FULL-LENGTH ARTICLE

Manufacturing

Technical challenges for complete implementation of automated growth-based methods for microbiological examination of advanced therapy medicinal products. What's wrong with *Candida albicans*?

Antonio Rodríguez-Acosta^{1,2,*}, Jesús Chaparro-García^{1,2,*}, Inmaculada De-Toro³,
 Rafael Maldonado-Sánchez^{1,2}, Raquel Muñoz-Fernández^{1,2}, Cristina Antúnez^{1,4},
 Cecilia Frecha^{1,2}, Laura Leyva^{1,2,5,a,**}

¹ Instituto de Investigación Biomédica de Málaga-IBIMA, Málaga, Spain

² Unidad de Terapia Celular, Hospital Regional Universitario de Málaga, Málaga, Spain

³ Servicio de Microbiología, Hospital Regional Universitario de Málaga, Málaga, Spain

⁴ Unidad de Expansión y Terapia Celular. Centro de Transfusión, Tejidos y Células, Málaga, Spain

⁵ UGC Neurología, Hospital Regional Universitario de Málaga, Málaga, Spain

ARTICLE INFO

Article History:

Received 23 June 2021

Accepted 15 November 2021

Keywords:

automated growth-based methods
 blood culture system
Candida albicans
 cell therapy products
 method suitability
 sterility testing

ABSTRACT

Background: Automated growth-based methods for sterility testing of cell-therapy products should be qualified to demonstrate that they are equivalent to, or better than, the conventional reference method. The aim of the present study was to assess the ability of the BACTEC FX40 system to detect low microbial contamination and to confirm the suitability of the method in the presence of seven different human mesenchymal cell-based products, according to Ph. Eur. 2.6.27. Additionally, a study to select the best vial to detect fungus contamination was performed.

Methods: Microorganisms representing Gram-negative, Gram-positive, aerobic, anaerobic, spore-forming, slow-growing bacteria, yeast and mold were prepared in either Dulbecco's PBS or seven biological matrices containing approximately 5, 10, and 15 colony-forming units (CFU) per sample. These preparations were inoculated to the specific media required for each test method: (i) BACTEC aerobic and anaerobic vials; (ii) aerobic and anaerobic media for direct inoculation; and (iii) Trypase soy 3P or Brucella blood agar plates. Colonies from cultures were identified using MALDI-TOF mass spectrometry.

Results: The BACTEC FX40 system, in both Dulbecco's PBS and the biological matrices with a 5-CFU inoculum, detected most of the microorganisms significantly faster than the conventional method, despite the presence of a matrix containing gentamicin and several matrices containing 10% DMSO. Conversely, it showed an extremely delayed detection of *Candida albicans* compared with the conventional method. The addition of a Mycosis IC/F (MYC) vial decreased radically the time to detection (TTD) of *C. albicans* (28.2 ± 1.8 h) compared with the conventional method (36 h).

Conclusions: When a MYC vial was added to the standard aerobic and anaerobic vials to test each sample, BACTEC FX40 was shown to be a superior alternative sterility method for cell-therapy products contaminated with low inocula, with a faster TTD for microbial growth compared with the conventional method (5 versus 14 days). The studies were carried out in different cell-based matrices with sensitivities and specificities of 100% for all the tested strains at 15-, 10- and 5-CFU inoculum, with the exception of *Kocuria rhizophila* at 5 CFU (90.48% sensitivity and 100% specificity).

© 2021 International Society for Cell & Gene Therapy. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

** Correspondence: Laura Leyva, MD, PhD, Unidad de Terapia Celular, Hospital Regional Universitario de Málaga, pab A, 7ª planta, Málaga 29010, Spain

E-mail address: laura.leyva@ibima.eu (L. Leyva).

* These authors contributed equally to this work.

^a Present address: Laboratorio de Investigación, Hospital Civil, pab 5, sótano, Málaga 29009, Spain

Introduction

Advanced therapy medicinal products (ATMPs) are a new generation of promising medicines that are expected to possibly meet one of the most complex set of regulations to make them available to patients. Most ATMPs are parenterally administered drugs and,

therefore, must be manufactured in aseptic conditions, complying with Good Manufacturing Practice (GMP) guidelines, as these medicinal products cannot undergo a terminal sterilization [1].

Pharmacopeial procedures established the conventional sterility test method, which consists of the development of turbidity in liquid culture media due to the growth of potential contaminants [2–4]. This test, following *Ph. Eur.* 2.6.1 "Sterility" [3], is not always suitable for ATMPs because of their short shelf-life, non-filterable properties, and limited amounts of sample available for testing, among other difficulties. Thus, a sterility method able to provide faster results and, at the same time, ensure the required degree of sterility is highly desirable. Rapid microbiological methods such as nucleic acid-based, immunological, and biosensor-based methods are in an early stage of development [5,6]; therefore, ATMPs are usually analyzed using automated growth-based methods (AGBMs). The general chapter *Ph. Eur.* 2.6.27 states: "These approaches may be applied when the test described in general chapter 2.6.1. Sterility, is required but cannot be performed for technical reasons or due to the characteristics of the specific cell-based preparation" [7]. Although these methods were initially designed to test human blood samples, in 2018, both Bact/Alert (BioMérieux, Durham, NC) and BACTEC FX (BD Diagnostics, Sparks, MD) systems obtained U.S. Food and Drug Administration (FDA) approval to test leukocyte-reduced apheresis platelet units [8,9].

According to *Ph. Eur.* 2.6.27 [7], it is not required to completely validate the microbiologic examination of cell-based preparations based on AGBM. However, the growth promoting capacities (growth promotion test) of each batch of culture media used for microbiological examination must be previously ensured. Additionally, the suitability of the method must be confirmed in the presence of the specific cell-based preparation, to demonstrate that the product does not interfere with the detection of ≤ 100 CFU of each of the suggested strains. Method suitability studies must also be performed on all new products, as well as whenever a change is made to the product formulation. These studies should be designed to assess the specificity, sensitivity, reproducibility and robustness of the test method in the presence of the specific sample composition. Only three replicates with ≤ 100 CFU of the selected microorganisms are required, and the routine sampling incubation period may be extended up to 14 days [7].

Numerous studies using AGBM with different matrix samples such as platelets [10], umbilical cord blood [11], chondrocytes [12], corneal products [13,14], hematopoietic progenitor cells [6,15,16] and human mesenchymal stromal cells (hMSCs) derived from bone marrow, cord blood or adipose tissue [17,18] suggest that both Bact/Alert and BACTEC FX systems are reliable and useful tools for the microbiologic control of ATMPs. Many of these studies revealed equivalent or better performances of AGBM compared with the conventional sterility method [19–25]. Furthermore, some of them clearly showed that cell-based samples, free of antibiotics and other well-known inhibitors of microorganism growth, do not interfere with the outcome of these methods [11,15].

Sensitivity and time to detection (TTD), defined as the minimum period required to detect microbial growth, depend on both the incubation temperature and the number of viable microorganisms inoculated for testing [11,26]. These days, traditional methods to prepare microbial suspensions, such as visual comparison with the McFarland scale or spectrophotometry, are being replaced by highly accurate commercial lyophilized strains with < 100 CFU. These methods face numerous challenges that prevent them from being standardized, including the type of vials used, the contact time of the microorganisms with the matrix sample before inoculating into vials, the incubation temperature and the number of viable microorganisms actually used.

The aim of the present study was to assess the ability of the BACTEC FX40 system to detect extremely low microbial contamination

(< 15 CFU) in seven different matrices prepared from hMSC-based products, alongside its capacity to ascertain potential differences in microorganism growth patterns when cell-based products are used as matrix samples. The first phase consisted of an extended growth promotion test to assess the culture media suitability of the vials in the BACTEC FX40 system compared with the conventional sterility test method vials by direct inoculation. For this purpose, we used a very low inoculum (≤ 5 CFU) of the microorganisms recommended by the general chapter 2.6.27 for growth promotion tests. These were used alongside *Kocuria rhizophila* to select the most appropriate combination of culture vials to test our medicinal products. The second phase included a method suitability test using the previously selected vials and the seven mentioned matrices, with a low number of the same microorganisms (15, 10 and ≤ 5 CFU). TTD was analyzed, along with the sensitivity of the vials and methods. Finally, after observing a very high dispersion in the TTD of *Candida albicans*, during the third phase we assessed which culture vials were able to detect fungus contamination with the shortest TTD and the highest sensitivity.

Materials and Methods

Strains

Strains used for the reproducibility study were selected from those listed in the *Ph. Eur.* 07/2017:20627, table 2.6.27.-1 for growth-promotion test and in table 2.6.27.-2 for method suitability studies. All of them were acquired as Bioball SingleShot (BioMérieux, Marcy l'Etoile, France) and contained 30 CFU per vial.

Staphylococcus aureus (NCTC 10788), *Bacillus subtilis* (NCTC 10400), *Pseudomonas aeruginosa* (NCTC 12924), *C. albicans* (NCPF 3179) and *Aspergillus brasiliensis* (NCPF 2275) were selected to test aerobic vials, whereas *Clostridium sporogenes* (NCTC 12935) and *Cutibacterium acnes* (DSM 1897) were used for the anaerobic vials. *C. acnes* was selected due to the lack of commercially available strains of *Bacteriodes fragilis* to prepare replicates with an accuracy of < 5 CFU. Instead of *Micrococcus spp.*, *K. rhizophila* (NCTC 8340), another micrococcaceae commonly isolated in our environmental monitoring program, was chosen. Strains used for inoculation were no more than five passages from the original master seed-lot, complying with the *Ph. Eur.* [27] and USP [28]. The strains were stored at -18°C or below until use.

Microbial inoculum preparation

With the purpose of obtaining samples with ≤ 5 CFU for the reproducibility study (growth promotion test), each 30-CFU Bioball was rehydrated with 3 mL of Sterile Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO) for 3 min, after which suspensions were carefully mixed through pipetting. To avoid the intrinsic inaccuracy of the needle-syringe method for measuring small volumes, aliquots of 0.5 mL were placed in sterile Eppendorf tubes using a micropipette. Then, the complete volume was aspirated and inoculated into vials or tubes by 1-mL syringes with 21G needles. A similar procedure was followed to prepare samples for the method suitability studies, replacing the DPBS with the matrix samples as the rehydration fluid. Suspension volumes were adapted to obtain inoculum of 15, 10 and ≤ 5 CFU.

The microbial load of suspensions was determined spreading, in duplicate (extended growth promotion) or triplicate (method suitability studies), 0.4 mL of the remaining volume of each set of samples onto irradiated Tryptic Soy 3P (TSA3) or Brucella Blood agar (BBA) plates, for aerobic and anaerobic microorganisms, respectively. Both plates were purchased from BioMérieux. The identification was confirmed through matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with a Microflex LT spectrometer (Bruker Daltonik, Bremen, Germany).

Culture methods and incubation conditions

For the conventional sterility test method by direct inoculation, 9-mL tubes of tryptic soy broth (TSB) and fluid thioglycolate medium with resazurin (THIO), both from BioMérieux, were used. Classic sterility testing consists of a parallel incubation of the sample in TSB broth under aerobic conditions at 20–25°C, and a replicate in the THIO broth under anaerobic conditions at 30–35°C, for ≤ 14 days. However, in our study, all tubes were incubated at $35 \pm 1.5^\circ\text{C}$ to match the incubation temperature at the BACTEC. Turbidity was checked twice a day through visual inspection to detect potential microbial growth. When detected, TTD was manually recorded, and an aliquot from the contaminated TSB or THIO broth was transferred to TSA3 or BBA plates, respectively, and incubated at $35 \pm 1.5^\circ\text{C}$, in aerobic or anaerobic conditions using an anaerobic gas generation system (AnaeroGen Compact and W-zip Plastic Pouches; Oxoid, Thermo Fisher Scientific, Waltham, MA) until macroscopic colonies were observed. Colonies were then identified using MALDI-TOF mass spectrometry; if more than one type of microorganism was recovered from the agar plates, the test was invalidated. The identification of positive cultures of *Aspergillus brasiliensis* was made through visual detection of mycelium spheres in TSB medium. Negative controls, comprising exclusively the matrix without microorganisms, for the TSB and THIO tubes were tested in parallel and should appear clear and transparent upon visual examination at the end of the incubation period.

The BACTEC FX40 is based on a fluorescence detection system that detects CO_2 production from active microbial metabolism. The media vials used were BACTEC Plus Aerobic/F (PAF) and Peds Plus/F (PPF) for aerobic growth, Lytic/10 Anaerobic/F (LAF) for detection of anaerobic bacteria and Mycosis IC/F (MYC) for detection of fungi, all from BD Diagnostics. PAF, PPF and LAF vials contained nonionic adsorbing and cationic exchange resins able to inactivate antibacterial and antifungal agents. MYC vials contained antibiotics to inhibit bacterial growth. All vials were incubated at $35 \pm 1.5^\circ\text{C}$. Automated readings were taken every 10 min throughout the incubation period. When the system detected an increase in CO_2 concentration within the vial, an audible and visual alarm was generated, and TTD was automatically recorded.

For identification purposes, positive PAF, PPF and MYC vials were punctured, and the fluid from the culture vial was spread onto TSA3 plates. Meanwhile, positive LAF vials were spread onto BBA plates. Subsequent incubation, recovery and microbial identification were performed as described above. To detect false-negative results, negative aerobic and anaerobic vials were sampled and seeded onto TSA3 or BBA plates at the end of incubation time and reincubated for 5 days at $35 \pm 1.5^\circ\text{C}$ in aerobic or anaerobic conditions, respectively.

Following Ph. Eur. 2.6.27., the incubation time for the growth promotion test should be of ≤ 7 days for all culture media; however, in the case of method suitability studies and routine samples, the incubation period could be extended up to 14 days. Based on previous results, BACTEC culture vials and TSB or THIO tubes in which no growth was detected at the end of day 14 underwent an extended incubation period of ≤ 21 days.

Extended growth promotion test

For every microorganism used to test aerobic vials, a total of 30 samples with ≤ 5 CFU and six negative controls, consisting exclusively of DPBS, were inoculated. For that purpose, six 30-CFU Bioballs from *S. aureus*, *B. subtilis*, *P. aeruginosa*, *C. albicans*, *A. brasiliensis* and *K. rhizophila* were resuspended in 3 mL DPBS in six different 15-mL Falcon tubes and aliquoted into Eppendorf tubes, as explained in Microbial inocula preparation. The total volume of each Eppendorf tube was aspirated and seeded into PAF vials ($n = 10$), PPF vials ($n = 10$) and TSB tubes ($n = 10$). Likewise, for each microorganism used to test anaerobic vials (*C. sporogenes* and *C. acnes*), a total of 20 samples with ≤ 5 CFU were seeded into LAF vials ($n = 10$) and THIO tubes ($n = 10$).

The remaining volume of each rehydrated Bioball was used to determine the microbial count in the inoculated samples by spreading 0.4 mL, in duplicate, onto TSA3 or BBA plates for aerobic or anaerobic microorganisms, respectively. Plates were incubated at $35 \pm 1.5^\circ\text{C}$ for ≤ 3 days for bacteria and 5 days for yeast and molds, reporting CFU at the end of the incubation time. For negative controls, two vials for every test microorganism and culture medium used were spiked with DPBS without microorganisms. The study design is described in Figure 1. A terminal subculture was performed with all negative cultures at the end of the incubation time to prove absence of microorganism growth. This included the removal of an aliquot of 0.3 mL from each vial or tube, which was spread on TSA3 or BBA plates and incubated at $35 \pm 1.5^\circ\text{C}$ for ≤ 5 days in aerobic or anaerobic conditions, respectively. If growth was detected, the vial was classified as a false negative (FN) and, if no CFU were recovered, the culture was classified as a true negative (TN) result.

Method suitability studies. Matrix samples preparation

The hMSCs were prepared in two different academic clean room facilities. The hMSCs used for this study derived from surplus cells during the validation of the manufacturing processes. All the volunteers who donated adipose tissue and umbilical cord for validation of the respective manufacturing processes gave written informed consent to allow the use of the surplus cells for the improvement of the manufacturing and quality control processes, as well as for further research.

Seven different matrix samples with a specific composition based on adipose- or cord blood-derived hMSCs were aseptically manufactured according to GMP guidelines. Some of them had excipients with potential interference properties, such as dimethylsulfoxide (DMSO) and gentamicin (Table 1). Each 30-CFU Bioball was rehydrated with the pertinent matrix sample, as described above. Furthermore, samples were seeded into PAF, PPF, and MYC vials and into TSB tubes for aerobic microorganisms, alongside LAF vials and THIO tubes for anaerobic bacteria, all while adapting the inoculated volume to the target number of CFU. Negative controls were included in all assays.

Statistics

Descriptive statistics for categorical variables were based on percentages and frequencies, and continuous variables were based on means, standard deviations (SDs), medians and interquartile ranges (IQRs). Data were analyzed using SPSS 25.0 for Windows (SPSS, Chicago, IL). Differences in the TTD for positive vials were analyzed using nonparametric tests. A P value < 0.05 was considered statistically significant. The growth of each strain among the different matrices was initially compared by means of Kruskal–Wallis test. Then, differences in the growth between two matrices were assessed with matrix pairwise comparison and adjusted for multiple testing with Bonferroni correction (P_c = corrected P). Sensitivity, specificity and positive and negative predictive values of each culture medium and strain were assessed with a contingency table. These were then compared using χ^2 test, considering 5% of significance.

Results

Extended growth promotion test

As mentioned, aliquots of each rehydrated Bioball were spread onto agar plates for the plate count method to obtain the microbial amount in the inoculated samples. Supplementary Table 1 shows that recovery after completion of the respective incubation times ranged from 2 to 6 CFU for the inoculation of suspensions containing ≤ 5 CFU.

For both the BACTEC FX system and direct inoculation methods, sensitivity after 7 days of incubation was 100% for all cases, with the

Figure 1. Study design of the extended growth promotion test. Each 30-CFU Bioball was rehydrated with 3 mL of DPBS. Aliquots of 0.5 mL were inoculated into BACTEC vials or TSB/THIO tubes. Aliquots of 0.4 mL of the remaining sample volume of each rehydrated Bioball were spread onto irradiated TSA3 or BBA agar plates for the plate count method, to determine the microbial load of the inoculated suspensions.

following exceptions: 90% for *S. aureus* in PAF vials and TSB tubes, 20% for *C. albicans* in PAF and PPF vials and 90% for *C. acnes* in THIO tubes, as depicted in Table 2. All positive vials confirmed growth of the inoculated microorganism, ruling out the possibility of cross-contamination during sample preparation and inoculation process. All noncontaminated samples remained negative at the end of the incubation time, and terminal subcultures onto agar plates confirmed them to be true negative results.

TTD at contamination levels ≤ 5 CFU, however, was different when comparing the conventional sterility test and the BACTEC FX instrument (Table 3). Significantly shorter TTDs for *B. subtilis*, *P. aeruginosa* and *A. brasiliensis* were found in both aerobic BACTEC vials compared with TSB tubes. PAF vials showed the shortest TTDs for *B. subtilis* and *A. brasiliensis*, whereas PPF vials showed the shortest TTDs for *P. aeruginosa* and *S. aureus*, whose growth experienced a clear advantage in PPF vials compared with PAF vials and TSB tubes. For this reason, the PPF vial was selected as the aerobic medium for the method suitability studies. Concerning the anaerobic microorganisms, LAF vials

detected 100% of *C. sporogenes* and *C. acnes* within the first 2 and 5 days of culture, respectively, showing a significantly lower TTD compared with the THIO tubes (Tables 2 and 3).

Regarding the ability to detect *C. albicans*, TSB tubes showed a significantly shorter TTD ($P < 0.0001$) compared with both of the aerobic BACTEC vials. Only 20% of the PAF and PPF inoculated vials were detected as positive at the end of the 7-day incubation period. Furthermore, to obtain a sensitivity of 100% with a 5-CFU inoculum, the length of incubation had to be extended up to 21 days (Figure 2).

In all cases, the identification of the TSA3 plates confirmed the growth of *C. albicans*. As the sensitivity of the PAF and PPF vials for *C. albicans* in the 7-day incubation was too low, we compared these data with results from previous growth promotion studies performed in our unit in 2015, with 5 CFU per vial (Figure 3). Surprisingly, for the study in 2015, sensitivity for the detection of *C. albicans* at the end of the day 7 was 100% for both PAF and PPF vials, and TTD for *C. albicans* was significantly shorter than in the current study ($P < 0.001$ for PAF and $P < 0.023$ for PPF vials), whereas no changes were found in the conventional method.

Method suitability study

Supplementary Table 2 shows the recovered CFU in the seeded agar plates that allowed an estimation of the microbial load in the suspensions for the different matrices used in the inoculation of the vials. The estimated mean of the microbial load provided 10 to 20 CFU for 15-CFU samples, 7 to 13 CFU for 10-CFU samples and 3 to 7 CFU for ≤ 5 -CFU samples.

One of the objectives of this study was to evaluate the ability of the seven matrices to allow the growth of a very low inoculum of the test strains in the selected vials (PPF and LAF). All cell-based matrices allowed the growth of the microorganisms despite the presence of gentamicin or DMSO (Supplementary Table 3). Furthermore, only

Table 1
Cell-based matrix sample composition used for method suitability studies.

Matrix	Product type	hMSC/mL	Excipients (% of total)
Ad-AS	Autologous	1×10^6	DMEM, 10% FBS, 1% L-glutamine, gentamicin ($10 \mu\text{g/mL}$)
Ad-FP	Autologous	1×10^6	RL, Glu (2.5%), HSA-20 (1%)
Ad-MCS	Allogeneic	10×10^6	HSA-5+ DMSO (10%)
Ad-WCS	Allogeneic	5×10^6	HSA-5+ DMSO (10%)
CB-MCS	Allogeneic	2×10^6	HSA-5+ DMSO (10%)
CB-WCS	Allogeneic	10×10^6	HSA-5+ DMSO (10%)
CB-FP	Allogeneic	10×10^6	HSA-5+ PLM (10%)

Glu, Glucocemim 50% (B. Braun Medical); HSA-5, human serum albumin (Albu-norm 5%, Octapharma); HSA-20, human serum albumin (Albutein 20%, Grifols); PLM, Plasmalyte (Viaflo, Baxter); RL, Ringer lactate (Viaflo Hartmann, Baxter).

Table 2
Extended growth promotion test.

Strain	Culture media	Days in culture								Sensitivity at day 7
		1	2	3	4	5	7	14	21	
Strains used to test aerobic vials (≤5 CFU)										
<i>S. aureus</i>	PAF	0/10		9/10			9/10	9/10	9/10	90%
	PPF	10/10								100%
	TSB	2/10		9/10			9/10	9/10	9/10	90%
<i>B. subtilis</i>	PAF	10/10								100%
	PPF	10/10								100%
	TSB	10/10								100%
<i>P. aeruginosa</i>	PAF	10/10								100%
	PPF	10/10								100%
	TSB	10/10								100%
<i>C. albicans</i>	PAF	0/10		0/10			2/10	8/10	10/10	20%
	PPF	0/10		1/10			2/10	9/10	10/10	20%
	TSB	1/10		10/10						100%
<i>A. brasiliensis</i>	PAF	0/10		10/10						100%
	PPF	0/10		10/10						100%
	TSB	0/10		5/10			10/10			100%
<i>K. rhizophila</i>	PAF	0/10		9/10			10/10			100%
	PPF	0/10		10/10						100%
	TSB	0/10		10/10						100%
Strains used to test anaerobic vials (≤5 CFU)										
<i>C. sporogenes</i>	LAF	10/10								100%
	THIO	0/10	10/10							100%
<i>C. acnes</i>	LAF	0/10	0/10		8/10	10/10				100%
	THIO	0/10	0/10		0/10	9/10	9/10		9/10	90%

Sensitivity was calculated as the accumulated number of vials flagged positive of the 10 seeded for each strain and culture media, at day 7 of incubation.

one of the 63 inoculated vials (seven matrices, with three different inoculum levels, in triplicate) with ≤ 5 UFC of *K. rhizophila* in adipose derived-finished product (Ad-FP) matrix showed no growth even after an extended incubation of 21 days. All negative controls remained sterile at the end of the incubation time, confirming that the matrices themselves did not interfere with the outcome of the test; terminal subcultures on agar plates confirmed that all of them were true negative results.

When the TTDs from the three inoculum levels were combined in each of the matrices, TTDs were shorter in PPF and LAF vials than in TSB or THIO tubes for *S. aureus*, *B. subtilis*, *P. aeruginosa*, *C. sporogenes* and *C. acnes*. Conversely, *C. albicans* exhibited a longer TTD in PPF vials, and *A. brasiliensis* and *K. rhizophila* showed no difference between the two methods for the majority of the matrices (Table 4).

Influence of inoculum size

Despite the small amounts of inocula seeded, when all the matrices were analyzed together, statistical differences in mean TTDs among the inocula were found: *S. aureus*, *B. subtilis* and *K. rhizophila*

showed statistically shorter TTDs with 15-CFU than with 10-CFU inocula. All the tested strains except *C. acnes* showed significantly shorter TTDs with 15-CFU than with 5-CFU inocula. In addition, *P. aeruginosa*, *C. albicans*, *A. brasiliensis*, *K. rhizophila* and *C. sporogenes* showed shorter TTDs with 10-CFU than with 5-CFU inocula, as shown in Table 5.

In general, *S. aureus*, *B. subtilis*, *P. aeruginosa* and *C. sporogenes* showed relatively homogeneous patterns of growth in the first 24 h after seeding. However, *C. albicans*, *A. brasiliensis*, *K. rhizophila* and *C. acnes* had longer TTDs, with a high dispersion in the case of *C. albicans* and *K. rhizophila*, especially for ≤ 5 -CFU inocula (Figure 4). This was especially evident for *C. albicans*, whose growth was detected in the first 7 days of incubation in only 47 of the 63 samples seeded in the PPF vials.

Composition of the matrices in cell-based preparations; influence of the excipients with potential interference properties: antibiotics and DMSO

To assess whether the composition of the different matrices interfered with the growth of microorganisms in the BACTEC vials, we

Table 3
Extended growth promotion test: comparison of TTD (h) between BACTEC vials and tubes from the conventional sterility method, when seeded with ≤ 5 CFU.

Strain	Aerobic vials			Anaerobic vials		TTD comparison in different vials (P^a)
	PAF	PPF	TSB	LAF	THIO	
<i>S. aureus</i>	41.7 [37.2–48.4]	16.3 [15.8–16.7]	48 [36–72]			PPF detected sooner than PAF and TSB ($P < 0.001$ in both cases)
<i>B. subtilis</i>	13.3 [12.9–13.9]	13.6 [13.4–14.1]	24 [24]			PAF and PPF detected sooner than TSB ($P < 0.0001$ in both cases); PAF detected sooner than PPF ($P < 0.05$)
<i>P. aeruginosa</i>	18.3 [18.1–18.7]	17.5 [17.1–17.6]	24 [24]			PAF and PPF detected sooner than TSB ($P < 0.0001$ in both cases); PPF detected sooner than PAF ($P < 0.01$)
<i>C. albicans</i>	263.1 [196.3–338]	216.3 [171.5–234.6]	48 [36–48]			TSB detected sooner than PAF and PPF ($P < 0.0002$ in both cases)
<i>A. brasiliensis</i>	47.0 [45.8–48.9]	51.2 [50.9–53.1]	84 [66–96]			PAF and PPF detected sooner than TSB ($P < 0.001$ and $P < 0.05$); PAF detected sooner than PPF ($P < 0.0003$)
<i>K. rhizophila</i>	41.8 [40–51.5]	42.1 [40.7–45.2]	48 [48]			PPF detected sooner than TSB ($P < 0.003$)
<i>C. sporogenes</i>				15.5 [15.3–16.1]	48 [48]	LAF detected sooner than THIO ($P < 0.0001$)
<i>C. acnes</i>				94.9 [93.7–96.3]	120 [120]	LAF detected sooner than THIO ($P < 0.0001$)

TTD is shown as median [interquartile range] of positive samples.

^aComparison of TTD in PAF, PPF and LAF culture vials and traditional TSB and THIO culture medium was performed using the Mann–Whitney test.

Figure 2. Comparison of results of TTD for *C. albicans* samples seeded at ≤ 5 CFU. 100% sensitivity was achieved at the end of 2 days for TSB tubes, 16 days for PPF vials, and 21 days for PAF vials. 100% specificity was attained at the end of the 7-day incubation time for both PPF and PAF vials, as well as for TSB tubes.

compared the TTDs of the eight microorganisms seeded in the seven matrices with those seeded in DPBS. The matrices did show an influence on the time of growth in all the spiked microorganisms, except for *A. brasiliensis* ($P = 0.057$) and *C. sporogenes* (n.s.), using Kruskal–Wallis tests for the analysis.

First, the presence of antibiotic-binding substances in BACTEC vials suppressed the inhibition of microorganism growth by gentamicin in the Ad-AS matrix, although the TTDs for *S. aureus*, *B. subtilis*, *P. aeruginosa* and *C. acnes* were significantly longer in this matrix compared with growth in the other matrices or DPBS. *S. aureus* showed a slower growth in Ad-SA than in CB-MCS matrix, and *B. subtilis* showed slower growth in Ad-SA than in Ad-MCS and the CB-derived matrices. Likewise, *C. acnes* displayed slower growth in Ad-SA compared with the rest of the Ad-derived matrices, as did *P. aeruginosa*, which additionally exhibited a slower growth than in CB-WCS and CB-FP, as pictured in Figure 5. Second, not only did the DMSO seem to not interfere with the detection of the different strains, but it appeared to even facilitate the growth of *P. aeruginosa*, as the TTD was significantly shorter in two of the matrices with 10% DMSO (Ad-MCS and CB-WCS) than in DPBS. Third, *K. rhizophila* was the strain most affected by the composition of the matrices, as significantly faster growth was detected in the acellular matrix DPBS than in the rest of the cellular matrices (only CB-WCS did not overcome Bonferroni correction), regardless of the presence of gentamicin or DMSO. In general, growth of the different strains in DPBS matrix was very homogeneous, with less dispersion in the TTD than in the cellular matrices. Finally, although Kruskal–Wallis test showed significant differences in the growth of *C. albicans* among the different matrices, the pairwise comparisons did not pass the rigor of the Bonferroni corrections (Figure 5).

Sensitivity, specificity and positive and negative predictive values were calculated for each matrix sample and inoculum group. Because

there was detectable microbial growth for the majority of the inoculated flasks, only the results per inoculum group and strain (collecting data from the seven different matrices in triplicate) were depicted as the most significant information. 100% sensitivity was achieved for all the test strains and the three inocula, except for *C. albicans* and *K. rhizophila*. When using PPF vials, *C. albicans* gave two FN results at 15-CFU inocula, four at 10-CFU and 10 at 5-CFU after 7 days of incubation (sensitivity 90.48%, 80.95% and 52.38%, respectively; negative predictive value 77.78%, 63.64% and 41.18%, respectively). Nevertheless, all the PPF vials grew during the extended 21-day period of incubation. Furthermore, *K. rhizophila* gave two FNs at 5-CFU inocula in the PPF vials at 7 days (sensitivity 90.48% and negative predictive value 77.78%). In one of the vials, growth was detected on day 10, and the other showed no growth after 21 days of incubation. Specificity and positive predictive value were of 100% for all the strains and inocula tested in PPF and LAF vials.

Optimization of fungus detection

Because of the delayed detection of *C. albicans*, especially using the lowest inoculum, an additional extended growth promotion study was designed using DPBS as matrix sample. For process implementation, BACTEC MYC vials were added under the same incubation conditions. The results of a set of 20 samples of each PAF, PPF, MYC and TSB vials inoculated with ≤ 5 CFU of *C. albicans* are shown in Figure 6. All samples became positive, except for five samples seeded in PAF vials, three of which exceeded a 21-day incubation and were considered FN, and two whose growth was detected at 14 days and 10 hours and 16 days and 11 hours of incubation. These confirmed the huge dispersion of the TTD for *C. albicans* strains when low inocula were handled. However, growth detection was confirmed within 32 hours in all MYC vials, with TTDs significantly shorter in these

A

Detection of *C. albicans* in 2015 vs. 2019

B

2015

2019

Figure 3. Comparison of TTD for *C. albicans* between growth promotion test validations in 2015 and 2019. (A) Median values of TTD for *C. albicans* (≤ 5 CFU/vial) in PAF, PPF and TSB vials in 2015 and 2019 validations, and comparisons between the two by Wilcoxon test. (The exact median values and interquartile ranges are depicted at the bottom of each column.) (B) Number of vials of 10 seeded with *C. albicans* that flagged positive on day 7 of incubation, in the 2015 and 2019 validations.

vials compared with TSB tubes ($P < 0.00000001$ [none of the TSB tubes showed turbidity at 24-h inspection, but all of them displayed turbidity at the next visualization at 36 h]). As expected, positive PAF and PPF vials continued showing a retarded growth of *C. albicans*, especially compared with the growth in the MYC vials ($P < 0.0000001$ and $P < 0.0000001$, respectively). Detection of *C. albicans* in MYC vials and TSB tubes had a sensitivity of 100% at 32 and 36 h of incubation time, respectively, whereas PPF vials needed 14-day incubation to obtain a sensitivity of 100%. In that same period, PAF vials achieved a sensitivity of only 75%.

Additionally, to confirm the improvement achieved by introducing MYC vials in the detection of yeasts and molds, and its suitability in the microbiological examination of cell-based products, three sets of 12 samples of PPF and MYC vials were inoculated with ≤ 5 CFU of *C. albicans* or *A. brasiliensis*. These were diluted in DPBS, 5×10^6 Ad-MSC/mL suspended in Ringer lactate supplemented with 2.5% glucose and 1% human serum albumin (Ad-MSC), or the supernatant from the culture of Ad-MSC obtained for bioburden assessment (SPN) and composed of α -MEM with 2 mM GlutaMAX supplemented with 5% viral-inactivated human platelet lysate and 0.6 IU/mL sodium heparin

Table 4
Method suitability

Strain	Vial	Ad-SA	Ad-FP	Ad-MCS	Ad-WCS	CB-MCS	CB-WCS	CB-FP
<i>S. aureus</i>	PPF	16.4 ± 0.4	15.7 ± 0.7	15.8 ± 0.8	16.3 ± 0.8	15.4 ± 0.5	15.8 ± 0.6	15.4 ± 0.7
	TSB	54.7 ± 13.6	48.0 ± 15.9	42.7 ± 16.0	42.7 ± 13.6	41.3 ± 10.6	46.7 ± 15.2	52.0 ± 15.9
	P	<0.0004	<0.0004	0.0004	<0.0004	<0.0004	<0.0004	<0.0004
<i>B. subtilis</i>	PPF	16.2 ± 1.6	13.7 ± 1.0	13.2 ± 0.8	13.7 ± 0.7	13.1 ± 0.5	13.3 ± 0.5	13.0 ± 0.4
	TSB	26.7 ± 5.3	22.7 ± 4.0	21.3 ± 5.3	22.7 ± 4.0	22.7 ± 4.0	22.7 ± 4.0	22.7 ± 4.0
	P	<0.0003	<0.004	<0.04	<0.004	<0.004	<0.004	<0.004
<i>P. aeruginosa</i>	PPF	18.8 ± 1.0	16.3 ± 0.5	16.3 ± 0.5	16.3 ± 0.6	16.7 ± 0.5	16.0 ± 0.6	16.6 ± 0.8
	TSB	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0	24.0 ± 0.0
	P	<0.0002	<0.0002	<0.0001	<0.0002	<0.0002	<0.0002	<0.0001
<i>C. albicans</i>	PPF	199.3 ± 72.1	180.6 ± 149.7	104.0 ± 99.8	99.5 ± 65.6	84.1 ± 90.4	85.0 ± 75.5	92.6 ± 76.7
	TSB	37.3 ± 7.2	37.3 ± 4.0	40.0 ± 8.5	37.3 ± 7.2	38.7 ± 8.0	38.7 ± 5.3	38.7 ± 5.3
	P	<0.0003	<0.0002	NS	<0.0005	NS	NS	NS
<i>A. brasiliensis</i>	PPF	52.4 ± 8.5	53.4 ± 11.7	62.0 ± 10.2	57.2 ± 12.5	64.5 ± 13.5	59.2 ± 6.6	56.1 ± 8.0
	TSB	58.7 ± 4.0	60.0 ± 13.4	70.7 ± 16.4	65.3 ± 19.1	73.3 ± 15.2	68.0 ± 10.4	58.7 ± 7.2
	P	<0.02	NS	<0.05	NS	NS	NS	NS
<i>K. rhizophila</i>	PPF	78.0 ± 27.5	88.2 ± 32.5	97.0 ± 58.7	81.5 ± 20.8	80.0 ± 26.1	69.8 ± 11.7	75.9 ± 24.6
	TSB	76.0 ± 22.4	92.0 ± 24.0	86.7 ± 20.6	82.7 ± 22.0	77.3 ± 22.5	70.7 ± 14.0	77.3 ± 21.7
	P	NS	NS	NS	NS	NS	NS	NS
<i>C. sporogenes</i>	LAF	16.0 ± 0.3	15.7 ± 0.4	15.5 ± 0.3	15.4 ± 0.4	15.8 ± 0.7	15.5 ± 0.7	15.8 ± 0.9
	THIO	49.3 ± 4.0	48.0 ± 6.0	45.3 ± 5.3	45.3 ± 5.3	48.0 ± 0.0	40.0 ± 6.0	48.0 ± 0.0
	P	<0.0002	<0.0003	<0.0003	<0.0003	<0.0002	<0.0003	<0.0002
<i>C. acnes</i>	LAF	102.5 ± 5.9	88.2 ± 2.1	90.9 ± 1.9	90.0 ± 1.4	98.2 ± 6.9	93.1 ± 2.3	94.6 ± 3.4
	THIO	125.3 ± 6.3	108.0 ± 12.0	110.7 ± 10.0	110.7 ± 10.0	122.7 ± 5.3	120.0 ± 0.0	120.0 ± 0.0
	P	<0.0003	<0.0003	<0.0004	<0.0004	<0.0003	<0.0002	<0.0002

Mean TTD (h) in BACTEC PPF or LAF vials and in direct inoculation method TSB or THIO tubes, for the 7 hMSC-based matrices. TTD is expressed as mean ± SD for the positive vials of the triplicates for the three inocula seeded of each microorganism and matrix. P values reflect the statistical difference in TTD for a specific microorganism and matrix depending on the seeded vial by means of a Mann–Whitney test.

(Table 6). The different matrices used to dissolve the strains did not affect the growth of *C. albicans* in PPF vials or MYC. In contrast, samples dissolved in Ad-MSC or supernatant showed a slightly prolonged TTD for *A. brasiliensis* in PPF vials compared with those dissolved in DPBS. When comparing TTDs between the two types of vials, in the case of *C. albicans*, TTD was significantly shorter in MYC vials than in PPF vials for the three matrices, but conversely, there were no significant differences in the growth of *A. brasiliensis*.

Discussion

Although microbial contamination is a rare event under GMP processes, the absence of microbial growth in ATMPs is still challenging, as the aseptic processes are less standardized than in traditional drug manufacturing [17,29,30]. Contamination may occur during collection, manufacturing, conditioning or administration of the product [6,17]. Furthermore, sterility of the source material cannot be guaranteed, and the ATMP cannot be sterilized at the end of the manufacturing process. Because of the extremely limited shelf-life, results of the sterility testing cannot be available before administration of the ATMP to the patient [29]. Therefore, sterility testing along with detection of Mycoplasmas and endotoxin assessment are critical to release the cell-therapy products. The direct inoculation method presents some limitations such as the amount of sample to be tested, the 14-day incubation period and that inoculation of some cell suspensions could itself cause turbidity in the growth media, leading to false-positive results. To circumvent these limitations, AGBM systems are supported by the FDA and the European Medicines Agency as alternative methods for cell-therapy products [7], as long as they are qualified and demonstrate their equivalence to the conventional reference method.

The use of AGBM has shown an improvement in recovery and sensitivity owing to the continuous monitoring and objective detection of microbial growth, not affected by the turbidity of cell suspensions [5,6]. Likewise, the implementation of specific software facilitates data compilation and traceability. AGBM also allows for sterility testing not only upon the final product release, but on bigger volumes of the supernatant obtained from the cell culture 48 to 72 h before

harvest and conditioning of the cell-therapy medicines. Therefore, they provide more opportunities to detect low levels of contamination in the medicinal products and facilitate more rapid decision making in the case that preventive or corrective actions need to be taken.

There are several commercial culture vials used by BD BACTEC FX40 and BacT/Alert systems, with no apparent effects on recovery results [31], but with a wide window of standardization opportunities. Nevertheless, as these systems have been developed to assess the microbial contamination of blood samples, their manufacturers do not have to comply with GMP validations guidelines and thus are unaware of resulting implications on their user validations for improvement of ATMPs. For example, some of the Certificates of Analysis do not comply with the recommended microorganisms for growth promotion tests listed in *Ph. Eur.* 2.6.27, which implies that the ATMP manufacturer must perform growth promotion tests for each batch of vials used. Furthermore, they fail to provide useful information such as the number of CFU used for their growth promotion tests or the expected TTD for each tested microorganism, as a reference for in-house studies.

The current study was prompted by our need to establish the equivalence of the BACTEC AGBM to the conventional method in detecting low levels of microorganisms when present in cell-therapy medicinal products. Our data demonstrate that the BACTEC FX40 system performance with aerobic and anaerobic vials is equivalent to, or better than, the direct inoculation method for general sterility testing of both DPBS and cell-therapy products contaminated with representative organisms at low inocula. This excludes the detection of *C. albicans*, for which these vials appear to be unsuitable. Five of the six aerobic microorganisms were detected with PAF and PPF vials within the first 2 days of incubation. Even *K. rhizophila*, a slow grower in nearly all systems, was detected in 100% of the PPF vials spiked with 15- and 10-CFU inocula within the first 3 and 4 days, respectively, and in 90.48% of the vials with ≤5 CFU within the first 5 days, with TTD slightly longer in cellular matrices than in DPBS. A previous study reported shorter TTDs for *K. rhizophila* in the BacT/Alert system at 33°C, but with an inoculum of ≤100 CFU, and established a mean limit of detection of 18 CFU [32]. In addition, although some authors have

Table 5
Influence of inoculum size on the method suitability study

CFU	PPF aerobic vials							LAF anaerobic vials		
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>	<i>A. brasiliensis</i>	<i>K. rhizophila</i>		<i>C. sporogenes</i>		<i>C. acnes</i>
TTD (h)										
15 CFU	15.35 ± 0.68 15.3 [14.8–15.95] 15.97 ± 0.55	13.30 ± 1.03 13.0 [12.65–13.65] 14.04 ± 1.74	16.55 ± 1.27 16.2 [15.90–16.50] 16.57 ± 1.02	72.22 ± 65.61 44.9 [28.75–93.75] 95.84 ± 81.26	52.38 ± 8.08 49.2 [48.0–56.75] 56.22 ± 10.31	62.63 ± 15.88 61.1 [49.95–72.7] 73.89 ± 17.23		15.47 ± 0.63 15.3 [15.1–15.65] 15.42 ± 0.35		92.77 ± 6.79 91.1 [88.7–95.5] 93.76 ± 5.26
10 CFU	16.1 [15.6–16.2] 16.19 ± 0.71	13.5 [13.15–14.25] 13.85 ± 1.08	16.2 [15.8–16.85] 17.06 ± 0.87	62.7 [32.9–132.0] 194.09 ± 105.9	52.8 [48.15–61.85] 64.95 ± 9.90	71.3 [64.1–83.55] 102.25 ± 43.40		15.3 [15.10–15.75] 16.07 ± 0.52		91.8 [90.45–96.30] 95.21 ± 5.90
5 CFU	16.2 [15.7–16.55]	13.4 [13.2–14.35]	17.1 [16.6–17.65]	154.3 [118.6–249.1]	62.2 [56.05–72.75]	97.4 [87.85–112.05]		16.1 [15.65–16.40]		94.2 [90.0–99.2]
<i>P</i>										
15 versus 10 CFU	≤0.005	≤0.05	NS	NS	NS	≤0.05		NS		NS
15 versus 5 CFU	≤0.001	≤0.05	≤0.005	0.00001	0.0001	0.00001		≤0.001		NS
10 versus 5 CFU	NS	NS	≤0.05	≤0.001	≤0.005	≤0.001		0.0001		NS

Mean time required to detect microbial growth for each level of contamination (15, 10 and 5 CFU) in BACTEC vials for the seven matrices globally. Data are mean ± SD or median (interquartile range) for the triplicates seeded of each microorganism and level of contamination in the seven matrices assessed. Because TTD values for half of the strains did not follow a normal distribution, *P* values reflect the statistical difference in TTD for a specific microorganism depending on the seeded inoculum through Mann–Whitney tests.

reported poor detection of anaerobic bacteria with other anaerobic vials in AGBM [15,33,34], in our study, LAF vials detected 100% of *C. sporogenes* within the first 20 h of culture and *C. acnes* within the first 5 days of culture, a shorter TTD than previously reported [35], supporting their high detection rate. Conversely, *C. albicans* was poorly detected at 7 days with PAF and PPF vials in comparison with the TSB tubes, corroborating previous studies [29]. A single incubation temperature to detect all of the microorganisms was used in both methods, which may introduce a small bias in the different performances. In spite of some authors claiming that incubation at 35 to 37°C may be too high for the growth of certain clinically relevant strains, providing possible FN results [6], in the current study, when suitable culture media were selected, sensitivities within 7 days of incubation were at 100% with inocula >5 CFU and almost 100% with ≤5 CFU for all the strains tested.

Notably, previous growth promotion assays conducted in our GMP facility obtained a sensitivity of 100% for the detection of *C. albicans* at the end of the day 7 in both PAF and PPF vials. These showed a significantly shorter TTD than the current vials. Meanwhile, no changes in the TTD using the conventional sterility test were detected. In this case, both PPF and PAF vials underwent several product improvements regarding reagent modifications, medium volume and algorithm adjustments, as well as the change from glass to plastic vials, all approved by the FDA [36–40]. Despite the company claims that the statistically detectable median difference in the TTD in PPF plastic and glass vials was not considered clinically relevant, as it was <10% with all microorganisms tested [37], from our point of view, the slight changes in the composition of both BACTEC vials could have influenced the growth of very low inocula of *C. albicans*, leading to different results in 2015 and 2019.

Alternatively, it is well known that the TTD also depends on the product matrix [5], highlighting the need for suitability studies for microbiological control of cell-based preparations. Cell concentrations in the matrices ranging from 1 to 10 million hMSC/mL did not affect the growth of the strains tested or provide false-positive results at the highest cellular concentrations. They instead prolonged the TTD for *K. rhizophila* compared with the growth in an acellular matrix such as DPBS. When assessed with PPF and LAF vials, the seven matrices allowed the growth of the different strains to reach a sensitivity of 100% during the 7-day incubation, except for *K. rhizophila* at ≤5 CFU and *C. albicans*, whose sensitivity decreased alongside the inoculum levels. When compared with the traditional method, PPF and LAF vials showed shorter TTD for *S. aureus*, *B. subtilis*, *P. aeruginosa*, *C. sporogenes* and *C. acnes*, a similar TTD for *A. brasiliensis* and *K. rhizophila* and a significantly longer TTD for *C. albicans* in all the matrices.

Pathogen recovery has been reported to be greatly reduced in those BACTEC vials inoculated with samples from products cultured with antibiotics, despite the presence of antibiotic-binding resins [41–43]. This suggest that these substances are not fully effective in inactivating residual concentrations of antibiotics, or that saturation might occur with high doses of antibiotics in the samples [15]. In our study, recovery of different strains was optimal in the matrix containing gentamicin (Ad-AS), although the growth of *S. aureus*, *B. subtilis*, *P. aeruginosa* and *C. acnes* was slightly delayed in this matrix compared with other cellular matrices. Ampicillin, gentamicin, piperacillin-tazobactam, and vancomycin have been reported to be neutralized effectively in AGBM systems, allowing the recovery of nearly all the tested strains [43]. In the manufacturing processes in which antibiotics are needed during cell expansion, the presence of antibiotics may interfere with the growth promotion test. This problem can be circumvented by preincubating samples with substances with inactivating properties, such as penicillinase, before inoculation into Bactec vials [44].

Similarly, some authors have reported that the presence of cryoprotectant DMSO in the matrices could impair the growth of microorganisms, leading to FN results in microbiological controls [45,46],

Figure 4. Method suitability: distribution of TTD for the different microorganisms seeded in PPF aerobic and LAF anaerobic vials, when all the matrices were analyzed together. Data distribution is displayed as mean $\pm$ 2 SD of TTD. Microorganisms in the upper graph showed relatively homogeneous TTD of <24 h, regardless of the inoculum. However, the four microorganisms in the lower graph showed longer TTD, with high dispersion in the case of *C. albicans* and *K. rhizophila*.

whereas others demonstrated that $\leq 10\%$ DMSO had no measurable effect on bacterial viability [6,23,47]. In the current study, we optimally recovered the different strains in matrices containing 10% DMSO, discarding a relevant inhibition of microorganism growth in the culture media caused by DMSO. We must clarify that, in our studies, the microorganisms were in contact with the matrix samples for ~ 3 min before spiking them in the vials. This was the lag time of potential interfering substances before being neutralized by resins or dilution with the culture medium. The contact time of matrix samples containing potential interference substances is an extremely important aspect to be considered when standardizing a technique, owing to the post-antibiotic effect; however, it is not reported in most of the revised studies.

Slightly different levels of low microorganism concentration in ATMPs, however, would also affect the TTD. The inoculum range of 5 to 15 CFU/vial was chosen based on previous studies quantifying the

degree of bacterial contamination in platelet units [10] and cord blood samples [47]. We demonstrated that small variations in inoculum levels had an effect on the strains' growth, as all the microorganisms showed shorter TTDs with the highest inoculum, with the exception of *C. acnes*. Currently, the *Ph. Eur.* formally states that during the confirmation of the suitability of the method, <100 CFU of each of the strains listed in Table 2.6.27.-2 should be inoculated into the medium in the presence of the product to be tested [7]. This inoculum represents a wide range of CFU, with upper limits very unlikely to mimic what would be expected to exist in accidental contamination during the aseptic manufacturing of ATMPs. The authors believe that the suitability of the method for ATMPs should be made with the lowest possible inoculum, to assess the sensitivity and microbial detection limit of the AGBM in real testing conditions. This is because small counts of residual microorganisms in ATMPs can be missed, growing up to

Figure 5. Method suitability: effect of the product matrix on the TTD of the different strains. Distribution of TTD is expressed as boxplots. "Box limits" indicate the range of the central 50% of the data (from the 25th to the 75th percentile, known as the interquartile range), with a central line marking the median value. Lines extend from each box to capture the range of the remaining data, with dots placed past the line edges to indicate outliers. Matrices based on adipose tissue-derived MSCs are colored in white, with the gentamicin-containing matrix [Ad-SA] filled with a dotted pattern. Those derived from cord blood MSCs are shown in light gray, and the acellular matrix exclusively composed of DPBS is displayed in dark grey. P_c = P value after Bonferroni correction.

huge numbers during the storage and transportation of these products [17,48].

The aim of the third phase was to optimize fungus detection. Although many studies suggest that *Candida spp.* is effectively recovered using BACTEC [49–53] and BacT/ALERT [54–56] systems, and compare their performance [57–59], others have reported limitations of these systems in detecting molds and yeasts [6,17,29,60,61]. Some authors circumvented this problem by simultaneously seeding samples onto Sabouraud dextrose agar, together with the use of aerobic and anaerobic vials, and incubating at 20 to 25°C for 14 days to improve the detection of molds [29,61]. Alternatively, gross mold contaminations could be detected through visual inspection of the BACTEC vials that failed to flag positive at the end of the incubation period [5]. Given the high dispersion of TTD obtained in our study for *C. albicans* with a low inoculum, for all the matrices in PAF and PPF vials, we tested the MYC vial, aimed at promoting fungal growth, and compared the results. The presence of antibiotics in MYC vials provides a significant benefit for the suppression of bacterial growth and detection of yeasts [49,59]. Because the inoculum size affected TTDs of *Candida spp.* growth in older BACTEC systems [62], we decided to test the lowest inoculum to compare the performance and sensitivity of routine sampling vials versus MYC ones, and repeated the suitability study with three different matrices. Our initial assumption was fulfilled: *C. albicans* could be detected more efficiently from cell therapy products if additional MYC vials were

introduced during the sampling procedure. With a 100% sensitivity in <30 h of incubation in MYC vials, *C. albicans* showed a significantly shorter TTD than in PAF and PPF vials and TSB tubes and a narrower dispersion than in both BACTEC aerobic vials, despite the low inoculum. These findings are in agreement with experiments aimed at detecting *Candida spp.* bloodstream infections [15,17,49,50,60,63,64]. All the revised studies showed a significantly shorter TTD for *C. albicans* detection in PAF vials than in our current study, where the long TTD in BACTEC vials displayed their limited value for the detection of yeast at a low inoculum in cell-therapy products and may reflect a composition change in the media compared with vials manufactured a few years ago.

In addition, based on previous findings suggesting that fungi preferentially secrete to cell fractions of processed umbilical cord blood [23], we evaluated the detection of fungi in different matrices with and without cells (DPBS, Ad-MSC, and supernatant of cultures of Ad-MSC). The yield of the MYC vials to detect ≤ 5 CFU of *C. albicans* was higher than the PPF vials in all three matrices, whereas *A. brasiliensis* showed similar positivity rates in both vials. This is a major issue of concern, because if the suitable vials are not selected correctly, potential fungal contamination of the ATMPs may not be detected in the early stages of the manufacturing process, where corrective measures could easily be taken to prevent further risks. Despite the loss of a limited and precious product, the use of an additional vial would be a reasonable strategy to ensure the sterility of cell-therapy medicinal products.

TTD (h)	PAF <i>n</i> =20	PPF <i>n</i> =20	MYC <i>n</i> =20	TSB <i>n</i> =20
Mean ± SD	238.3 ± 80.5	195.1 ± 67.3	28.2 ± 1.8	36
Median	245.2	185.7	27.8	36
[IQR]	[177.6 - 293.0]	[136.5 - 254.4]	[26.6 - 29.6]	[36 - 36]

Figure 6. Sensitivities of the different vials to detect *C. albicans* in the extended growth promotion study. This yeast was diluted in DPBS at 5-CFU inocula.

In conclusion, aside from a complex control of the whole manufacturing process, a continuous personnel training program and an exhaustive environmental monitoring control in the GMP facility, repeated sterility testing is essential to confirm the absence of contamination in cell therapy products. Our data support the general use of pediatric aerobic PPF, anaerobic LAF, and mycosis MYC as the best

vial combination for routine microbiological control of ATMPs using the BACTEC system and its ability to detect a very low number of the microorganisms suggested from the *Eur. Ph*. These vials optimize the sensitivity of the BACTEC FX40 system in detecting the microorganisms contaminating cell-therapy products, at a single temperature, in a 7-day incubation period. Sensitivities of 100% were achieved in <24

Table 6
Optimization of fungi detection

Strain and matrix	TTD in PPF vials			TTD in MYC vials		
	DPBS	Ad-MSC	SPN	DPBS	Ad-MSC	SPN
<i>C. albicans</i>						
Mean ± SD	188.9 ± 45.2	189.7 ± 107.9	170.8 ± 67.9	28.2 ± 1.8	27.8 ± 1.4	28.5 ± 1.0
Median (IQR)	185.7 (154.8–222.5)	158.5 (113.0–239.0)	192.5 (100.0–213.0)	27.8 (26.6–29.6)	27.4 (27.1–29.1)	28.5 (27.7–29.2)
Statistical differences between matrices ^a	Not significantly different			Not significantly different		
Statistical differences between PPF and MYC vials ^b	PPF versus MYC in	DPBS, <i>P</i> = 0.000003; Ad-MSC, <i>P</i> < 0.000003; SPN, <i>P</i> < 0.00003				
<i>A. brasiliensis</i>						
Mean ± SD	54.9 ± 6.2	59.0 ± 4.3	61.2 ± 7.8	56.2 ± 5.4	57.9 ± 6.7	59.4 ± 5.4
Median IQR	52.7 (51.0–57.6)	58.5 (58.0–59.7)	60.0 (54.5–69.7)	54.5 (52.5–59.0)	57.5 (52.0–59.0)	60.0 (56.0–61.0)
Statistical differences between matrices ^a	<i>P</i> = 0.042	DPBS versus Ad-MSC, <i>P</i> = 0.025; DPBS versus SPN, <i>P</i> = 0.039; Ad-MSC versus SPN, NS		Not significantly different		
Statistical differences between PPF and MYC vials ^b	Not significantly different					

Comparison of TTD (h) for 5-CFU inoculum of *C. albicans* and *A. brasiliensis* dissolved in three matrices in PPF and MYC vials.

IQR, interquartile range.

^aStatistical differences in the TTD between the three different matrices seeded in the same type of vials by means of Kruskal–Wallis test. Comparison of two matrices by Mann–Whitney test.

^b Comparison of the TTD in the same matrix seeded in the PPF or MYC vial, by Mann–Whitney test.

h for *S. aureus*, *B. subtilis* and *P. aeruginosa* in PPF vials and for *C. sporogenes* in LAF, as well as ≤ 30 h for *C. albicans* in MYC vials. However, several other microorganisms, such as *A. brasiliensis*, *K. rhizophila* and *C. acnes*, needed 3 to 5 days to be detected when seeded at 5 to 15 CFU/vial, and therefore, these vials should be incubated for ≥ 7 days to detect other potential slower-growing contaminants.

ATMP manufacturers proved to have adapted a rapid and effective technique in the field of microbiological diagnosis. Nonetheless, it still lacks the necessary robustness to be used extensively in the field of cell therapy and needs to meet the regulatory requirements for investigational cell-based medicines. Complying with the requirements of regulatory agencies in terms of traceability, data integrity, change control and instrumental validation is impossible without the involvement of the manufacturers of these instruments. This leaves a huge effort in the hands of ATMP manufacturers, most of which are academic institutions who lack the necessary resources to undertake this responsibility. In our opinion, it would be beneficial to conduct a primary validation of the method according to Ph. Eur. 5.1.6 or similar. This primary validation, along with the complete observation of the pharmaceutical regulation, could finally introduce this method to the ATMP field.

Declaration of Competing Interests

We declare that we have no conflicts of interest.

Author Contributions

Conception and design: LL, JCG and ARA; Collection of data: JCG, IDT, RMS, RMF, CA, CF; Assembly of data: ARA, JCG, RMS, RMF, CF; Data analysis and interpretation: ARA, JCG, LL; Writing—Original Draft: ARA, JCG and LL; Revision and final approval of manuscript: all the authors

Acknowledgments

We thank Ana Tozer and Claudia Corazza for their help in English language revision.

Funding

A. Rodríguez-Acosta, R. Maldonado and C. Antúnez hold a "Linking of Technicians to Common Research Facilities" contract (RF2-0004-2020, F2-0002-2018 and RF2-0006-2019, respectively) from the Andalusian Ministry of Health and Family. J. Chaparro holds a "Promotion of youth employment" research contract (PEJ 2018-002289-A) and R. Fernández-Muñoz and C. Frecha hold a "Technical staff supporting R + D + I" research contract (PTA 2017-14556-I and PTA2018-015571-I, respectively), all of them from the Spanish Ministry of Science and Innovation. L. Leyva holds a Nicolás Monardes research contract (RC-002-2019) from the Andalusian Ministry of Health and Family.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jcyt.2021.11.002](https://doi.org/10.1016/j.jcyt.2021.11.002).

References

- [1] Code of Federal Regulations 2012. Title 21-Food and drugs. Chapter I-Food and Drug Administration, Department of Health and Human Services. Subchapter F-Biologics. Part 610-General biological products standards. U.S. Government Publishing, pp. 2018–2019.
- [2] USP71. Sterility tests. In: United States Pharmacopoeia. 41th ed. National Formulary. Rockville (MD): The United States Pharmacopoeial Convention, Inc; 2018.
- [3] Sterility (04/2011:20601). In: European Directorate for the Quality of Medicines & HealthCare (EDQM), editor. European Pharmacopoeia. 9.8th ed 07/2019, Strasbourg, France.
- [4] The Japanese Pharmacopoeia - Seventeenth Edition. 4.06 Sterility Test. 2016.
- [5] Gebo JET, Lau AF. Sterility Testing for Cellular Therapies: What Is the Role of the Clinical Microbiology Laboratory? J Clin Microbiol 2020 Jun 24;58(7):19. e01492-.
- [6] Störmer M, Wood EM, Schurig Schurig, Karo O, Spreitzer I, McDonald CP, Montag T. Bacterial safety of cell-based therapeutic preparations, focusing on haematopoietic progenitor cells. Vox Sang 2014;106(4):285–96.
- [7] Microbiological examination of cell-based preparations (07/2017:20627). In: European Directorate for the Quality of Medicines & HealthCare (EDQM), editor. European Pharmacopoeia. 9.2th ed 2017, Strasbourg, France.
- [8] FDA, "Biological Device Application Approvals: BK180266. BACT/ALERT® BPA and BPN Culture Bottle. <https://www.fda.gov/vaccines-blood-biologics/development-approval-process-cber/2018-biological-device-application-approvals>." 2018.
- [9] FDA, "Biological Device Application Approvals: BK180211.BD BACTECTM Platelet Aerobic/F Culture vial and Platelet Anaerobic/F Culture Vial. <https://www.fda.gov/vaccines-blood-biologics/development-approval-process-cber/2018-biological-device-application-approvals>." 2018.
- [10] Brecher ME, Means N, Jere CS, Heath D, Rothenberg S, Stutzman LC. Evaluation of an automated culture system for detecting bacterial contamination of platelets: an analysis with 15 contaminating organisms. Transfusion 2001 Apr;41(4):477–82.
- [11] Hocquet D, Sautet M, Roussel S, Malugani C, Pouthier F, Morel P, Gbaguidi-Haore H, Bertrand X, Grenouillet F. Validation of an automated blood culture system for sterility testing of cell therapy products. Cytotherapy 2014 May;16(5):692–8.
- [12] Kielpinski G, Prinzi S, Duguid J, du Moulin G. Roadmap to approval: use of an automated sterility test method as a lot release test for Carticel, autologous cultured chondrocytes. Cytotherapy 2005;7(6):531–41.
- [13] Schroeter J, Wilkemeyer I, Schiller RA, Pruss A. Validation of the Microbiological Testing of Tissue Preparations Using the BACTEC™ Blood Culture System. Transfus Med Hemother 2012 Dec;39(6):387–90.
- [14] Gain P, Thuret G, Chiquet C, Vautrin AC, Carricajo A, Acquart S, Maugery J, Aubert G. Use of a pair of blood culture bottles for sterility testing of corneal organ culture media. Br J Ophthalmol 2001 Oct;85(10):1158–62.
- [15] Khuu HM, Stock F, McGann M, Carter CS, Atkins JW, Murray PR, Read EJ. Comparison of automated culture systems with a CFR/USP-compliant method for sterility testing of cell-therapy products. Cytotherapy 2004;6(3):183–95.
- [16] Golay J, Pedrini O, Capelli C, Gotti E, Borleri G, Magri M, Vailati F, Passera M, Farina C, Rambaldi A, Introna M. Utility of routine evaluation of sterility of cellular therapy products with or without extensive manipulation: Best practices and clinical significance. Cytotherapy 2018 Feb;20(2):262–70.
- [17] Lysák D, Holubová M, Bergerová T, Vávrová M, Cangemi GC, Ciccocioppo R, Krzuliak P, Jindra P. Validation of shortened 2-day sterility testing of mesenchymal stem cell-based therapeutic preparation on an automated culture system. Cell Tissue Bank 2016;17(1):1–9.
- [18] Viganò M, Budelli S, Lavazza C, Montemurro T, Montelatici E, de Cesare S, Lazzari L, Orlandi AR, Lunghi G, Giordano R. Tips and Tricks for Validation of Quality Control Analytical Methods in Good Manufacturing Practice Mesenchymal Stromal Cell Production. Stem Cells Int 2018 Sep 4;2018:3038565.
- [19] Bugno A, Saes DPS, Almodovar AAB, Dua K, Awasthi R, Ghisleni DDM, Hirota MT, de Oliveira WA, de Jesus Andreoli Pinto T. Performance Survey and Comparison Between Rapid Sterility Testing Method and Pharmacopoeia Sterility Test. J Pharm Innov 2018;13(1):27–35.
- [20] Murray L, McGowan N, Fleming J, Bailey L. Use of the BacT/alert system for rapid detection of microbial contamination in a pilot study using pancreatic islet cell products. J Clin Microbiol 2014 Oct;52(10):3769–71.
- [21] Mastroratti C, Yang L, Halpenny M, Toye B, Ramírez-Arcos S. Evaluation of the sterility testing process of hematopoietic stem cells at Canadian Blood Services. Transfusion 2012 Aug;52(8):1778–84.
- [22] Akl S, Lorenz J, Regan D. Sterility testing of minimally manipulated cord blood products: validation of growth-based automated culture systems. Transfusion 2013 Dec;53(12):3251–61.
- [23] Ramírez-Arcos S, Kou Y, Yang L, Perkins H, Taha M, Halpenny M, Elmoazzen H. Validation of sterility testing of cord blood: challenges and results. Transfusion 2015 Aug;55(8):1985–92.
- [24] Khuu HM, Patel N, Carter CS, Murray PR, Read EJ. Sterility testing of cell therapy products: parallel comparison of automated methods with a CFR-compliant method. Transfusion 2006 Dec;46(12):2071–82.
- [25] Liu C, Weber C, Sempek DS, Grossman BJ, Burnham CA. Sterility testing of apheresis hematopoietic progenitor cell products using an automated blood culture system. Transfusion 2013;53(11):2659–66.
- [26] Parveen S, Kaur S, David SA, Kenney JL, McCormick WM, Gupta RK. Evaluation of growth based rapid microbiological methods for sterility testing of vaccines and other biological products. Vaccine. 2011 Oct 19;29(45):8012–23.
- [27] Microbiological examination of non-sterile products: microbial enumeration test (07/2010:20612). European Directorate for the Quality of Medicines & HealthCare (EDQM). France: Strasbourg; 2017 editor. European Pharmacopoeia. 9.2th ed.
- [28] USP 61 Microbiological examination of nonsterile products: microbial enumeration test. United States Pharmacopoeia. 41th ed. Rockville (MD): The United States Pharmacopoeial Convention, Inc; 2018 National Formulary.
- [29] Panch SR, Bikkani T, Vargas V, Procter J, Atkins JW, Gupta V, Frank KM, Lau AF, Stronck DF. Prospective Evaluation of a Practical Guideline for Managing Positive Sterility Test Results in Cell Therapy Products. Biol Blood Marrow Transplant 2019 Jan;25(1):172–8.
- [30] Flory E, Reinhardt J. European regulatory tools for advanced therapy medicinal products. Transfus Med Hemother 2013 Dec;40(6):409–12.

- [31] Riley JA, Heiter BJ, Bourbeau PP. Comparative recovery of microorganisms from BacT/ALERT plastic and glass FA and FN blood culture bottles. *J Clin Microbiol* 2005 Jul;43(7):3244–6.
- [32] Jimenez L, Rana N, Amalraj J, Walker K, Travers K. Validation of the BacT/ALERT(R) 3D System for Rapid Sterility Testing of Biopharmaceutical Samples. *PDA J Pharm Sci Technol* 2012 Jan-Feb;66(1):38–54.
- [33] Mueller-Premru M, Jeverica S, Papst L, Nagy E. Performance of two blood culture systems to detect anaerobic bacteria. Is there any difference? *Anaerobe* 2017 Jun;45:59–64.
- [34] Almuhayawi M, Altun O, Abdulmajeed AD, Ullberg M, Özenci V. The Performance of the Four Anaerobic Blood Culture Bottles BacT/ALERT-FN, -FN Plus, BACTEC-Plus and -Lytic in Detection of Anaerobic Bacteria and Identification by Direct MALDI-TOF MS. *PLoS One* 2015 Nov 10;10(11):e0142398.
- [35] Sanabria A, Røkeberg MEO, Johannessen M, Sollid JE, Simonsen GS, Hanssen AM. Culturing periprosthetic tissue in BacT/Alert® Virtuo blood culture system leads to improved and faster detection of prosthetic joint infections. *BMC Infect Dis* 2019 Jul 10;19(1):607.
- [36] U.S. Department of Health and Human Services. Food and Drug Administration (FDA), Premarket Notification 510 (k) Number K151866 (2016). BACTEC Peds Plus/F culture vials (plastic). Summary, 2016.
- [37] U.S. Department of Health and Human Services. Food and Drug Administration (FDA), Premarket Notification 510 (k) Number K173873 (2018). BACTEC Peds Plus/F culture vials (plastic). Summary
- [38] U.S. Department of Health and Human Services. Food and Drug Administration (FDA), Premarket Notification 510 (k) Number K173873 (2018). BACTEC Peds Plus/F culture vials (plastic). Substantial equivalence determination decision.
- [39] U.S. Department of Health and Human Services. Food and Drug Administration (FDA), Premarket Notification 510 (k) Number K083572 (2008). BACTEC Aerobic/F culture vials. Substantial equivalence determination decision"
- [40] U.S. Department of Health and Human Services. Food and Drug Administration (FDA), Premarket Notification 510 (k) Number K113558 (2012). BACTEC Aerobic/F culture vials (plastic). Substantial equivalence determination decision.
- [41] Chen IH, Nicolau DP, Kuti JL. Effect of Clinically Meaningful Antibiotic Concentrations on Recovery of *Escherichia coli* and *Klebsiella pneumoniae* Isolates from Anaerobic Blood Culture Bottles with and without Antibiotic Binding Resins. *J Clin Microbiol* 2019 Nov 22;57(12):e01344–19.
- [42] Chen IH, Nicolau DP, Kuti JL. Recovery of Gram-Negative Bacteria from Aerobic Blood Culture Bottles Containing Antibiotic Binding Resins after Exposure to β -Lactam and Fluoroquinolone Concentrations. *J Clin Microbiol* 2019 Sep 24;57(10):e00849–19.
- [43] Chung Y, Kim IH, Han M, Kim HS, Kim HS, Song W, Kim JS. A comparative evaluation of BACT/ALERT FA PLUS and FN PLUS blood culture bottles and BD BACTEC Plus Aerobic and Anaerobic blood culture bottles for antimicrobial neutralization. *Eur J Clin Microbiol Infect Dis* 2019 Dec;38(12):2229–33.
- [44] Lotfi R, Rojewski MT, Zeplin PH, Funk W, Pullig O, Nöth U, Schrezenmeier H. Validation of Microbiological Testing of Cellular Medicinal Products Containing Antibiotics. *Transfus Med Hemother* 2020 Apr;47(2):144–51.
- [45] Dyrda G, Boniewska-Bernacka E, Man D, Barchiewicz K, Stota R. The effect of organic solvents on selected microorganisms and model liposome membrane. *Mol Biol Rep* 2019 Jun;46(3):3225–32.
- [46] Tarrand JJ, LaSala PR, Han XY, Rolston KV, Kontoyiannis DP. Dimethyl sulfoxide enhances effectiveness of skin antiseptics and reduces contamination rates of blood cultures. *J Clin Microbiol* 2012 May;50(5):1552–7.
- [47] Honohan A, Olthuis H, Bernards AT, van Beckhoven JM, Brand A. Microbial contamination of cord blood stem cells. *Vox Sang* 2002 Jan;82(1):32–8. PMID: 11856465.
- [48] Montag T, Nicol SB, Schurig U, Heiden M, Huber H, Sanzenbacher R, Flory E, Schwanig M, Schneider CK. Microbial safety of cell based medicinal products—what can we learn from cellular blood components? *Clin Chem Lab Med* 2008;46(7):963–5.
- [49] Köck R, Eißing LC, Boschin MG, Ellger B, Horn D, Idelevich EA, Becker K. Evaluation of bactec mycosis IC/F and Plus Aerobic/F blood culture bottles for detection of *Candida* in the presence of antifungal agents. *J Clin Microbiol* 2013 Nov;51(11):3683–7.
- [50] Nawrot U, Kowalska-Krochmal B, Sulik-Tyska B, Kozak M, Świętek K, Pajączkowska M, Piątkowska E, Rosiak D, Swoboda-Kopeć E. Evaluation of blood culture media for the detection of fungi. *Eur J Clin Microbiol Infect Dis* 2015 Jan;34(1):161–167.
- [51] Cobos-Trigueros N, Morata L, Torres J, Zboromyrska Y, Soriano A, Pitart C, De La Calle C, Marco F, Hernandez C, Almela M, Mensa J, Martinez JA. Usefulness of time-to-positivity in aerobic and anaerobic vials to predict the presence of *Candida glabrata* in patients with candidaemia. *J Antimicrob Chemother* 2013 Dec;68(12):2839–41.
- [52] Spanu T, Posteraro B, Fiori B, D'Inzeo T, Campoli S, Ruggeri A, Tumbarello M, Canu G, Trecarichi EM, Parisi G, Tronci M, Sanguinetti M, Fadda G. 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 Jan;50(1):176–9.
- [53] Pryce TM, Palladino S, Price DM, Gardam DJ, Campbell PB, Christiansen KJ, Murray RJ. Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions. *Diagn Microbiol Infect Dis* 2006 Apr;54(4):289–97.
- [54] Bourbeau PP, Pohlman JK. Three days of incubation may be sufficient for routine blood cultures with BacT/Alert FAN blood culture bottles. *J Clin Microbiol* 2001 Jun;39(6):2079–82.
- [55] Spanjaard L, Kuijper EJ, Dankert J. Clinical comparison of two commercial blood culture systems. *Eur J Clin Microbiol Infect Dis* 2000 Nov;19(11):881–5.
- [56] Kim SH, Yoon YK, Kim MJ, Sohn JW. Clinical impact of time to positivity for *Candida* species on mortality in patients with candidaemia. *J Antimicrob Chemother* 2013 Dec;68(12):2890–7.
- [57] Horvath LL, George BJ, Murray CK, Harrison LS, Hospenthal DR. Direct comparison of the BACTEC 9240 and BacT/ALERT 3D automated blood culture systems for candida growth detection. *J Clin Microbiol* 2004 Jan;42(1):115–8. PMID: 14715740; PMCID: PMC321727.
- [58] Fiori B, D'Inzeo T, Di Florio V, et al. Performance of two resin-containing blood culture media in detection of bloodstream infections and in direct matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) broth assays for isolate identification: clinical comparison of the BacT/Alert Plus and Bactec Plus systems. *J Clin Microbiol* 2014 Oct;52(10):3558–67.
- [59] Ericson EL, Klingspor L, Ullberg M, Ozenci V. Clinical comparison of the Bactec Mycosis IC/F, BacT/Alert FA, and BacT/Alert FN blood culture vials for the detection of candidemia. *Diagn Microbiol Infect Dis* 2012 Jun;73(2):153–6.
- [60] Meyer MH, Letscher-Bru V, Jaulhac B, Waller J, Candolfi E. Comparison of Mycosis IC/F and plus Aerobic/F media for diagnosis of fungemia by the bactec 9240 system. *J Clin Microbiol* 2004 Feb;42(2):773–7. PMID: 14766852; PMCID: PMC344513.
- [61] England MR, Stock F, Gebo JET, Frank KM, Lau AF. Comprehensive Evaluation of Compendial USP<71>, BacT/Alert Dual-T, and Bactec FX for Detection of Product Sterility Testing Contaminants. *J Clin Microbiol* 2019 Jan 30;57(2):e01548–18.
- [62] George BJ, Horvath LL, Hospenthal DR. Effect of inoculum size on detection of *Candida* growth by the BACTEC 9240 automated blood culture system using aerobic and anaerobic media. *J Clin Microbiol* 2005 Jan;43(1):433–5.
- [63] Chiarini A, Palmeri A, Amato T, Immordino R, Distefano S, Giammanco A. Detection of bacterial and yeast species with the Bactec 9120 automated system with routine use of aerobic, anaerobic, and fungal media. *J Clin Microbiol* 2008 Dec;46(12):4029–33. Epub 2008 Oct 15. PMID: 18923011; PMCID: PMC2593249.
- [64] Klingspor L, Muhammed SA, Ozenci V. Comparison of the two blood culture systems, Bactec 9240 and BacT/Alert 3D, in the detection of *Candida* spp. and bacteria with polymicrobial sepsis. *Eur J Clin Microbiol Infect Dis* 2012 Nov;31(11):2983–7. Epub 2012 Jun 6. PMID: 22669561.