Detection of _Aspergillus_ species in BACTEC blood cultures

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Invasive aspergillosis is associated with high morbidity and mortality rates; nevertheless, blood cultures almost invariably yield a negative result. The recovery and detection time of _Aspergillus fumigatus_, _Aspergillus flavus_ and _Aspergillus terreus_ were studied in BACTEC Plus Aerobic/F, Mycosis-IC/F and Myco/F Lytic vials, incubated in the BACTEC 9240 and 9000 MB automated systems. Two different approaches were used for subculture in solid medium: (i) the routine method, using a sterile airway needle/subculture unit, and (ii) a novel procedure, using instead a tuberculin disposable syringe and collecting a larger aliquot (100 μl), following vigorous agitation of the vials. _A. fumigatus_ was detected at inoculum concentrations of >3 conidia per 10 ml after 21–40 h, in both BACTEC Plus Aerobic/F and BACTEC Mycosis-IC/F vials. A few more hours were needed to detect _A. flavus_ and _A. terreus_. The novel subculture procedure of BACTEC culture vials on solid medium resulted in several positive results that were not detected by the routine sampling procedure. BACTEC Plus Aerobic/F vials show an advantage particularly in patients under antifungal treatment. In cases of polymicrobial bloodstream infections (concurrent bacterial growth), the inoculation of blood samples into a BACTEC Mycosis-IC/F vial achieved the best results. Further multicentre studies are needed to validate this improved automated detection of _Aspergillus_ spp. from blood cultures in clinical laboratories, as this diagnostic procedure allows antifungal susceptibility testing of moulds.

INTRODUCTION

Invasive aspergillosis (IA) is a potentially life-threatening infection, caused mainly by _Aspergillus fumigatus_. In serious cases, _A. fumigatus_ may spread from the lung to other organs, resulting in haematogenously disseminated fungal disease (Dagenais & Keller, 2009; Segal, 2009). Detection of cell-wall components such as galactomannan and (1,3)-β-D-glucan is being used increasingly for IA diagnosis, but some false-positive reactions, which lead to inappropriate invasive diagnosis and overtreatment with antifungals, have been reported (Del Bono _et al._, 2008). Nucleic acid detection assays represent a valid alternative, but are not yet clinically validated and still require standardization (Perlin & Zhao, 2009). At present, isolation of the fungal organism represents the most important evidence of its viability, whilst also allowing determination of its antifungal-susceptibility pattern. Furthermore, the classification of ‘possible’, ‘probable’ and ‘proven’ IA is based on host factor, clinical manifestations and mycological evidence (De Pauw _et al._, 2008).

Considerable efforts were made to improve the detection of micro-organisms from blood specimens using rapid and...
reliable automated systems. BACTEC 9240 and BACTEC 9000 MB automated systems (Becton Dickinson) were conceived for continuous monitoring and detection of micro-organisms in broth culture (Fuller et al., 2001; Meyer et al., 2004). At present, blood cultures are useful for diagnosis of some invasive fungal diseases (Nucci & Anaissie, 2007). It is possible that Fusarium spp., Aspergillus terreus and other moulds would be detectable in blood cultures by the formation of small adventitious spores in tissue. Although A. fumigatus is able to grow in BACTEC culture vials, blood cultures of patients with IA are very frequently negative, and the reasons for this are not clearly established (Thuret et al., 2005). Girmenia et al. (2001) reported a restricted subset (10%) of positive blood cultures in patients with deep-seated aspergillosis, thus questioning the perceived lack of value of this strategy for the diagnosis of aspergillosis.

The purpose of the present study was to evaluate the detection of A. fumigatus, Aspergillus flavus and A. terreus in three distinct BACTEC culture media (Becton Dickinson): BACTEC Plus Aerobic/F, BACTEC Mycosis-IC/F and BACTEC Myco/F Lytic vials. Variables such as conidial concentration, type of inoculum (conidia or hyphae), presence of blood in BACTEC culture vials and presence of antifungals or other microbial agents were tested.

**METHODS**

**Fungal strains and growth conditions.** Three clinical isolates of A. fumigatus (strains F09, G77 and H01) belonging to the fungal collection of the Department of Microbiology, Faculty of Medicine, University of Porto, and the reference strain A. fumigatus ATCC 46645 were used in this experimental study. A. fumigatus strains were genetically distinct, as confirmed by microsatellite-based multiplex PCR (Araujo et al., 2009). One clinical isolate each of A. flavus and A. terreus were also tested. Fungal conidia were kept at 70°C in brain-heart infusion with 5% glycerol before testing. After thawing, the organisms were cultivated on Sabouraud agar slants at 35°C for 7 days. Conidia were harvested by flooding the agar surface with PBS and the conidial concentration was adjusted with a Densimat photometer (bioMérieux), as described previously (Araujo et al., 2004).

**Inoculation of conidial suspensions into BACTEC culture vials.** Serial suspensions of Aspergillus conidia, ranging from 50 to 10^5 conidia per 10 ml, were prepared in sterile PBS and viability was confirmed by c.f.u. counts on Sabouraud agar medium. The conidial inoculations were inoculated (in triplicate) into three distinct BACTEC culture media (Becton Dickinson) at final concentrations of 1, 3, 25, 250 and 2500 conidia per 10 ml. The media were: (i) BACTEC Plus Aerobic/F, used for the recovery of several aerobic and microaerophilic micro-organisms from blood specimens; (ii) BACTEC Mycosis-IC/F, with a selective culture medium for the recovery of yeast and moulds from blood specimens; and (iii) BACTEC Myco/F Lytic, designed for the detection of Mycobacterium spp. from blood specimens. BACTEC Plus Aerobic/F and Mycosis IC/F vials were incubated in the BACTEC 9240 automated system and BACTEC Myco/F Lytic vials were incubated in BACTEC 9600 MB automated system, as recommended by the manufacturer (Becton Dickinson). A distinct set of BACTEC culture vials with PBS (without Aspergillus conidia) was prepared in each assay as a negative control. BACTEC culture vials were incubated under continuous agitation at 35°C, according to the manufacturer’s instructions: 7 days for BACTEC Plus Aerobic/F vials, 14 days for BACTEC Mycosis IC/F vials and 42 days for BACTEC Myco/F Lytic vials.

**BACTEC culture vial in vitro test conditions**

**Addition of blood.** A set of BACTEC culture vials (Plus Aerobic/F, Mycosis IC/F and Myco/F Lytic) was inoculated (in triplicate) with fresh whole blood collected from healthy adult volunteers; 8 ml blood was added to BACTEC Plus Aerobic/F and Mycosis-IC/F vials, whilst 4 ml blood was added to BACTEC Myco/F Lytic vials, as recommended by the manufacturer. A. fumigatus conidial suspensions were then inoculated into the vials, resulting in final concentrations of 1, 3, 25, 250 and 2500 conidia per 10 ml.

**Inoculation of hyphae and hyphal fragments.** A concentration of 10^7 conidia per 10 ml of a strain of A. fumigatus (F09) was incubated in RPMI 1640 medium (Sigma-Aldrich) at 37°C for 15 h under agitation, allowing both conidial germination and hyphal growth, but preventing the formation of conidiophores and new conidia (hyphal clumps could be confirmed by optical microscopy). The hyphal suspension was then separated into two aliquots; one of the aliquots was homogenized by sonication for separation of hyphal clumps and fragmentation of A. fumigatus hyphae, as described by Sande et al. (2009). Serial dilutions were performed for both aliquots, resulting in final concentrations of 3 and 25 hyphal fragments per 10 ml, and then, both were inoculated into BACTEC culture vials (Plus Aerobic/F, Mycosis IC/F and Myco/F Lytic).

**Presence of bacteria or yeasts (polymicrobial cultures).** A single strain of A. fumigatus (F09) was inoculated into BACTEC culture vials simultaneously with a 24 h culture of each of Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 35218 and Candida albicans ATCC 10231. The final concentration of bacteria and yeast ranged from 3 to 30 c.f.u. per 10 ml – such microbial concentrations have been described from bloodstream infectious episodes (Bouza et al., 2007).

**Presence of antifungals.** Amphotericin B, caspofungin, posaconazole or voriconazole, at a final concentration of 1 µg ml⁻¹, was added to a set of BACTEC culture vials inoculated with serial conidial suspensions of A. fumigatus. The antifungal concentration represented the minimal serum concentration of antifungal agent usually considered necessary for antifungal treatment (Bajjoka et al., 1999; Freifeld et al., 2007). The A. fumigatus strain (F09) used in this assay was considered susceptible to all the tested antifungals, showing MICs of 0.5 µg ml⁻¹ to amphotericin B, 0.25 µg ml⁻¹ to posaconazole and 0.5 µg ml⁻¹ to voriconazole and a minimal effective concentration of 0.5 µg ml⁻¹ to caspofungin.

**Subculture of positive BACTEC culture vials.** According to the manufacturer’s recommendations, a positive result in the BACTEC automated systems suggested the presence of at least one micro-organism that must be subcultured in solid medium for further confirmation and characterization (Fuller et al., 2001; Meyer et al., 2004). Culture vials signalled as presumptively positive by the automated systems were therefore subcultured on Columbia agar +5% sheep blood in a 5–10% CO₂ atmosphere for 48 h at 35°C. Two distinct procedures were used for the recovery of moulds from the BACTEC culture vials: (i) the routine recommended method, using a sterile airway needle/subculture unit (bioMérieux) for collection of one or two drops of culture medium (about 25 µl), and (ii) a novel proposed procedure, using instead a tuberculin disposable syringe for collection of 100 µl, after vigorous agitation by hand of the culture vials (approx. 10 s) in order to fragment fungal balls. The plates were examined for 48 h and then discarded.
Data analysis. Time to positivity or detection time was defined as the time that elapsed from the time of initial incubation of BACTEC culture vials to the time of first indication of positivity for each culture vial by the automated systems; the mean value for detection time was obtained from three positive replicates. Excel 2003 and SPSS 16.0 applications were employed for data analysis, particularly for calculation of SD values. The ANOVA test, using the Bonferroni correction, and Student’s t-test for paired samples were used for data comparison. Data were compared at a significance level (P) of 0.05.

RESULTS AND DISCUSSION

Detection of Aspergillus spp. by BACTEC automated systems

A. fumigatus was detected by BACTEC automated systems soon after 20.7 ± 0.6 h and up to 99.9 ± 0.5 h (Fig. 1). No statistically significant differences (P>0.05) were observed between BACTEC Plus Aerobic/F and Mycosis-IC/F vials regarding the detection of A. fumigatus in PBS; A. fumigatus growth was detected significantly later in BACTEC Myco/F Lytic vials (P<0.001). These results are similar to those described by Nucci & Anaissie (2007). The detection of A. fumigatus by BACTEC automated systems was dependent on conidial concentration – the highest inoculum corresponded to faster detection results (Fig. 1). Nevertheless, low conidial concentrations of A. fumigatus, such as 3 conidia per 10 ml, could still be detected by BACTEC culture vials; the sole exception was the concentration of 1 conidium per 10 ml, which could not be detected by such devices. A. flavus and A. terreus were also detected by the BACTEC automated systems, ranging from 50.9 ± 0.3 to 37.3 ± 0.5 h, respectively. As A. fumigatus conidia germinate and grow significantly faster than those of the other Aspergillus species in liquid culture medium (Araujo & Rodrigues, 2004), it is comprehensible that A. fumigatus isolates could be detected a few hours earlier. BACTEC Plus Aerobic/F and Mycosis-IC/F vials showed similar results regarding Aspergillus detection, whilst the BACTEC Mycosis-IC/F vial has been described as more effective for the isolation of yeasts from blood specimens (Meyer et al., 2004). The BACTEC Myco/F Lytic vial was a reliable medium for the recovery of A. fumigatus, but no advantage was found in comparison to the other BACTEC culture vials, as suggested by Vetter et al. (2001). Macroscopic growth of A. fumigatus could be appreciated as a ‘fungal ball’ floating in culture medium (similar fungal balls could be observed in vials containing A. flavus or A. terreus).

In vitro testing conditions

Blood samples. Detection of A. fumigatus in BACTEC culture vials with human blood was similar to the results described above in PBS (P>0.05): detection time ranged from 21.8 ± 0.5 to 41.7 ± 0.8 h in BACTEC Plus Aerobic/F and BACTEC Mycosis-IC/F vials, respectively, and from 40.2 ± 1.5 to 102.0 ± 1.8 h in BACTEC Myco/F Lytic vials (the ‘fungal balls’ could not be observed clearly due to the opacity of blood samples). Thus, conidial growth is not inhibited by the presence of blood cells in BACTEC culture vials.

Type of inoculum. Hyphal clumps or hyphal fragments produced similar results to those observed with Aspergillus conidia. The presumptively positive results were detected soon after 26.9 ± 0.8 h – the equivalent conidial concentration was positive in a similar detection time. According to our results, the type of inoculum is irrelevant, as both conidia and hyphae are able to produce presumptively positive results in BACTEC culture vials.

Polymicrobial cultures. Curiously, the presence of bacteria or yeast in BACTEC culture vials frequently resulted in faster presumptive signals of a positive culture (ranging from 10.5 ± 0.2 to 25.7 ± 1.2 h), without the previously observed fungal ball growth. Such early detection is caused by the bacteria or yeast in culture, which prevent the growth of A. fumigatus. BACTEC Mycosis-IC/F vials inoculated with A. fumigatus and bacteria were an exception, resulting in similar detection patterns as described above for A. fumigatus in PBS (P>0.05). In fact, BACTEC Mycosis-IC/F medium contains the antibiotics chloramphenicol and tobramycin, which restrict bacterial growth, and a lysing agent (saponin), which induces haemolysis of blood cells and the subsequent release of phagocytosed fungi. In clinical settings, the diagnosis of A. fumigatus in polymicrobial bloodstream infections may be problematic if conducted in BACTEC culture vials other than BACTEC Mycosis-IC/F vials. Thus, these culture vials can be advantageous in the case of simultaneous inoculation of A. fumigatus and bacteria, similarly to results shown previously for yeasts and bacteria (Meyer et al., 2004).

Antifungal agents. Interestingly, BACTEC Plus Aerobic/F vials provided a similar detection of A. fumigatus in the...
presence of amphotericin B, caspofungin, posaconazole and voriconazole; positive results were detected starting at 21.6 ± 0.8 h up to 41.0 ± 1.0 h. However, no growth was found in BACTEC Mycosis-IC/F or Myco/F Lytic vials containing amphotericin B or posaconazole; both vials could detect A. fumigatus significantly later when caspofungin or voriconazole was present (ranging from 49.9 ± 1.1 to 226.6 ± 1.0 h) (P<0.001). Antifungal agents prevent the growth of moulds in culture vials; however, BACTEC Plus Aerobic/F vials showed a considerable advantage in the presence of antifungals. In fact, BACTEC Plus Aerobic/F vials contain polymeric adsorbing and cationic-exchange resins that probably block the activity of the antifungal agents, similarly to results described previously for antibacterial agents (Vetter et al., 2001). This aspect is pertinent as a significant number of at-risk patients are undergoing prophylactic or empiric antifungal treatment that might prevent mould detection.

**Recovery of Aspergillus spp. on solid culture medium**

The method recommended for subculture of presumptively positive BACTEC culture vials did not result in the recovery of Aspergillus spp., even when such vials presented visible ‘fungal balls’. When using the classic subculture technique, A. fumigatus, A. flavus and A. terreus were recovered soon after 48 h from all positive BACTEC culture vials initially inoculated with the highest concentration of conidia (2500 conidia per 10 ml), and occasionally at intermediate concentrations (250 conidia per 10 ml). Aspergillus spp. were never cultured from positive vials initially inoculated with 25 conidia per 10 ml or lower concentrations. However, the novel procedure succeeded in the isolation of A. fumigatus, A. flavus and A. terreus from all presumptively positive BACTEC culture vials, even with the lowest concentration of conidia (3 conidia per 10 ml), soon after 48 h. The results from subcultures of presumptively positive BACTEC culture vials containing human blood or polymicrobial cultures were similar to those obtained from subcultures of vials inoculated with A. fumigatus suspensions in PBS.

It was clear throughout this study that the subculture strategy presently employed by the clinical laboratories rarely recovers Aspergillus spp. from blood culture and, when it does, it is often considered a contaminant, with the exception of a few studies such as that by Girmenia et al. (2001). Therefore, such results are almost always interpreted as false-positive results when, in fact, they could represent a positive culture of A. fumigatus in a blood culture that is poorly analysed. We propose a novel procedure involving the vigorous agitation of BACTEC culture vials by hand (approx. 10 s) in order to fragment fungal balls, the use of tuberculin disposable syringes and subculture of a larger aliquot (100 µl) to overcome the limitations of the standard procedure. The true value of Aspergillus fungaemia for IA diagnosis should be rethought, considering the proper use of BACTEC cultures, and it is recommended that more studies similar to that of Girmenia et al. (2001) should be performed. It is probable that the correct use of BACTEC culture vials and the application of an improved subculture strategy to those cultures will increase the frequency of detection of Aspergillus fungaemia in patients with deep-seated aspergillosis.

The detection of moulds by automated systems in blood samples cannot replace early diagnostic methods, such as the detection of galactomannan or (1,3)-β-D-glucan, but it can complement such tests. The high number of false-positive results by both diagnostic methods may justify the need for an additional approach be used simultaneously. At present, and despite the fact that culture of the fungal organism may not represent the best tool for early diagnosis of IA, its unique features, such as evidence of viability and ability to perform susceptibility testing, should not be underestimated. BACTEC culture vials represent a reliable resource for the recovery of Aspergillus spp. from blood.

In conclusion, BACTEC automated systems may allow sensitive and fast detection of A. fumigatus, A. flavus and A. terreus in the blood of some patients. These Aspergillus species could be isolated from blood cultures, especially using the novel subculture methodology proposed in this paper. BACTEC Plus Aerobic/F vials may be more advantageous for Aspergillus detection in blood samples collected from patients under antifungal treatment, whilst BACTEC Mycosis-IC/F vials may be more advisable for cases of polymicrobial bloodstream infection. Further multicentre studies are still necessary to validate the potential use of such automated methods for Aspergillus detection in clinical laboratories.

**ACKNOWLEDGEMENTS**

The BACTEC culture vials were kindly provided by Quilaban – Química Laboratorial Analítica, Lda (Sintra, Portugal). The authors thank Bristol-Myers Squibb, Merck Sharp & Dohme, Schering-Plough Farma and Pfizer Inc. for providing the antifungal powders. R. A. was co-financed by the European Social Fund. IPATIMUP is partially supported by Fundação para a Ciência e a Tecnologia, Programa Operacional ‘Ciência, Tecnologia, Inovação’ (POCTI) and Programa Operacional do Potencial Humano (POPH-QREN).

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