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The BD BACTEC FX blood culture system with the gentlemacs dissociator is suitable for sterility testing of heart valve and vascular allografts—A validation study

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Abstract To present our validation study of the BD BACTEC FX blood culture system for sterility testing of cardiovascular tissues aimed for human application. For operational qualification, we performed temperature mapping of the system, vacuum test using non-inoculated BACTEC vials, and growth promotion tests by injecting contaminant strains into aerobic and anaerobic bottles. For performance qualification, negative control, assessment of method suitability, evaluation of sensitivity limits, control of neutralization of antibiotics in biopsy samples from allografts and tissue toxicity effects, were performed. Tissue samples and transport/cryopreservation solutions were homogenized in GentleMACS Dissociator and injected into BACTEC Plus aerobic and anaerobic vials for incubation at 35 °C for 14 days. Tissues were spiked with aerobic and anaerobic bacteria and fungi. Growth of contaminants appeared in all aerobic and anaerobic vials for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*; in anaerobic vials for Cutibacterium (Propionibacterium) acnes and *Clostridium sporogenes*; and only in aerobic vials for *Candida albicans* and *Aspergillus brasiliensis*. The majority of bacterial strains were detected within two days (59–100%), exceptionally between 3 and 14 days. In contrast, fungal contaminations were detected within 2, 3–6, 7–10 and after 10 days of incubation in 33.3, 71.6, 96.6 and 99.9% of cases,respectively. Uninhibited growth appeared in the tissue biopsies and homogenized tissues with and without antibiotics and in other solutions. BD BACTEC blood culture system with GentleMACS Dissociator is a rapid and efficient tool for detection of contamination in cardio-vascular tissues aimed for human application.

Keywords Sterility · BACTEC · Cardiovascular allografts · Contamination · Tissue banking · Validation

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Introduction

Sterility testing of human cardiovascular tissues (CVT) presents a critical step in tissue banking, necessary to guarantee safety of allografts (heart valves, blood vessels) aimed for human application (Fan 2012). Following the recommendations of the European Directives on human tissues aimed for human application, allografts must be tested for possible contaminants at least at the beginning of processing (tissues and/or transport solution) as well as prior to sealing the primary packaging of allograft before its preservation and storage (cryopreservation solution) (Jashari 2007,Van Kats 2010). In order to stimulate growth of aerobic and anaerobic bacteria as well as mould and yeasts, test samples have to be inoculated in nutritional media and incubated for 14 days. From all positive samples, identification of the isolate is mandatory by sub-culturing of contaminated material (Tabaku 2004, Van Kats 2010).

For rapid and reliable management of infections in clinical settings, blood culture systems were introduced in the '80 s and '90 s as highly sensitive systems to culture and identify bacteria and fungi in blood and other body fluids (Appelbaum et al. 1982; Kirihara 1985; Fuller and Davis, 1997). At the present time automated blood culture systems continue to improve (Funke 2004) and they currently offer the preferred method for rapid identification of blood infections in patients worldwide with fewer cases of sampling introduced contamination (Minassian 2014). Many laboratories currently use the blood culture system for their routine bacteriology testing because of the ease of use of such a system, the rapid detection of contaminants, and the minimal manipulation required that might otherwise result in sample contamination and thus false positive results (Minassian et al., 2014). Among the current marketed automated systems, the BD BACTEC FX Blood Culture System (Becton Dickinson, USA), the BacT/ALERT (BioMerieux, France) and VersaTrek (Trek Diagnostic Systems, USA) (Dreyer 2014), are the most widely used systems in clinical microbiology laboratories. Blood culture vials have been successfully used as a more rapid and efficient tool for the detection of possible contaminants in biological fluids other than blood (Fuller 1997; Minassian 2014). In view of these results, some tissue establishments (TE) have adopted this technique for testing tissue culture media and/or tissue preservation fluids for the possible presence of contaminants (Paolin 2017; Van Kats 2010). Hence, in recent years some eye banks have worked on the validation and implementation of the BD BACTEC blood culture system as a tool for routine sterility testing of cell culture solutions and cornea preservation solutions (Schroeter 2012; Skenderi 2018).

In tissue banking, confirmation of the lack of contaminats on CVT (heart valves, blood vessels) is one of the most important concerns. The majority of the cardiovascular TE's report a significant discard rate annually of CVT allografts due to the failure to eliminate contamination (Zahra 2019). A worrying high failure rate in identifying the presence of microbiological contamination (about 50%) is observed in tissue banks when only swabs or tissue biopsies are used for testing (Varettas K 2014; Vehmejer et al. 2001; Veen 1994). In contrast, the sensitivity to identify the presence of contamination increases to over 90%, if samples of the filtered transport fluid are tested together with the tissue biopsy (Diaz Rodriguez 2016; Zahra 2019). Accordingly, implementation of closed systems might be more suitable for testing of tissues, aimed for human application (Van Kats 2010). To be able to test tissues in a closed system like the BD BACTEC FX blood culture system, the tissue sample needs to be homogenised into an injectable substance. For this we have used the GentleMACS Dissociator with heaters (Miltenyi Biotec B.V. and Co. KG, Germany); this allows samples of tissue suspended in a sterile liquid (DMSO (dimethyl- sulfoxide, WAK Chemie, Germany), normal saline, RPMI (Roswell Park Memorial Institute, USA)) to be homogenized into a solution that can than be injected into the BD BACTEC Plus blood culture vials, similar to blood samples.

This paper presents our validation study using the BD BACTEC FX blood culture system with the GentleMACS Dissociator for the complete microbiology testing (transport solution/cryopreservation solution together with the tissue samples (before and after antibiotic treatment,respectively) of the human heart valves and blood vessels aimed for human application. Implementation of this technique in our TE were based on the following statements:

(i) The technique is simple, fast and proven to be most sensitive and accurate in detecting

bacterial and fungal presence in blood and bodily fluids and tissues,

(ii) False positive results due to lab introduction of contamination is expected to decrease significantly due to the fact that the testing is carried out in a closed system,

(iii) The system does not require daily control of samples during the incubation period and terminal sub-culturing (Minassian 2014).

Furthermore, our estimations are that this system is more cost-effective when compared to the open technique used in many TE's and laboratories.

The study was designed and carried out in accordance with the European Pharmacopoeia (EP) 2.6.1 and 2.6.27 and the Belgian Superior Health Council (BSHC) requirements for the quality, safety and security of human body material (HBM), aimed for human application. Although this system was already validated for testing of solutions, used during processing of other tissue types (tissue culture medium, storage solutions for cornea banking, cell cultures, etc., (Paolin 2017; Van Kats 2010; Schroeter 2012, Skenderi 2018)), so far no CVT establishment has validated and implemented this system for sterility testing of the heart valves and blood vessels, involving both tissue and liquid samples used during the processing of these products.

Material and methods

Material

For the validation procedure presented in this paper, we used CVT (heart valves) that were identified as not being suitable for human application. After the initial sterility testing of the donated tissues, the sterile samples, not treated with antibiotics, were temporarily stored in normal saline of (0.9%) and frozen at −18 °C before their usage for this validation.

Microbial strains used

The strains used for this validation included: *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 27,853), *Escherichia coli* (ATCC 8739), *Enterococcus faecalis* (ATCC 29,212), *Staphylococcus epidermidis* (ATCC 12,228), *Clostridium sporogenes* (ATCC 19,404), *Cutibacterium (Propionibacterium) acnes* (ATCC11827), *Candida albicans* (ATCC 10,231) and *Aspergillus brasiliensis* (ATCC 16,404). The *Enterococcus faecalis* was used instead of a *Streptococcus* due to the clinical problems that can result should there be contamination with Enterococci and this micro-organism's resistance against many antibiotics used by tissue banks to decontaminate CVT, contrary to the susceptibility of streptococci, which is considered easier to eradicate from tissue products if present prior to decontamination.

Fluids and media

Transport solution (normal saline 0.9%), cryopreservation solution (DMSO 10% in RPMI), decontamination antibiotic cocktail in RPMI (Vancomycin 50 µg/mL, Lincomycin 120 µg/mL, Polymyxin B 100 µg/mL), BACTEC vials with medium (BD BACTEC Plus Anaerobic/F Vials and Aerobic/F Vials, Becton, Dockinson and Co, USA), TrypticaseSoyAgar and TrypticaseSoyBroth as well as nutritional angar plates (Biomerieux, France) and sheep blood agar plates (Columbia) and Brucella agar (Becton Dickinson, USA).

Technical tools

(a) *BD BACTEC FX blood culture system (Becton, Dockinson and Co, USA)*

(b) *GentleMACS Dissociator with Heaters (Miltenyi Biotec B.V.* and *Co. KG, Germany)*, a closed sterile system with disposable component, for homogenization of tissue products with transport medium and cryopreservation medium, respectively. This equipment is usually used in pathology laboratories. The GentleMACS makes use of a disposable M-Tube in which tissue can be homogenized with liquid and than drawn up by a syringe.

(c) All work was done under proper environmental conditions in a Class II Biohazard Safety Cabinet (ESCO Group, Singapoore).

(d) Anoxomat provides an enriched or anaerobic atmosphere for culturing specific microorganisms.

(e) Densitometer was used for measuring the concentration of an inoculum in transparent fluid.
(f) Maldi-Tof (MBT smart, Bruker, Germany) was used for identification of bacterial/fungal strains.

The validation was carried out in two steps: The *operational qualification* and the *performance qualification*.

Operational qualification

For the operational qualification, prior to the validation tests some preliminary tests had to be performed (in accordance with EP and Belgian Superior Health Council (BSHC) advice 8698 from 2014):

Temperature Mapping (TM)

The BD BACTEC FX blood culture system works on the principle of incubation so as to accelerate the multiplication of micro-orgabnisms. Therefore, it is essential to ensure the stability of the temperature within the device. For this test, the temperature probes were placed in different locations within the device, which is set at the temperature of 35° C. The temperature was recorded for a maximum of 36 h.

Vacuum Tests (VT)

For this test, the non-inoculated BACTEC Plus Aerobic/F and Anaerobic/F Vials, were introduced into the BD BACTEC FX blood culture system cells, in the different locations and incubated at 35 °C for a maximum of 14 days. All bottles are meant to remain negative after 14 days of incubation.

Growth Promotion Test (GPT)

According to EP and BSHC advice, these tests are to ensure that the culture media and culture conditions are suitable for the growth of bacteria/fungi, as well as to check for false negative results.

In a Class II Biohazard Safety Cabinet, 10 ATCC strains (see above) were each inoculated (100 µL from a 10–100 CFU/mL normal saline solution) into BACTEC Plus aerobic and anaerobic blood culture vials. The bacterial and fungal strains used are the most common isolates in CVT as previously published (Fan 2012) and suggested by EP and BSHC in 2014. The inoculated vials were introduced into the BD BACTEC FX blood culture system for incubation at a temperature of 35 °C for a maximum of 14 days. All positive bottles were removed from the incubator the day they gave a positive sign ("flagged" in the system). From each positive bottle, the fluid was subcultured on TSA (Tryptic Soy Agar) and TSB (Tryptic Soy Broth) nutritional agar plates (Biomerieux, France), and than sent to the Microbiology Laboratory for micro-organism identification. These tests were repeated 9 times for each of the selected contaminant strain.

Performance qualification

For performance qualification we carried out the following**:**

Negative Control (NC)

This test was carried out to ensure that the samples tested by the Tissue Bank (normal saline 0.9%, DMSO and antibiotic cocktail) do not interfere with the results of the analysis in the BACTEC system. The BACTEC system continually checks for evidence of growth in any of the bottles, and signals ("flags") as soon as a positive sample is identified. However, false positive results can occur if the injected sample interferes with the sensor. In order to confirm that the fluids added to the tissue products do not lead to false positive results, these negative control tests were performed. Normal saline is a transport medium, while the DMSO is the cryopreservative that is added during processing after allograft has been decontaminated with the antibiotic cocktail used in the TE. These sterile solutions (without added tissue products) were injected into the BACTEC Plus aerobic and anaerobic vials, and then incubated in the BD BACTEC FX blood culture system for 14 days. These tests were repeated three times in accordance with the EP to ensure the results were reproducible. The expectation was that these culture bottles would remain negative after incubation.

Method Suitability Testing (MST)

Method suitability testing was performed to ensure that the injected medium does not contain agents that inhibit bacterial/fungal growth or interferes with the detection of that growth.

Volumes of 10 mL of sterile physiologic solution, as equivalents to the test solutions that would normally be added to the BACTEC vials, were contaminated with 100 µL of a low inoculum of 10–100 CFU per mL of the micro-organism to be tested and were injected aseptically into the BD BACTEC Plus aerobic and anaerobic vials, then incubated at 35 °C for maximum of 14 days in the BD BACTEC FX blood culture system. This test was repeated three times (according to EP) for each of the bacterial and fungal strains. The expected results were an uninhibited growth of the inoculated contaminating strain (Fig. 1).

Evaluation of Sensitivity Limits (SL)

For this part of the validation process, the maximum concentration of the bacterial strain used was 100 CFU/mL. It was important to also ensure that low concentrations of contamination could be detected, down to concentrations of < 10 CFU/mL. Therefore, lower concentrations such as 5–50 CFU/mL and 1–10 CFU/mL, were evaluated as well. Again, the test was repeated three times (according EP) for each solution and mycroorganism, as well as for the three inocula concentrations. These tests were done in 2 solutions: transport solution (normal saline 0.9%) and cryopreservative (RPMI with DMSO).

Control of antibiotic neutralization in the allograft biopsy samples

Many tissue samples that are tested contain bacteriotoxic or bacteriostatic factors or antibiotic residues used for decontamination of allografts (Buzi 2014; Jashari 2011). Verification of neutralization of this toxicity and antibiotic residues in the different fluids (transport/DMSO/cocktail of antibiotics and negative control) by the BACTEC Plus aerobe/F and anaerobe/F vials was one of the aims of this study. Various tests were performed in order to demonstrate this. After decontamination of the donated tissue (myocardium, heart valve) in an antibiotic cocktail for 40–48 h (standard EHB protocol, (Jashari 2007)), the tissue biopsies (5 × 5 mm, average 7–9 g, including myocardium from the left ventricle, right ventricle and septum, tricuspid valve, mitral valve and a sample from the aortic wall) were collected during the heart dissection in the cleanroom. Subsequently, the biopsies were tested as follows:

1. Following decontamination, two biopsy specimens, were inoculated directly on blood agar plates to

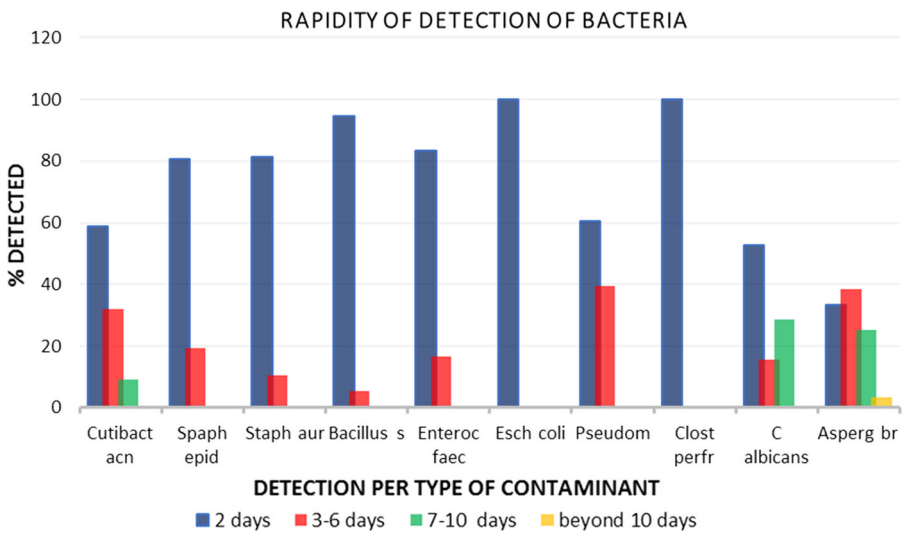

Fig. 1 Rapidity of positive detection in BACTEC System. Majority of contaminants were detected within 2 days and only few cases beyond 10 days (see Table 2)

measure the concentration of antibiotics in the tissue/fluid,

2. Two biopsy specimens were inoculated into the BACTEC Plus vials after aseptic removal of the vial cover in the Class II Biohazard Safety Cabinet,

3. Following decontamination, two biopsy specimens were homogenized and the homogenate was put directly in the cavities/holes made in the blood agar plates (as demonstrated in Fig. 2),

4. Two further biopsy specimens were introduced into an M-Tube together with 10 mL of sterile normal saline 0.9%, homogenized in the GentleMACS Dissociator (Fig. 3), and then injected into a BD BACTEC Plus Aerobe / F Vial, and.

5. Two final biopsy speciments were introduced into an M-Tube together with 10 mL of sterile 10% DMSO (concentration used for cryopreserevation of CV allografts). The homogenized solution in the GentleMACS Dissociator was subsequently injected into a BACTEC Plus Anaerobe / F Vial (Fig. 3).

The bottles with injected material were incubated in the BD BACTEC FX blood culture system at 35 °C for 24 h. To check for the presence of toxic or antibiotic residues in fluid and tissue, all bottlesfrom 2, 3, 4 and 5 were sub-cultured after 24 h onto two different TSA agar plates (including negative controls). On these plates, prior to applying the tissue biopsy and BACTEC Plus Vial-fluid samples onto the plates, *Escherichia coli* and a Coagulase Negative Staphylococcus were respectively streaked over the whole plate (one micro-organism per plate; Fig. 4). Subsequently, biopsy material from the bottle was transferred onto the TSA plates and fluid from the bottle was put in a small hole made in the agar. Any antibiotics present in the biopsy or in the fluid would diffuse into the agar and, thereby inhibit the growth of the bacteria added. This should appear as a visible circle around the tissue biopsy or around the holes containing the homogenate/BACTEC fluid/saline/DMSO/only tissue. Tissue biopsies and homogenate without added antibiotics served as negative controls. After 48 h of incubation at 35 °C, the diameters of the circles of failed growth on the TSA plates were measured so as to define the difference in the antibiotic concentration before (biopsy 1) and after (biopsies 2, 3, 4) incubation in the BACTEC medium.

Tissue toxicity testing

The potential of tissue toxicity was tested by adding a very low inoculum (1–10 CFU/mL) of Escherichia coli, Coagulase Negative Staphylococci or Candida albicans into a broth with and without the presence of heart valve tissue. Both broths, with and without tissue, should show the same growth to confirm that the presence of the tissue does not interfere with bacterial/fungal growth.

Complementary evaluation of the negative control vials

As a complementary negative control, 100 µL were aspirated from 100 randomly selected negative aerobic and anaerobic vials following 14 days incubation in the BD BACTEC blood culture system, and were re-tested to check for possible presence of contamination in the vials (control for possible false negative result) including a Gram stain, to visually confirm that no microorganisms were present. The control was

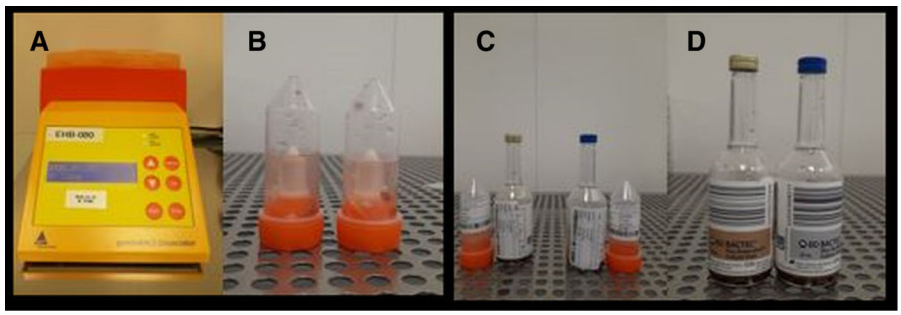

Fig. 2 **a** GentleMACS Dissociator for homogenization of the tissue samples and transport/cryopreservation media. **b** M–tubes for collecting the samples of tissues and solutions (transport/cryopreservation). **c** homogenate for inoculation in the BACTEC Plus vial; **d** BACTEC Plus Aerobic/F and Anaerobic/F vials

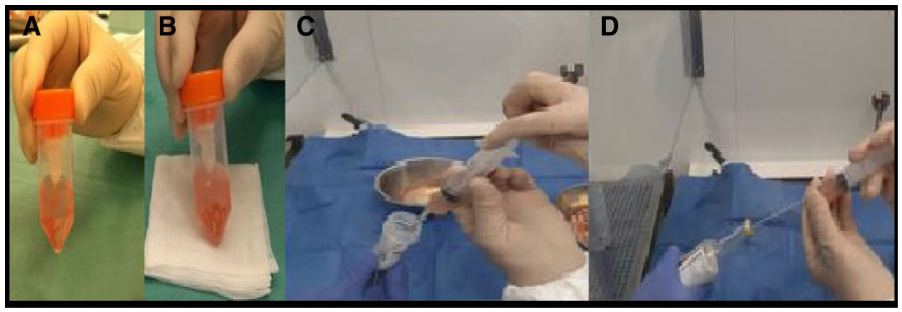

Fig. 3 Collected material for homogenization (tissue pieces and solution (**a, b**)), to be processed in GentleMACDissociator. Subsequent injection of homogenate in the BACTEC Plus Vials (**c, d**) for incubation for maximum 14 days

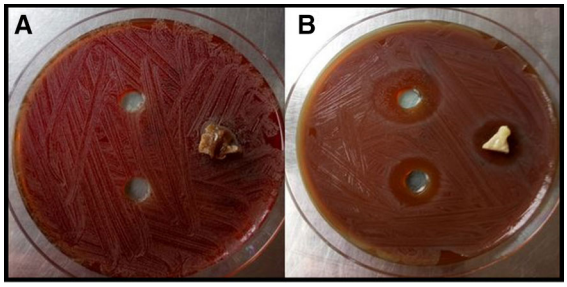

Fig. 4 Blood agar plate. **a Escherichia Coli**, growth (no inhibition by antibiotics); **b** coagulase Negative Staphylococcus, circles around biopsies/homogenate (inhibition by antibiotics)

performed by automated aerospray (Elitech, Molecular Diagnostics) onto Columbia5% sheep blood agar and Brucella agar (Becton Dickinson, USA), for aerobic and anaerobic vials, respectively. Plates were incubated for 72 h at 35 °C in 5% CO_2 enriched atmosphere or anaerobic atmosphere (anoxomat system) for aerobic and anaerobic cultures, respectively and examined for bacterial growth every 24 h. All growing microorganisms were identified by mass spectrometry MALDI-TOF MS (mass spectrometry).

Results

Mapping the BDBACTEC FX blood culture system showed a constant temperature of 35 °C ± 2.5 °C during the monitored period of up to 36 h. There were no fluctuations of the temperature registered during the whole period of monitoring.

The ***Vacuum Tests*** showed that all BD BACTEC Plus /F Vials (three aerobic and three anaerobic), that had not been spiked with contaminants, were negative after 14 days of incubation (Table 1).

The ***Grow Promotion Test*** showed positivity within 14 days for each of the tested microorganism (Table 1). Depending on the type of microorganism, growth was detected either in the BD BACTEC Plus aerobic/ F Vials or anaerobic/ F Vials, or in both. A hundred percent of Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa grew in both aerobic and anaerobic vials. All Cutibacterium acnes and Clostridium sporogenes grew only in anaerobic vials. On the contrary, all tested fungal strains (Candida albicans and Aspergillus brasiliensis) grew only in aerobic vials, (Table 1).

Performance Qualification

Negative Controls showed no positivity after 14 days of incubation. There was no interference caused by DMSO, RPMI, normal saline or antibiotic cocktail with the BACTEC vials in the BD BACTEC FX blood culture system.

Method Suitability Tests for the samples taken from the transport solution and tissues ("test A" or sterility test of donated material), after incubation of the tissues in the antibiotic cocktail, and from the cryopreservation solution ("test B" or sterility test of the "in process" and the "final product"): each microorganism was detected within 14 days of incubation. The detection time of growth was mostly within 2 days, but it varied depending on the tested micro-organism (Table 2, Fig. 1).

Evaluation of the ***Sensitivity Limits (SL)*** showed that all strains in transport and in cryopreservation

Table 1 Operational qualification: Vacuum Test (VT), Growth Promotion Test (GPT), Method Suitability Test (MST), Environment preference of microorganisms (EP), and Sensitivity Limits (SL). For VT, the non-inoculated aerobic and anaerobic vials were incubated for 14 days. For GPT, the vials were inoculated with selected aerobic/anaerobic bacteria and fungi. T/P: transport/preservation solution; MO (Ae/An): Aerobic/Anaerobic microorganisms; O/Ø: Preference for environment (aerobic/anaerobic); NIV: non-inoculated vials; SL: Lowest concentration of microorganisms not inhibiting microbial growth

Test	VT	GPT	M. Suitability (MST)(T/P solution)	EP O/Ø	SL		
MO (Ae/An)					1–10 MO/mL	5–50 MO/mL	10–100 MO/mL
Cutibacterium acnes	–	+	+ / +	Ø	+	+	+
Staphylococcus epidermidis	–	+	+ / +	O + Ø	+	+	+
Staphylococcus aureus	–	+	+ / +	O + Ø	+	+	+
Bacillus subtilis		+	+ / +	O + Ø	+	+	+
Clostridium sporogenes	–	+	+ / +	O + Ø	+	+	+
Enterococcus faecalis	–	+	+ / +	O + Ø	+	+	+
Escherichia coli	–	+	+ / +	O + Ø	+	+	+
Pseudomonas aeruginosa	–	+	+ / +	0 + Ø	+	+	+
Candida albicans		+	+ / +	O	+	+	+
Aspergillus brasiliensis		+	+ / +	O	+	+	+
	NIV	9 × for each MO	3 × for each MO		Repeated 3 × for each MO		

Table 2 Rapidity of detection of contamination by the BD BACTEC FX blood culture system: in the majority of samples, contamination was detected in the first 48 h of incubation (between 33 and 100%). Only in few cases (2.6–3.3%), was contamination detected only after day 10 of incubation (only for Staphylococcus aureus, Candida albicans and Aspergillus brasiliensis)

Contaminant used	Growth detected (%)			
	Within 2 days	3–6 days	7–10 days	After 10 days
Cutibacterium acnes	59	32	9	–
Staphylococcus epidermidis	80.6	19.4	–	–
Staphylococcus aureus	81.4	10.6	5.3	2.6
Bacillus subtilis	94.6	5.4	–	–
Enterococcus faecalis	83.5	16.5	–	–
Escherichia coli	100	–	–	–
Pseudomonas aeruginosa	60.5	39.5	–	–
Clostridium sporogenes	100	–	–	–
Candida albicans	52.8	15.7	28.6	2.8
Aspergillus brasiliensis	33.3	38.3	25	3.3

fluid were detected from contaminated tissues at the lowest concentrations (1 to 10 CFU/mL; Table 1).

Neutralization of antibiotics

The ***Bacteriostatic or Toxicity effect*** of tissues on the experimental contaminants, as presented in Table 3 demonstrate that the tissue biopsies (1) taken after incubation of allografts in the antibiotic cocktail, contain the different antibiotics in therapeutic high concentrations (clear circles of 30 mm in Table 3). However, the level of detectable antibiotics decreases significantly (to 0–2 mm) after 24 h of incubation in the BD BACTEC FX blood culture system. After two days of incubation, the biopsies and homogenate did not contain any detectable antibiotics anymore (0 mm clearance; Table 3, Fig. 4), either in the tissue biopsy or homogenized in transport fluid or DMSO fluid.

Tissue toxicity was non present. The growth of a low inoculum of *Escherichia coli*, Coagulase Negative Staphylococcus or *Candida albicans* was equal in broth with or without heart valve tissue.

Final ***re-testing of the 100 negative vials*** on TSA plates showed growth of *Cutibacterium acnes* only in one vial.

Discussion

Confirmation of lack of contamination of cardiovascular allografts is very important step in tissue banking activity. Despite significant differences in techniques used by different TE-s (Diaz Rodriguez 2016; Fan 2012; Hensen Suss 2018; Jashari 2007; Zahra 2019), all are aiming for the same goal: the release of allografts with a minimum risk for transmission of contamination at the moment of transplantation. TE-s report a very significant variation in their ability to detect and prevent or eradicate contamination in the solutions and tissue samples they process. Some TE-s fail to detect and identify one or more of the contaminants present, due to the use of culture media that do not support growth of all types of potential contaminants that may have been present, or misidentify some of the microorganisms (Zahra 2019). Furthermore, using open techniques for sterility testing of human tissues might introduce contamination of the samples from the environment or cause cross contamination during inappropriate handling in the laboratory (Zahra 2019). This would be avoided if the whole process is performed in a closed system (from the transport solution to the DMSO before final packaging of allograft) as is done with homogenized

Table 3 Diffusion tests for antibiotic residues in tissue and homogenates, after decontamination, directly on agar plates and after incubation for 24 h in BACTEC. *E. coli: Escherichia coli*; CNS: Coagulase negative staphylococcus.

Testing	Substance	Time	M.O.	Zones (in mm)	Zones after 24 h					Disk-diffusion
DIRECT	Biopsy	Agar plate	*E.coli*	25						Polymixin: 17 mm
DIRECT	Biopsy	Agar plate	CNS	23						Vancocin: 15 mm
BACTEC	Biopsy	24 h in BACTEC	E.coli		1	2	0	0	1	
BACTEC	Biopsy	24 h in BACTEC	CNS		0	1	1	0	0	Lincocin: 21 mm
DIRECT	Homogenate	Agar plate	E.coli	30						
DIRECT	Homogenate	Agar plate	CNS	30						
BACTEC	Transp + biopt	24 h in BACTEC	E.coli		0					
BACTEC	Transp + biopt	24 h in BACTEC	CNS		0					
BACTEC	DMSO + biopt	24 h in BACTEC	E.coli		0					
BACTEC	DMSO + biopt	24 h in BACTEC	CNS		0					

tissue that can be injected into BD BACTEC Plus blood culture vials. Introduction of automated systems that use rich media has enhanced the detection and identification of possible contaminants (Minassian 2014; Schroeter 2012; Skenderi 2018).

For over 30 years of tissue banking activity, we have used open techniques for sterility testing of CVT with moderate success. Each year approximately 10–15% of heart valves and approximately 15–20% of vascular allografts were discarded due to the presence of contamination (EHB Yearly Reports, internal data). Different aspects of our decontamination strategy and results thereof were presented and published in the past (Diaz Rodriguez 2016; Fan 2012; Jashari 2007; Tabaku 2004).

Following the relocation of our TE in May 2015 and change of the laboratory used for sterility testing of our allografts, we have experienced a significant increase in positive culture results of CVT (heart valves, blood vessels). This led us to consider a review and modification of the techniques used for sterility testing in our TE. There was a management decision to use the BD BACTECT FX blood culture system as the preferred system for our classical (open) system of testing. The validation procedure was prepared and performed during 2017 and 2018, with its implementation as per 1 May 2019.

The BACTEC blood culture system has already shown excellent performance in the detection of highly virulent bacteria, published in some clinical studies (Appelbaum 1982; Flahyart 2007; Minassian 2014; Paolin 2017) and in some validation studies by other TE (Schroeter 2012; Skenderi 2018; Van Kats 2010).

As per tissue banking recommendations (EU Directives, EP, National Competent Authority requirements), the optimal duration of incubation for sterility confirmation is 14 days. However, during our validation study, the majority of the contaminants were recognized by the BD BACTEC FX blood culture system within two days, whereas only a limited number of vials were identified as positive only after 10 days of incubation (2.6, 2.9 and 3.3% for *Staphylococcus aureus, Candida albicans* and *Aspergillus brasiliensis*,respectively; Table 2, Fig. 1). These results correspond quite closely with the results of a similar study in a clinical setting, carried out by Minassian et al. (Minassian 2014). In the case of slow growing bacteria (e.g. *Cutibacterium acnes*), 59% was detected within 2 days, 32% within 3–6 and 9% within 10 days of incubation. In contrast to our results, the group of Minassian detected all Propionibacterium (Cutibacterium) infections in the contaminated liquid from the knee joint within five days of incubation. This finding might be understandable, due to the likely very high bacterial load in infected material, contrary to the low bacterial load expected to be identified in CVT-s (retreived and processed under clean/sterile environment). However, our finding is in accordance with that of other TE-s and with clinical experience (Bossard 2016; Van Kats 2010), where it has been identified that more than 20% of contaminations are detected after 7 days of incubation. Further, a French group published a validation paper for the BacT/ALERT system (Plantamura 2012), showing that the growth of Propiobacterium was faster (within 3 days) than in our study, with almost all their cultures (9 out of 10) being positive after only three days of incubation. The anaerobic bacteria (Clostridium and Propionibacterium) grew only in the anaerobic vials, indicating a correct anaerobic environment in the BD BACTEC FX blood culture system. The fungi (*Candida albicans* and *Aspergillus brasiliensis*) grew better at temperatures of 20 °C. In our validation study with the BD BACTEC blood culture system, growth of the fungi (*C. albicans, A. brasiliensis*) was assured at 35 °C, but exclusively under aerobic conditions. The study of Minassian (a clinical setting) (Minassian 2014), showed predominantly growth of fungi in the aerobic vials, but also in the anaerobic vials (insignificant numbers). A similar validation study done by another TE, testing only the solutions used for cornea processing and preservation (Schroeter 2012), reported growth of Staphylococcus aureus only in anaerobic vials, in contrast with our study, where the *Staphylococcus aureus* grew in both aerobic as well as anaerobic vials as can be expected with this facultative aerobic microorganism.

The BD BACTEC Plus Vials were able to detect all the bacterial strains selected for this study including all concentration used (1–10, 5–50 and 10–100 CFU/mL). Similarly, Plantamura's group (Plantamura 2012) spiked cell cultures with a concentration of bacteria between 6 and 100 CFU/mL, using only 5 mL as spiking solution. This is in contrast to the present study, in which we used a total volume of 10 mL (to conform with the validated instructions of use of BACTEC system), including three different

inocula (see above), with no difference in the growth of contaminating micro-organisms between the lowest and the highest inoculum. All three concentrations were sufficient for achieving growth of bacteria in the BACTEC Plus vials (Table 1). Furthermore, all vials not containing bacteria remained negative after 14 days of incubation (under the same conditions). These two tests confirm the sensitivity and specificity of 100% of the BD BACTEC FX blood culture system. Schroeter et al. validated the media used in processing of corneal allografts by contaminating solutions with aerobic and anaerobic bacteria and fungi (Schroeter 2012). Similar to our study, all contaminants were detected after various incubation times depending on the type of contaminant used.

One of the problems reported in the literature is the (potential) presence of antibiotics in donated tissues such as heart valves and blood vessels. Indeed, in our TE more than 95% of the donated substances of human origin (hearts, blood vessels), are retreaved from heart beating donors (thus, mainly organ and tissue donors), with potential use of antibiotics before organ and tissue recovery (Jashari 2010). Further, allografts after decontamination and rinsing practices still contain a small residue of antibiotics in the tissues (Buzi 2014; Jashari 2011), or high concentration after decontamination as shown in this study (Table 3). Inactivation of antibiotics and toxic substances (i.e. DMSO) in tissues and solutions as tested in our study with the BD BACTEC Plus vials is a necessity in order to ensure the detection of any contaminant present in the tissue product. All tissue biopsies and homogenates containing even high concentrations of antibiotics, did not have a bacteriotoxic or bacteriostatic effect when introduced in the BD BACTEC Plus vials for incubation. The group of Flayhart reported a much broader spectrum of antibiotic inactivation by BD BACTEC FX system comparing to BacT/ALERT for cefoxitin, piperacillin-tazobactam, vancomycin, gentamycin, penicillin, ampicillin and cefepime (Flayhart 2007). This is very important since most TE-s use antibiotics other than betalactams (penicillines and cephalosporins). Rinsing the human tissue in normal saline is often not sufficient to remove all residual antibiotics. Therefore, the BD BACTEC Plus medium is very useful to solve this problem without the necessity to manipulate samples in other ways. Blood culture bottles have a very rich medium containing a diversity of substances that make it possible for fastidious micro-organisms to grow and to neutralise any toxins and/or antibiotics that may be present. Dilution is one way to achieve this, but also active coal, gelatin, proteins and anticoagulating drugs such as SPS (sodium polianethole sulphonate; Palarasah 2010). On top of that, resins in blood culture vials neutralise toxic substances and most antibiotics. This is contrary to how betalactamases work, because these only break down penicillines and cefaloporines, which are the beta-lactam antibiotics. It was shown by Spaargaren et al. that the activities of some generally used antibiotics decreased by 80 to 90% within 2 h in the BACTEC Plus Aerobic/F resin-containing culture medium (Spaargaren 1998).

The GentleMACS Dissociator is a suitable system for homogenization of cardiac and/or vascular biopsies with the transport solution and cryopreservation solution, in order to facilitate their direct injection into the BACTEC Plus vials. This technique allowed us to combine in each procedure two components (transport solution and cardiac/vascular tissue biopsies for the donated material; cryopreservation solution and tissue biopsies of the allograft, before its final packaging and cryopreservation). In our earlier "standard way" of testing, tissues and solutions were prepared and tested separately, with extensive manipulations and work being carried out using open method, prone for inadvertent contamination. This method of sample preparation is much safer, as all the manipulation and injecting of the homogenized samples into the M-Tubes and, subsequently into the BACTEC Plus vials, is carried out in the Cleanroom Class A environment with practically no risk of inadvertent contamination. Testing carried out in this way makes it possible to confirm that only the donor (donation procedure) is the likely source of any contamination identiofied, unlike open system testing when any contamination present may also have been introduced through the manipulation carried out by the TE (processing carried out in a Cleanroom Class A) or during transport of the product or by the microbiological laboratory. Hence, our method was planned to create a more reliable testing system that tests both tissue and liquid samples before ("test A") and after decontamination ("test B") of the donated product. Use of the GentleMACS Dissociater together with the BD BACTEC FX blood culture system has essentially facilitated establishing more accurate and effective testing method of CVT's. Once the product is

homogenised, the samples (tissue and liquid), are very easily injected into the aerobic/anaerobic vials for incubation in the BD BACTEC FX blood colture system and the device "flags" any sample that shows growth of micro-organisms. If the culture bottles remain negative after14 days of incubation the culture is confirmed to be negative. As far as we are awre, there is no previous publication reporting the use of this device for preparation of CVT for testing in the BD BACTEC FX blood culture system.

One hundred negative vials (aerobic/anaerobic) that served as control of the "possible" contamination were confirmed negative in 99 cases (99%). *Cutibacterium acnes* was only detected from one bottle (1%). This finding has raised the question whether detection of contamination with Cutibacterium (Propionibacterium) might improve with an eventual extension of the duration of incubation by a few more days. However, all contamination of tissues with Cutibacterium in this validation study was detected in the BD BACTEC blood culture system after 2 to 10 days of incubation. Hence, despite this one (unexplained) positive case, we have decided to keep the duration of incubation to maximum of 14 days, in compliance with the validated standard bacteriology testing. In the comparable study of Minassian, who sub-cultured 1000 negative vials that were inoculated with the infected liquid from joint infection after 14 days of incubation in the BACTEC System, only three bottles (0.3%) of the negative vials tested positive for *Pseudomonas aeruginosa* and only one bottle (0.1%) for *Propionibacterium acnes* (Minassian 2014). As mentioned before, the bacterial load in the contaminated liquid from the infected joints might have been much higher than in the tested (donor) tissue in our study, which is normally, retrieved under strictly sterile conditions. Other reasons for *Cutibacterium* to survive decontamination are that, decontamination is an aerobic process and will not be very effective for anaerobic bacteria (they do not grow under aerobic circumstances). Furthermore, Vancomycin is a slow acting, poor penetrating agent (Van Kats 2010). However, Lincomycin, as one of the components of the antibiotic cocktail used in our TE, is effective against Cutibacterium, and this could make a difference. Last, but not least, *Cutibacterium* causes also the most inadvertent contaminations in TE-s and the microbiology lab samples, as usually the skin from all people walking around in the working areas, is the source of this micro-organism.

In view of the slow growing characteristics of the Cutibacterium, further investigation of its detectability in the donated CVT, tested in the BD BACTEC FX blood culture system, will need to be considered (on a larger number of samples).

Conclusion

The results of the validation study presented in this paper have shown that the BD BACTEC FX blood culture system is a suitable system for microbiological testing of the CVT, aimed for human application. It can easily and rapidly detect aerobic and anaerobic bacteria as well as fungi that may be present in CVT (heart valves/blood vessels) and in the transport and preservation fluid. The BD BACTEC FX blood culture system is a closed, automated system with negligible risk of inadvertent contamination being introduced when compared to the open, manual manipulations of historical systems.

Use of the Gentle MACS Dissociator with M-Tubes for homogenization of the biopsies and solutions, has made it possible to safely inject combined samples of tissue and fluid into the BACTEC Plus vials to stimulate the growth of any contaminant in the tissue. Tissue Establishments, as well as pathology and microbiology laboratories could make good use of such system for all clinical "sterile" biopsies taken for isolating bacterial and fungal causes of infection, including mycobacteria.

This work has been carried on in compliance with tissue banking Ethical Standards.

Funding There was no external funding used for this validation paper. As this work was carried out for validation of the system identified to be used for sterility testing of cardio-vascular allografts that are processed by our TE, all costs of the tests were covered by our TE.

Compliance with ethical standards

Conflict of interest The author declare that there is no conflict of interest for any of them. Dr. Jashar is a Director and Manage of Human Body Material of the EHB.

Ethical approval The cardio-vascular tissues used for this validation study, were assessed for processing as allografts for clinical application. However, as they were morphologically not suitable for clinical application and subsequently identified for

discard, they were used as "validation material" in the study. Informed consent was available for each donor of these tissues.

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