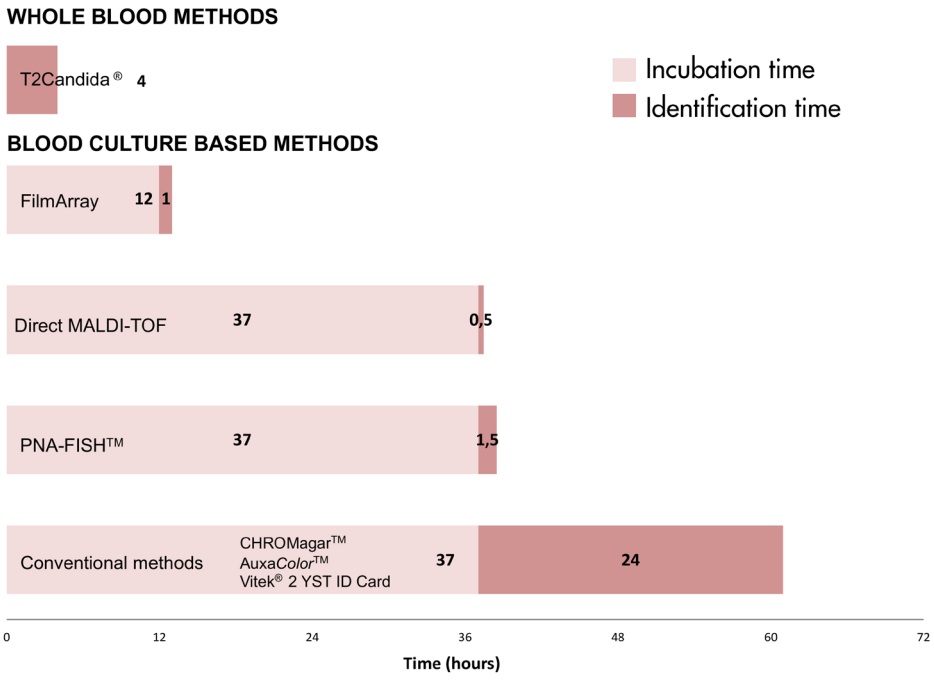

Figure 2 Graphic representation of time (hours) to yeast identification depending on employed technique.

Figure 3 T2Candida® instrument

cols available for the Bruker Biotyper MALDI-TOF MS (Bruker Daltonik GmbH Leipzig, Germany) and for the VITEK® MS (bioMérieux, Marcy l'Etoile, France) systems comprise several centrifugations, washings and treatments with 0.1% Tween 80, ethanol, formic acid and acetonitrile in order to concentrate the sample, precipitate proteins and remove completely

all blood cells that can interfere with the yeast spectra. Results of this protocol make MALDI-TOF one of the more promising alternatives to accelerate species-level identification of yeasts from blood cultures, with a sensitivity of 95.9% for *C. albicans* and 86.5% for non-*albicans Candida* species, being *C. guillermondii* the specie most frequently missed. The main limitation of this process is that it does not identify polifungal infections and that sample preparation requires time and expertise[7].

Other possibilities that are currently being developed and used in clinical settings are **multiplex-PCR platforms**. In this area one of the available tools nowadays is the FilmArray platform (FA; BioFire, Salt Lake City, UT), a closed diagnostic system allowing high-order multiplex PCR analysis with automated readout of results in one hour directly from positive blood cultures or after a 12 hours incubation. The FilmArray BCID panel for blood cultures targets 24 pathogens: eleven Gram-negative bacteria, eight Gram-positive bacteria and three antibiotic resistance genes, as well as five *Candida* species: *C. albicans, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*[8]. Filmarray has demonstrated a sensitivity of 99.2% with a 99.9% of specificity for all yeasts and 99.8% specificity for *C. albicans* in a multicentre controlled trial with 2,207 positive blood cultures[9]. The major advantages of this tool are that it covers a wide number of common pathogens with a good sensitivity and specificity, requires very little technical preparation and a very short time: it provides results in one hour with only 5 minutes for assay setup; and it detects polimicrobial infections. However, the fact that only one sample can be run at a time might be a rate-limiting step for a rapid diagnostic method. On the other hand, the emergence of pathogens such as *C. auris* in some hospitals make it necessary to evaluate the usefulness of this multiplex PCR techniques according to local epidemiology.

The main disadvantage of all these three methods of identification is that they still rely on a positive blood culture, which can take from 20 to more than 60 hours. As time in sepsis is gold, a new approach has been developed to shorten the time needed for invasive candidiasis diagnosis: **T2 magnetic resonance (T2MR)** is an assay that can be performed in whole blood without any previous incubation or DNA extraction. It lyses the *Candida* cells, amplifies the DNA with pan-*Candida* PCR primers, and finally detects the amplified product directly in the whole-blood matrix by agglomeration of superparamagnetic nanoparticles bearing target-complementary probes. Nanoparticle clustering yields changes in the T2 relaxation time, making it detectable by magnetic resonance. T2Candida® can detect five *Candida* species (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei*) and results in a >10-fold decrease in time to result while achieving detection sensitivities of ~1 colony-forming unit (CFU)/mL. It only needs 2-4 mL of whole blood, reason why it can be used in paediatric patients too. Findings in a 12 hospitals multicentre study, with 1,801 samples, endorse the utilization of T2Candida, with a sensitivity and specificity of 91.1% and 99.4% respectively, a limit of detection ranging from 1-3 CFU/mL depending on the *Candida* specie and a NPV of 99.4% in a 5% prevalence population, with a meantime to negative result of 4.2 hours. The ability to rapidly exclude candidemia can have a significant impact in clinical practice decreasing the number of patients on empiric antifungal therapy and thus the incidence of resistant strains, the potential side effects of antifungal treatment, and a substantial reduction in healthcare costs[10].

T2Candida® has also proved to detect deep-seated invasive candidiasis (IC) with negative blood cultures that were confirmed later by a positive tissue biopsy or by culture of a normally sterile site, in patients who were on antifungal therapy. In some cases, the discovery of the focus was performed even a week later and in one case, 12 sets of blood cultures were negative prior to diagnosis by biopsy. These findings highlight the potential of T2Candida in detecting not only candidemia but also deep-seated candidiasis[11].

An economical model has showed that T2Candida® has the potential to significantly reduce costs and mortality rates in patients at high risk for candidemia. In a hospital admitting 5,100 high-risk patients per year, assuming a 3% prevalence rate, the estimated potential savings per patient with candidemia is $26,887, a 48.8% reduction in hospital costs in candidemia. The application of species-specific therapy enabled by rapid *Candida* identification demonstrated potential savings of over 30 lives per year in a typical hospital setting, corresponding to a 60.6% reduction in mortality. Moreover, the potential savings in empirical therapy in non candidemic patients would be $4,521,081 (42.8%) in total costs or $886 per tested patient[12].

Therefore T2Candida® is a rapid and simple diagnostic tool with a response time of less than 4 hours and sample preparation of less than 10 minutes. It requires a minimum amount of blood allowing pediatric use, has a very low limit of detection (1-3 UFC/mL) and it is capable of detecting IC without candidemia, with the potential to save almost 50% of candidemia related-costs. However, it only detects five species, grouped in four options, the reagents have a very short expiration date and it costs 300 € per sample, cost that has its counterpart in total hospital savings but that needs to be very well justified to the hospital management[10-12].

INVASIVE FILAMENTOUS FUNGAL INFECTIONS

Classical mold IFI diagnosis relies on culture of samples such as sputum or bronchoalveolar lavage (BAL), along with histopathologic detection of the fungus on biopsy specimens. But culture is slow and has a low PPV (around 72%), which can be even lower in non-haematologic patients or those on antifungal therapy[13].

There had been advances in the diagnosis of invasive aspergillosis (IA), the most widespread being **galactomannan** (GM) in serum or BAL. However, GM is far from being a perfect diagnostic tool, its sensitivity and specificity do not exceed 85%, antifungal prophylaxis reduces specificity, and antifungal therapy reduces the sensitivity of the test[13]. Nowadays IFI diagnosis relies on late non-pathognomonic radiologic findings, reason why there is still a need of new diagnostic tools to improve mold IFI diagnosis.

There are multiple **PCR techniques** targeting *Aspergillus* spp. or *A. fumigatus*. The lack of standardization of these systems has prompted an European initiative for standardization of *A. fumigatus* PCR (EAPCRI) which, through the distribution of quality control panels, has leaded to the creation of recommendations for PCR protocols and a standardization of the technique[14]. Although PCR was excluded from EORTC/MSG definitions of IA because of the lack of a standard methodology, it will probably be included in next editions[15]. There are now several commercial assays for *A. fumigatus* PCR: MycoGENIE (Ademtech), AsperGenius (PathoNostics), Fungiplex (Renishaw) and Septifast (Roche). They are validated for blood, bronchoalveolar lavage, and even for biopsies (MycoGENIE). Most commercial assays dispose of a standardized PCR amplification system that when combined with EAPCRI recommendations provide a fully standardized approach[16].

The most interesting advance in PCR techniques is AsperGenius (PathoNostics, Maastricht, the Netherlands), a new multiplex real-time PCR assay consisting of two multiplex real-time PCRs, one that identifies clinically relevant *Aspergillus* species (*A. fumigatus* complex, *A. terreus*, *A. flavus, A. niger, A. nidulans*), and one that detects the TR34, L98H, T289A, and Y121F mutations in CYP51A and differentiates susceptible from resistant *A. fumigatus* strains. Its overall sensitivity, specificity, PPV, and NPV are 84.2%, 91.4%, 76.2%, and 94.6%, respectively[17].

The diagnostic odds ratio for *A. fumigatus* PCR is comparable to that of GM, with a sensitivity of 84-88% in blood and 76.8-79.6% in BAL and a specificity of 75-76% and 93.7-94.5% respectively. Its specificity in BAL is greater than GM's, while in blood is significantly lower. Sensitivity appears higher for PCR in blood than for GM. It looks like combining PCR in blood for screening and PCR in BAL for a diagnostic test is an interesting approach[16].

Moreover, early diagnosis and preemptive therapy of IA with a combination of PCR and GM compared to GM alone has showed a relative risk reduction of 68.1% in proven or probable IA and a reduction in time to diagnosis of one week in a large multicenter randomized trial conducted in 13 Spanish centers. This fact suggests that DNAemia precedes the release of fungal GM into the bloodstream, therefore a strategy combining diagnosis with both PCR and GM looks quite appealing[18].

Both PCR and GM are oriented to the detection of *A. fumigatus*, but the emergence of other filamentous fungus like Mucorales or *Scedosporium* species urge the development of panfungal diagnostic systems[2]. In this area, both PCR amplification followed by Electrospray Ionization/Mass Spectrometry (PCR/ESI-MS) and detection of Volatile Organic Compounds (VOCs) in exhaled air by Gas Chromatography/Mass Spectrometry (GC-MS) are the latest approaches for invasive mold infection diagnosis:

A broad-range multilocus **PCR/ESI-MS** to detect and identify fungal organisms directly from clinical specimens has showed to provide a rapid and sensitive detection and identification of fungal organisms directly from BAL specimens. It even detected and identified at least one fungal organism in 47.3% of the specimens where the standard culture method failed, and shows an agreement in identification with standard procedures of 62.7% at species level and 81.3% at genus level[19]. There is already a commercial platform of PCR/ESI-MS: Iridica™ (Abbott, USA). It detects more than 200 fungal species; performance time is less than six hours and can be run both in whole blood and in BAL, with a cost of only $30 per sample.

On the other hand, there is an increasing interest in the use of biomarkers like **VOCs** in exhaled breath for clinical diagnosis and management of diseases such as asthma, COPD or lung cancer. There is, too, an incipient research in the diagnosis of infectious diseases using these volatile biomarkers. The biggest advantage of exhaled breath is that it is characteristically non-invasive, reason why invasive techniques such as BAL and bronchial biopsy could be avoided[20,21].

2-pentylfuran appears to be a good marker of invasive aspergillosis (IA), with a sensitivity of 77% and a specificity of 78% compared to BAL or sputum culture[22]. However, *A. fumigatus* produces a great variety of characteristic VOCs in vitro, that can vary depending on the environment conditions or antifungal therapy[23,24] . It looks like a set of metabolites, rather than a very specific one, can be the "breath-signature" of each fungus, and it has been proved that 4 metabolites (α-trans-bergamotene, β-trans-bergamotene, β-vatirenene, and trans-geranylacetone) can differentiate patients with and without IA with 94% sensitivity and 93% specificity[24]. Further investigation needs to be carried out in this very interesting and applicable technique.

It should not be forgotten that a traditional technique such as direct microscopic examination of a sample with calcofluor white remains the most economic and rapid diagnostic tool and, although its sensitivity is low, it can detect mold IFI in less than 15 minutes. However, it requires expertise and it is not available in hospitals were there are no clinical microbiologists on call.

Although at the moment there is no perfect diagnostic method for invasive fungal infection, significant advances have been made in the last few years. Timely diagnosis of IFI is necessary to prevent its high morbidity and mortality, reason why further studies standardizing the already developed technologies and deepening in the knowledge of novel tools such as MALDI-TOF, T2Candida®, PCR/ESI-MS or VOCs detection with GC-MS are needed.

REFERENCES

1. Pemán J, Salavert M. Epidemiología general de la enfermedad fúngica invasora. Enferm Infecc Microbiol Clin 2012;30(2):90–8.
2. Slavin M, van Hal S, Sorrell TC, et al. Invasive infections due to filamentous fungi other than *Aspergillus*: Epidemiology and determinants of mortality. Clin Microbiol Infect 2015;21(5):490.e1-490.e10.

3. Pemán J, Quindós G. Aspectos actuales de las enfermedades invasoras causadas por *Candida* y otros hongos levaduriformes. Rev Iberoam Micol 2016;33(3):133–9.
4. Ostrosky-Zeichner L, Kullberg BJ, Bow EJ, et al. Early treatment of candidemia in adults: a review. Med Mycol 2011;49(2):113–20.
5. Hall L, Le Febre KM, Deml SM, Wohlfiel SL, Wengenack NL. Evaluation of the Yeast Traffic Light PNA FISH probes for identification of *Candida* species from positive blood cultures. J Clin Microbiol 2012;50(4):1446–8.
6. Forrest GN, Mankes K, Jabra-Rizk MA, et al. Peptide nucleic acid fluorescence in situ hybridization-based identification of *Candida albicans* and its impact on mortality and antifungal therapy costs. J Clin Microbiol 2006;44(9):3381–3.
7. Spanu T, Posteraro B, Fiori B, et al. Direct MALDI-TOF mass spectrometry assay of blood culture broths for rapid identification of *Candida* species causing bloodstream infections: An observational study in two large microbiology laboratories. J Clin Microbiol 2012;50(1):176–9.
8. Altun O, Almuhayawi M, Ullberg M, Özenci V. Rapid identification of microorganisms from sterile body fluids by use of FilmArray. J Clin Microbiol 2015;53(2):710–2.
9. Salimnia H, Fairfax MR, Lephart PR, et al. Evaluation of the FilmArray Blood Culture Identification Panel: Results of a Multicenter Controlled Trial. J Clin Microbiol 2016;54(3):687–98.
10. Mylonakis E, Clancy CJ, Ostrosky-Zeichner L, et al. T2 Magnetic Resonance Assay for the Rapid Diagnosis of Candidemia in Whole Blood: A Clinical Trial. CID 2015;60(892):899.
11. Pfaller MA, Wolk DM, Lowery TJ. T2MR and T2Candida: novel technology for the rapid diagnosis of candidemia and invasive candidiasis. Futur Microbiol 2015;11(1):103–17.
12. Bilir SP, Ferrufino CP, Pfaller MA, Munakata J. The economic impact of rapid *Candida* species identification by T2Candida among high-risk patients. Future Microbiol 2015;10(7):1133–44.
13. Arvanitis M, Mylonakis E. Diagnosis of invasive aspergillosis: Recent developments and ongoing challenges. Eur J Clin Invest 2015;45(6):646–52.
14. White PL, Mengoli C, Bretagne S, et al. Evaluation of *Aspergillus* PCR protocols for testing serum specimens. J Clin Microbiol 2011;49(11):3842–8.
15. De Pauw B, Walsh T, Donnelly J, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008; 46:1813–21.
16. White PL, Wingard JR, Bretagne S, et al. *Aspergillus* Polymerase Chain Reaction: Systematic Review of Evidence for Clinical Use in Comparison With Antigen Testing. Clin Infect Dis 2015;61(8):1293–303.
17. Chong G-LM, van de Sande WWJ, Dingemans GJH, et al. Validation of a new Aspergillus real-time PCR assay for direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. J Clin Microbiol 2015;53(3):868–74.
18. Aguado JM, Vazquez L, Fernandez-Ruiz M, et al. Serum galactomannan versus a combination of galactomannan and polymerase chain reaction-based *Aspergillus* DNA detection for early therapy of invasive aspergillosis in high-risk hematological patients: A randomized controlled trial. Clin Infect Dis 2015;60(3):405–14.
19. Shin JH, Ranken R, Sefers SE, et al. Detection, identification, and distribution of fungi in bronchoalveolar lavage specimens by use of multilocus PCR coupled with electrospray ionization/mass spectrometry. J Clin Microbiol 2013;51(1):136–41.
20. van de Kant KDG, van der Sande LJTM, Jobsis Q, van Schayck OCP, Dompeling E. Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. Respir Res 2012;13:117.
21. Sethi S, Nanda R, Chakraborty T. Clinical application of volatile organic compound analysis for detecting infectious diseases. Clin Microbiol Rev 2013;26(3):462–75.
22. Chambers ST, Syhre M, Murdoch DR, McCartin F, Epton MJ. Detection of 2-pentylfuran in the breath of patients with *Aspergillus fumigatus*. Med Mycol 2009;47(August):468–76.
23. Heddergott C, Latgé JP, Calvo AM. The volatome of *Aspergillus fumigatus*. Eukaryot Cell 2014;13(8):1014–25.
24. Koo S, Thomas HR, Daniels SD, et al. A breath fungal secondary metabolite signature to diagnose invasive aspergillosis. Clin Infect Dis 2014;59(12):1733–40.