Minireview

Experimental procedures for decontamination and microbiological testing in cardiovascular tissue banks

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Impact statement

Sterility testing is a critical issue in the recovery, processing, and release of tissue allografts. Contaminated allografts are often discarded, increasing costs, and reducing tissue stocks. Given these concerns, it is important to determine the most effective methodology for sterility testing. This work provides an overview of microbiological methods for sampling and culturing donor grafts for cardiovascular tissue banking.

Abstract

Several methods for sampling, culturing, and decontaminating donor grafts are available for cardiovascular tissue banks. Most of these protocols are not standardized, leaving tissue banks to adapt their microbiological procedures to their tissue processing routines. Effective microbiological testing and decontamination procedures are essential to ensure tissue quality and safety for human application. This review presents the different procedures currently available for microbiological testing in cardiovascular tissue banks.

Keywords: Biomedical, experimental, cardiovascular tissue banking, cardiovascular allografts, microbiological testing, decontamination

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Introduction

Progress in medical science has allowed for the transplantation of tissue from one person to another, enhancing the quality of life by restoring form and function.[1] For more than 60 years, society has recognized the medical and humanitarian value of donating and transplanting organs and tissues. Tissue banks are entities that provide tissue from living or deceased persons for transplantation purposes. The process whereby tissues and organs from a potential donor are retrieved involves assessing donor eligibility and obtaining authorization and/or informed consent, as well as tissue recovery, collection, acquisition, processing, storage, labeling, distribution, and dispensing.[2] Transplantation of human tissue allografts exposes recipients to the risk of complications, including both fatal and non-fatal transmission of infectious organisms such as bacteria, fungi, viruses, parasites, and prions.

There are several reports of infections associated with allograft use, mostly in bone tissues. However, the rate of bacterial infections following allograft use is reported to be similar to that in major orthopedic surgeries using prosthetic devices.[3–5] Furthermore, there are no recent reports of infections in recipients of donated tissues resulting from the transplants they received.[6] Contamination and subsequent infection by cardiovascular allograft are less common than those by bone tissues. Some cases have been reported, mainly involving endocarditis from contamination of aortic allograph valves.[7,8]

The absolute incidence of infections associated with contaminated allographs is unknown. However, millions of tissue transplants are performed annually, increasing the importance of routine screening practices for tissue donors.[9] Donor screening protocols should be designed to reduce the likelihood that tissues or organs will be procured that would transmit infection, while enhancing availability. Such protocols merit continuous evaluation in the face of new scientific data and new microbiological assays.

Despite the development of many effective culture methods, these have not been standardized into common practice guidelines, rendering culture methods that vary

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among tissues and tissue banks. Several methods for sampling and culturing are available; certain aspects of these processes will be discussed in this review.

Historical aspects

Transplantation of human allogeneic aortic and pulmonary heart valves started over 50 years ago.[10,11] However, microbiological contamination of retrieved tissues continues to be of critical concern to the safety of human tissues intended for clinical use.[12]

Prior to the advent of tissue preservation, transplantation of cardiac valves was required shortly after recovery to reduce the incidence of contamination and tissue damage.[13] Originally, the heart valves were procured aseptically and kept in nutrient solutions for implantation within hours or days of recovery.[14–16] Since then, several decontamination and storage techniques have been developed that minimize microbial contamination of heart valves. The use of ethylene oxide, freeze-drying,[17] and irradiation[18,19] were prevalent. Other forms of decontamination have also been tested including high concentration antibiotic incubation and chemical decontamination using formaldehyde, glutaraldehyde, or beta-propiolactone.[20–23] However, these allograft sterilization methods resulted in poor clinical outcomes.[20,24,25] Since the mid-1970s, the most common method has involved decontamination with low-dose antibiotics, followed by cryopreservation and storage of the allografts in liquid nitrogen.[26] Currently, heart valves are treated with a decontamination solution, followed by cryopreservation or decellularization, allowing preservation for six months or more. Sampling for microbial testing occurs at critical points during heart valve processing. If technology moves toward providing decellularized cardiac tissue instead of cryopreserved cardiac tissue, the impact of the decellularization process on bioburden will have to be assessed.

Source of contamination

The contamination of cardiovascular allografts can occur at different stages of the process, from retrieval through dispensation, brought on by hidden donor infections, and contamination from the environment, including contamination from supplies and reagents used during processing.[27,28] Monitoring clean rooms at rest as well as in operation during processing of tissue products enhances the microbial safety of tissue products.[29] Therefore, the decontamination process aims to reduce or eliminate the expected level of microorganisms on the cardiovascular allografts. A representation of several conventional and rapid sterility testing methods used in tissue banking are summarized in Table 1.

Another source of contamination is the microbiology laboratory. Different media, materials, and other content can be contaminated during the process of tissue or solution culture. The biosafety level (BSL) used for routine culture is the BSL-2. BSL-3 and BSL-4 are less prone to contamination but are usually available for research. In BSL-2, people are not dressed for sterile work, potentially carrying and spreading microorganisms compared to other BSLs.

The effectiveness of each method used to eradicate microorganisms differs.[12,30] Standards for microbiological analysis in tissue banking are not available, leaving each tissue bank to validate its own methods.[31] The American Association of Tissue Banks' (AATB) guidance document "Microbiological Process Validation & Surveillance Program" requires validation of procedures related to tissue processing. This document cites that the outcome of a disinfection or sterilization process is related to the capability of that process to reduce or eliminate an expected level and mix of microorganisms on the particular tissue type undergoing that process. If pre-sterilization/pre-disinfection microbiological load exceeds what the process has been validated to remove or inactivate, there is a lack of assurance the process will result in an expected reduction of microorganisms. Pre-sterilization/pre-disinfection microbiological cultures play a critical role in indicating whether the capability of the validated process will be exceeded. It is equally important that in-process and final culture methods are not inhibited or influenced by residual processing agents, testing materials, or other factors.[32]

Microbiological culture can guide interventions to minimize the risk of pathogen transmission to a recipient, beyond initial serum tests on donors (HIV, HCV, HTLV, etc.). Additional tests may be performed to evaluate or provide information prior to allograft processing or storage in a tissue bank.[33] Proper management of the risks must include the use of validated microbiological methods with accurate sensitivity and specificity to detect pathogenic microorganisms in cardiovascular allografts, which may affect the safety of the tissue to be transplanted.[32] Due to the limited shelf-life of some tissue products, the use of alternative microbiological methods aims to accelerate microbiological testing, optimizing process workflow, and rapid product release.[29]

Pre-recovery conditions and initial contamination rate

The AATB advocates that recovery sites must be evaluated for suitability using pre-established criteria designed to control contamination and cross-contamination.[32] Microbial contamination rates can vary based on the unique conditions of the room, wherein the donated tissues are recovered, the exact recovery procedures, the status of the donor's skin, as well as potential risks from the staff. In the study of Gall et al., postmortem valves retrieved in open mortuary areas are associated with a 54% risk of microbial contamination, while those retrieved in an operating room had a rate of only 12%.[34] However, Paolin et al. found a contamination rate of 84% even in operating room.[12] These authors attribute the increased contamination rate in the morgue to the atmosphere of the room. Some recovery sites have controlled environments and reduced risk of airborne contamination. Other recovery sites, such a hospital morgues, were not built to control microbial contamination. Studies have indicated that, in general, a reduction in time from asystole to tissue recovery may be an important factor

Table 1. Representative conventional and rapid sterility testing methods.

Sample	Method for culture testing	Parameters	Advantage(s)	Disadvantage(s)
Environmental				
Passive air	Agar plate	Number and size of particles in the cleanroom air	Simplicity, effectiveness, low cost	Incubation times are long for monitoring media (three to five days)
Active air	Particulate counts	Number of viable microorganisms in the air	Root-cause analysis for source of contamination	Cost
Surface	Agar plate	Number of viable microorganisms on the surfaces in a cleanroom	Evaluate the effectiveness of operations, cleaning, and disinfection procedures	Only flat surfaces
	Swabs	Number of viable microorganisms on irregularly shaped surfaces	More qualitative than quantitative	Area covered should be at least 24 m^2 but no larger than 30 m^2, require additional processing such as subculture
Water	Membrane filtration test	Number of viable microorganisms in water used for processing	Isolate discrete colonies of bacteria, concentration of larger samples on a membrane filter	May miss quantities of water-borne organisms
Personnel	Agar plate	Number of microorganisms on personnel in the cleanroom	Uncover the source of contamination, evaluate the effectiveness of operations procedures	Largest risk factor in aseptic manufacturing processes
Human tissue donor				
Postmortem blood	Blood culture	Occult ante mortem bacteremia, translocation of organisms from mucosal surfaces postmortem, contamination of blood samples during sample collection	Sensitive, specific, well-described, reliable, low turnaround time	Expensive, can be affected by amplification inhibitors and sources of external contamination, not well-described for cadaveric-blood samples
Transport solution	Media	Represent the microbiological quality of the tissues	Simple, effective, low cost	Dependent on incubation of microbial growth to detect microorganisms, results obtained in tissue and related liquid samples did not always match
Transport solution and Tissue	Immersed in a growth medium	Indicates the presence or absence of organisms by turbidity	Sensitive	Limited to either positive or negative for growth, labor intensive and time consuming, viable but non-culturable organisms, slow-growing organisms and organisms with complex culturing conditions will not be detectable using this method
Transport solution and Tissue	Automated system	Measure changes in impedance, CO_2 (via color change in media) or pressure (headspace pressure) that signal growth of microorganisms	Results in significantly less time, reduced hands-on time and labor costs, objective results even for viscous/cloudy samples	Qualitative test; the presence of microorganisms in turbid samples was confirmed by subculture on agar plates or in fresh culture media
Filterable samples	Fluorescence detection (Milliflex Quantum)	Fluorescence-based quantitative detection of microorganisms	Results in significantly less time, non-destructive; can continue to incubate media to obtain colonies for microbial identification	Validation and comparison with classical methods
Transport solution and Tissue	qPCR Bacterial DNA extraction from contaminated culture media and subsequent DNA amplification	Qualitative and quantitative indication of microbial DNA presence or absence	Faster than traditional culture methods; can detect DNA from samples undergoing treatment with antibiotics; sensitive	Expensive, specific; cannot easily distinguish viable from non-viable contamination; can be affected by amplification inhibitors and sources of external contamination; few studies at cardiovascular tissue banks

to decrease contamination. Jashari et al. found that cardiac tissue recovered in the operating room from multi-organ donors had contamination rates of 21.7%, while tissue recovered from non-heart-beating donors in the morgue had a higher contamination rate of 33.3%. Some studies suggest that contamination is less associated with room but classification of the donor.[35,36]

Heart retrieval from the thoracic and abdominal cavities of donors who present with trauma-induced hemorrhagic effusion can facilitate passive and active cross-contamination. The presence of gastrointestinal tract organisms in postmortem valves is likely a result of microbial migration through the blood stream and diaphragm in the postmortem interval. Moreover, non-heart-beating donors are often transferred from the site of death to the hospital hours after cardiac arrest, with a prolonged warm ischemic time interval (the time before cross-clamping in HB donors) that might favor the growth and migration of bacteria into the blood prior to body refrigeration in the morgue of the referring hospital. Heart-beating tissues were retrieved immediately after the removal of organs, only a few hours after circulatory arrest, whereas tissues from non-heart-beating donors were retrieved on average 17 h after cessation of circulation.[12]

The recovery of tissue should be performed using methods that restrict or minimize contamination with microorganisms from the donor, environment, recovery team, and/or equipment. Studies have indicated that a reduction in the time period between asystole and tissue recovery is an important deterrent to contamination. The relationship between retrieval time and risk of contamination has been reported in various studies. The lowest contamination rate was found when warm ischemic time was maintained below 24 h.[33] Van Kats et al. also confirmed a significant relationship between warm ischemic time and contamination at retrieval.[37] Paolin et al. demonstrated that tissue contamination was statistically correlated with gender, type of donor, cadaver time, number of people attending the retrieval, and season.[38] These authors proposed that to minimize the risk of bacterial contamination, aseptic techniques should be used at retrieval, including restrictions on the number of team members allowed in the room. In addition, cadaver time should be as short as possible, and the donor should be refrigerated within a few hours after death.[38]

Effective tissue recovery methods such as minimizing recovery times (<24 h after death) and the number of experienced team members involved in recovery are examples of factors that can affect the rate of tissue contamination. Additional factors such as minimizing the time after asystole, the total time leading up to tissue recovery, the type of recovery site, the efficacy of the skin prep method,[34,39] and other technical procedures may also impact the rate of contamination.[33]

The European Association of Tissue Banks states that the maximal time limit for tissue retrieval is 24 h postmortem as long as the body has been refrigerated within 4 to 6 h of death.[40] Postmortem translocation is not a problem if the body is appropriately stored.[41]

Transport and storage parameters of recovered cardiovascular tissue

Currently, the heart is recovered using aseptic techniques and placed into a sterile transport solution (TS). Transport to the tissue bank is conducted at a low temperature to suppress microbial proliferation.[42] Immediately following recovery, cardiovascular tissues are stored in a variety of different solutions prior to processing, such as normal saline, lactated Ringer's solution, PlasmaLyte®, transplant organ perfusate (e.g. Belzer's UW solution, Collin's solution), or tissue culture media.[33]

The volume of the TS should be adequate to cover the entire heart, including the vessels and valves. The type, lot number, manufacturer, and expiration date should be documented. The transport container should be fluid tight, designed to prevent contamination of the contents, and allow for aseptic delivery of the specimen at the time of processing. Transportation can be of short or long duration, as long as storage conditions are appropriate.

The time at which the tissue is accepted at the processing center should be documented. Cardiac and vascular tissues should be received at the processing location within a sufficient time interval following recovery to allow for disinfection within the established cold ischemic time limit (the interval between start of heart retrieval and arrival in the tissue bank). Tissue should be transported in a manner established by the tissue bank that conforms to the required environmental conditions for the duration of transport necessary to maintain the integrity of the tissue for its intended use.

Pre-disinfection culture

Microbiological culture prior to treating the tissue with antibiotics is important to identify the "pre-disinfection" bioburden to determine whether the microorganisms isolated at this time meet established acceptability criteria. The degree of effectiveness depends on the number of microorganisms, the type of microorganism, their physiological state, such as the stage of growth or formation of endospores, and the environment in which they are growing.

Disinfection is a process that reduces the number of viable microorganisms on the tissue, but may not destroy all microbial forms, such as spores and viruses. For this purpose, an agent that reduces the number of viable cellular microorganisms is used. A disinfectant might not kill spores; therefore, the use of general antimicrobials in tissue processing is also included in the disinfection process.

Microbiological culture methods

Microbiological growth media should be carefully selected to provide optimal conditions to support the growth of microorganisms sampled from TS, as well as samples from the different steps of valve processing. The culture should be able to grow the aerobic and anaerobic bacteria, as well as fungi.[43] Microbial testing can be both qualitative and quantitative.[44]

Identification of both genus and species should be performed in positive cultures. One variable that was not consistently evaluated or considered in most studies was the influence that microbial culturing technique has on specificity and sensitivity, regardless of the sampling method employed.[42] Furthermore, programs should conduct both qualitative and quantitative assessments of microorganisms to determine their sources of contamination, the effectiveness of their procedures, monitoring requirements of their quality assurance system, and the sensitivity of their culturing methods.

Many tissue banks are cultivating tissue samples in culture broths and subculturing fragments on agar plates as methods to determine the microbiological safety of dissected heart valves. However, only 17% of cultivated myocardial tissue fragments exhibited the presence of microorganisms, whereas 64% of the transport medium samples of the incoming hearts tested positive.[37] This is a remarkable difference, given that both types of sample had been collected prior to antibiotic treatment. Conventional and automated incubation conditions for sterility testing used in tissue banking are summarized in Table 2.

Qualitative testing

There are several methods for sampling and culturing microorganisms. However, it is difficult to assess the efficacy of most of these methods without baseline indicators such as the initial bioburden or contamination rate following recovery. Most studies used media types that are presumed capable of capturing the majority of organisms that might contaminate the tissues, but the use of only one culturing medium or incubation parameter may exclude important pathogens that might affect transplantation outcomes.

The European Pharmacopoeia has determined that the appropriate culture medium for the detection of anaerobic bacteria is thioglycollate broth; it recommends soybean casein digest broth for aerobic bacteria and fungi.[43] The principle of these growth-based methods involves a change in turbidity indicating the presence of viable microorganisms. The optimal incubation time is 14 days. The results obtained are limited to a qualitative assessment, either positive or negative for growth, of the presence or absence of microorganisms, once it has been determined that exact enumeration of residual microbes is not possible.[32] The direct inoculation method is suitable for both solutions and tissue samples.

Most tissue banks use the conventional method[45,46]; however, a faster microbial method for sterility testing increases the available pool of tissue products testing negative for contamination, thereby improving the identification of guaranteed adequate and assured product supplies.[29] Automated culture systems can be used for cell suspensions and other liquids[47] and are suitable for microbiological testing of tissue preparations.[12,37,48] In one automated method, the CO_2 produced from microbial growth is detected, promptly alerting the microbiologist that the culture is positive. The conventional method depends on the daily visual analysis of turbidity in the culture bottle, delaying the availability of results for consideration by the tissue bank.[49] Some of the liquid culture media used in the automated method contain antibiotic chelating molecules (resins) added to increase the sensitivity of microbial growth, minimizing the risk of false negatives.[50] It is important to ensure that the automated/rapid method used provides the proper level of sensitivity and specificity, so that the results yield the necessary information. An additional advantage of automated system is the lower risk of samples contamination due to lesser manipulation by the staff in the microbiology laboratory.

In our research group, we compared the conventional and automated culture methods in human cardiac tissue using an artificial contamination model. Myocardial samples were contaminated with sequential concentrations (10^4 to 10^{-1} CFU/mL) of ATCC control strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and

Table 2. Conventional and automated incubation conditions for sterility testing.

Type of culture	Methodology	Agent	Culture medium	Incubation temperature, °C	Incubation period	Sample
Conventional culture	Brazilian Pharmacopeia	Aerobic	Soybean casein digest medium	22.5 ± 2.5	14 days	Transport fluid directly
		Anaerobic	Fluid thioglycollate medium	32.5 ± 2.5		Tissue biopsy directly
		Fungi	Soybean casein digest medium	22.5 ± 2.5		Filtering the transport
	United States Pharmacopeia	Aerobic	Soybean casein digest medium	22.5 ± 2.5	14 days	fluid and culturing
		Anaerobic	Fluid thioglycollate medium	32.5 ± 2.5		the filter (membrane)
		Fungi	Soybean casein digest medium	22.5 ± 2.5		
	European Pharmacopeia	Aerobic	Soybean casein digest broth	20–25	14 days	
		Anaerobic	Thioglycollate broth	30–35		
		Fungi	Soybean casein digest broth	20–25		
Automated culture	Becton Dickinson- BACTEC™	Aerobic	Plus aerobic/F medium	35	5–15 days	Transport fluid directly
		Anaerobic	Plus anaerobic/F Medium			Tissue macerated
		Fungi/ Mycobacteria	Myco/F Lytic medium			
	Biomerieux-BacT/ALERT®	Aerobic	BacT/ALERT FA plus aerobic	35	5–15 days	
		Anaerobic	BacT/ALERT SN Anaerobic			
		Fungi	BacT/ALERT MB Mycobacteria/Fungi			

Candida albicans. The automated and conventional methods were compared using artificially contaminated solutions containing myocardial fragments, both before and after routine decontamination of the solution. *S. aureus* presented significantly higher levels and faster rates of growth in the automated method compared to the conventional method. Growth levels for the automated method were higher in more concentrated inoculum (10^2 CFU/mL). The growth rates for the automated method were significantly higher than for the conventional method when the inoculum concentration was above 10^3 CFU/mL. The automated culture method is faster than the conventional method, with higher detection rates in a contaminated model of myocardial fragments in TS used in tissue banks.[51]

Suessner et al. proposed an alternative qualitative microbiological testing method involving a shorter incubation time compared to the European Pharmacopoeia method.[29] As proposed by these authors, advantages of the alternative microbiological method include earlier product release dates associated with lower product storage times against higher product quality and greater numbers of products in stock for release. Optimization of sterility testing workflow can be achieved with a decreased period of incubation. The potential risk of implementing the alternative method might be false-negative results due to the shorter incubation period.

Quantitative testing

A quantitative test is usually performed using plate counts to enumerate starting and ending bioburden. This method uses membrane filters (MFs) with a nominal pore size $\leq$0.45 μm and an established effectiveness to retain microorganisms. The bioburden calculation is important to understand the possible mechanisms of contamination and to determine the bacterial burden from in vitro decontamination studies, as well as disinfection strategies during valve processing.[52,53] Microbial identification should be performed on all positive cultures to determine the genus and species present during tissue recovery, processing, environmental monitoring, and final sterility testing as part of a program's quality management monitoring system.[42]

In another study developed by our group, we determined the bioburden from the TS of heart valves obtained from non-heart-beating and heart-beating donors using different culture methods. The bioburden from TS was determined in 20 hearts donated for valve allograft tissue using MF and direct inoculation. Tryptic soy agar (TSA) and Sabouraud plates were incubated, and colonies were counted. Ninety-five percent of the samples for this study were obtained from heart-beating donors. The warm ischemic time period for heart was 1.06 ± 0.74 h, and the cold ischemic time period was 25.66 ± 11.16 h. The mean TS volume was 232.68 ± 96.67 mL. Out of 20 samples directly inoculated on TSA plates, two (10%) were positive. However, when MF was used, out of 20 samples in TSA, 13 (65%) were positive with a mean count of 1.36 ± 4.04 CFU/mL. In Sabouraud plates, the direct inoculation was positive in five samples (25%), with a mean count of 0.24 ± 0.56 CFU/mL. The use of MF increased the proportion of positive results to 50% (10 samples from a total of 20) with a mean of 0.28 ± 0.68 CFU/mL. The detection rate was superior using MF in comparison with direct inoculation ($p < 0.05$). Since the bioburden of TS is low, MF is the technique of choice due to consistently higher detection rates. Results from this study indicate that heart-TS can be contaminated with a wide variety of microorganisms. The MF technique provides a quantitative measure of the inherent bioburden of heart-TS. The results obtained in this study will allow for the development of improved antibiotic cocktail compositions, time and temperature incubation conditions, and microbiological analysis methods.[52,54]

Microorganisms detected immediately following recovery

The spectrum and frequency of bacterial contamination in tissues is highly heterogeneous, depending mostly on tissue and donor type.[55] Despite differences among the bacterial strains isolated in the tissues, decontamination nonetheless proved effective in musculoskeletal and cardiovascular tissues.[56] There was no discussion regarding the acceptable levels of TS contamination that would allow for allograft release. Not all organisms would necessarily be pathogenic. Allografts were often discarded based on the presence of positive cultures following antibiotic intervention; the identification of these remaining organisms might elicit additional antimicrobial treatments that could further reduce the proportion of allografts discarded,[13] thereby impacting the suitability of the tissue for implantation.[57] Comparison between studies is difficult, as different procurement procedures and bacterial screening protocols were used.

In our experience with heart valve banking, the overall contamination rate at retrieval was 52.0% in 1001 TS samples. *Staphylococcus* spp. was the most prevalent microorganism, identified in 32% of 770 isolates. Bacteria from skin microbiota were the most commonly identified (*Staphylococcus* spp., *Cutibacterium* spp., *Corynebacterium* spp., *Bacillus* spp.); bacteria from endogenous microbiota were less frequent, like *Streptococcus* spp. and *Enterobacteriales*. Other less common bacteria are commonly found in the environment (personal communication).

According to the AATB's recommendation, the following are considered to be pathogenic and highly virulent microorganisms that result in tissue discard if detected at any stage of processing: *Clostridium* spp. (notably *C. perfringens* or *C. tetani*); *Enterococcus* spp.; *Flavobacterium meningiosepticum*; *Klebsiella rhinoscleromatis*; *Listeria monocytogenes*; MRSA (methicillin-resistant *S. aureus*); *Mucor* spp.; *Mycobacterium* spp.; *Neisseria gonorrhoeae*; *Nocardia* spp.; *Penicillium* spp.; *P. aeruginosa* or *Pseudomonas. pseudomallei*; *Salmonella* spp.; *Shigella* spp.; *Streptococcus pyogenes* (Group A); *Aspergillus* spp.; *Candida* spp.; and other yeasts and fungi.

Disinfection of cardiovascular allografts

The contamination rate represents the proportion of tissues with bacterial or fungal contamination and the bioburden denotes the quantity of organisms on each sample.[13] For bioburden reduction, cardiovascular allografts may be decontaminated with homemade antibiotic cocktails, but such mixtures differ considerably in composition, concentration, temperature tolerance, and required length of incubation among tissue banks; to date, no consensus exists on an optimal formula.[34,37,45,58–63] Another option is to treat human tissues with ready-to-use solutions for decontamination purposes.[31]

To maximize safety and prevent the loss of tissue allografts, tissue banking professionals have implemented procedures and decontamination methods to control, reduce, or eliminate bioburden; where possible, they have established minimum sterility assurance levels for sterilization methods, with a focus toward reducing the likelihood of providing contaminated allografts for transplantation.[33] A Canadian report recommends that disinfection procedures for cardiac tissue be validated with quantification of log reduction using challenge organisms. Qualitative analysis such as the calculation of discard and/or contamination rates is acceptable for process verification but should not be used as a surrogate for the quantitative validation of log reduction.[42]

The effectiveness of the antibiotic solutions used should be validated against the range of bacterial species normally recovered from such tissues; furthermore, the tissue bank should develop a list of species exclusion criteria based on an assessment of the clinical risk of serious infection in the recipient. The most commonly found contaminating bacteria included *Staphylococci*, *Cutibacterium* (formerly *Propionibacterium*), *Streptococci*, and *E. coli*.[32] Homemade antibiotic cocktails are made with a combination of broad-spectrum antibiotics, including but not limited to penicillin, streptomycin, cefoxitin, vancomycin, amikacin, and gentamicin. Among these, the most commonly used antibiotic is vancomycin.[64] All antibiotics are reconstituted with sterile water and pre-mixed with the appropriate nutrient medium.

The decontamination protocol at the Human Tissue Bank of the Pontifical Catholic University of Paraná (Pontifícia Universidade Católica do Paraná (PUCPR)) uses low temperatures in the range of +2–8°C. The heart grafts are decontaminated in RPMI 1640 medium with low concentrations of antibiotics (240 mg/L cefoxitin, 50 mg/L vancomycin, 120 mg/L lincomycin, and 100 mg/L polymyxin B) and kept for 24 h at 2–8°C. This antibiotic solution has a shelf life of 72 h when stored at 4°C.[65]

Older studies reported incubation of allografts in a solution containing penicillin, streptomycin, and amphotericin B for 24 h at 37°C.[66] Some banks still use antifungal agents to reduce fungal bioburden. However, such antifungal agents have deleterious effects on cell viability during the cryopreservation process[34] and have therefore been removed from some decontamination protocols. The time period for which samples were incubated in the disinfection solution varied from 12 h up to six weeks. However, extension of the incubation period beyond 24 h does not appear to increase the effectiveness of the antibiotic treatment and can impact on tissue integrity.[13]

Most antibiotics require that microorganisms multiply in order to eliminate them, while cell replication does not happen at 4°C. If the tissue is immersed at a temperature of 4°C, the tissue will simply absorb the antibiotics, giving a false-negative result in the post-antibiotic incubation tissue culture.[64] In the Canadian report, the recommendation for optimal bioburden reduction suggests that the temperature used during cardiac antimicrobial incubation should be 37°C. While antimicrobials may exhibit some activity at lower temperatures, they are not nearly as effective, rendering a lower microorganism kill rate.[42]

The use of antimicrobial agents in the decontamination process reduces the risk of microorganism proliferation. However, the antimicrobial agents may induce bacteriostasis in the samples that undergo sterility tests and lead to false-negative results during microbiological analysis. According to the European Pharmacopoeia,[47] US Pharmacopoeia,[67] and ISO 11737–1,[68] the sterility test must be preceded by complete removal of any possible interfering antimicrobial agents.[69] Most tissue banks use extremely high concentrations of antibiotics for decontamination, much higher than those used in clinical situations. However, high concentration can be associated with precipitation and loss of activity. This should be considered before defining antibiotic concentrations for decontamination solutions. After decontamination, the tissues should ideally be rinsed; moreover, the residual antibiotics on the tissues should be neutralized. Failure to remove or neutralize such residues allows the presence of antibiotics to induce bacteriostasis of microorganisms, leading to false-negative results in all tested samples.[31,70] Perhaps even worse, there is a risk that the antibiotics can cause severe allergic or toxic reactions in tissue recipients.[71,72] We were unable to find current studies that reported on this issue and its potential to cause serious clinical consequences. There is a risk that recipients who have an allergy to specific antibiotics could develop allergic reactions after transplantation as a result of extremely high concentrations of antibiotics in the tissues.[70,73] Rinsing of grafts is standard practice in most tissue banks as well as clinically prior to implantation.[6] The European Pharmacopoeia,[47] US Pharmacopoeia,[67] and ISO 11737–1[68] recommend eliminating any factor that may interfere with microbial growth during sample sterility testing. Practical strategies to effectively dilute, neutralize, or remove these bacteriostatic and fungistatic residues can be incorporated into the sterility testing workflow using procedures that detect interfering substances via bacteriostasis and fungistasis analysis. False-negative results generated by insensitive sampling techniques, culture media, and testing methods can be potentially debilitating, if not fatal, to transplant recipients.

Due to virulence mechanisms such as facultative intracellular parasitism, biofilm formation, and persistence at a resting metabolic state without growth (which is reversible upon withdrawal of antimicrobial agents or stress conditions), microbial cells can escape these treatments.[74]

Effectiveness of the disinfection process

Different decontamination protocols do not exhibit the same level of bactericidal activity; they may also render varying effects on the structural integrity of the tissue, which could lead to significant differences in clinical outcomes in recipients. Ideally, a disinfection protocol should achieve the highest bioburden reduction with the lowest possible effect on tissue integrity. Despite the antiseptic measures adopted in all processing phases, some tissues remain contaminated, prohibiting their clinical use.

Significant differences in allograft contamination were observed before and after the disinfection process. A recently published review demonstrated that out of 1094 cardiovascular tissues analyzed, 919 (84%) were contaminated upon retrieval, while 459 (42%) and 69 (6%) proved positive after the first decontamination and after the second decontamination, respectively.[12] Fan et al. reported that cardiovascular tissues had a decontamination rate of 80.1%. Many of the studies did not address the bioburden reduction capabilities of the disinfection methods used.[60] The effectiveness of human cardiac valve allograft disinfection processes in tissue banking is summarized in Table 3. Different experimental models to test the antimicrobial efficacy of solutions in vitro are summarized in Table 4.

Table 3. Disinfection and processing practices of human cardiac valve allografts in tissue banking.

Composition of antibiotic cocktail	Concentration (μg/mL)	Time/ temperature	% contamination rate after antimicrobial intervention	Microorganisms after antimicrobial intervention	Reference
Cefoxitin Lincomycin Polymyxin B Vancomycin	0.528 0.048 0.02 0.05	20–72 h/4°C	6	–	Tabaku et al.[62]
Streptomycin Penicillin	50 30	6–8/35°C	15.1	*Staphylococcus aureus, Diphtheroids Streptococcus* spp., *Bacillus* spp. Coagulase-negative *Staphylococcus, Escherichia coli, Cutibacterium acnes, Lactobacillus* spp., *Enterococcus faecalis,* Other Gram-negative *bacilli, Clostridium perfringens, Candida albicans*	Ireland and Spelman[57]
Cefoxitin Lincomycin Polymyxin B Vancomycin	240 120 100 50	20–48 h/4°C	6.25	Gram-positive *cocci, Arthrobacter spp., Streptococcus viridans, Staphylococcus aureus*	Jashari et al.[61]
Ciprofloxacin Amikacin Metronidazole Vancomycin Flucytosine	3 12 12 12 30	5–6 h/37°C	18	Coagulase-negative *Staphylococci, Bacillus* spp., *Cutibacterium* spp., *Corynebacterium* spp., *Streptococcus* spp., *Staphylococcus aureus, Clostridium* spp. *Bacteroids* spp.	van Kats et al.[37]
Gentamicin Metronidazole Flucloxacillin	200 200 200	12h/4°C	0.9	*Mycobacterium chelonae, Candida famata*	Soo et al.[69]
Lincomycin Polymyxin B Vancomycin	120 124 50	20–48h/4°C	19.9	*Acinetobacter haemolyticus,* Gram positive *Bacillus, Candida albicans, Corynebacterium* subspecies, *Enterobacter faecalis,* Gram positive *cocci, Peptostreptococcus sp, Cutibacterium acnes, Serratia plymuthica, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Streptococcus mitis, Streptococcus parasanguinis, Streptococcus viridans* group	Fan et al.[60]
Gentamicin Cloxacillin Ceftriaxone AmphotericinB Streptomycin	80 640 750 200 250	24 h/24°C	13	*Staphylococcus* spp., *Klebsiella* spp.	Dashti-Khavidaki et al.[30]
Ceftazidime Lincomycin Polymyxin B Vancomycin	240 120 100 50	24–48h/4°C	42% first decontamination 6% second decontamination	Coagulase-negative *Staphylococcus, Staphylococcus aureus, Streptococcus* spp., *Clostridium* spp., *Escherichia* spp., *Enterococcus* spp., Others (18 genera) Coagulase-negative *Staphylococcus, Staphylococcus aureus,* Streptococcus spp., *Clostridium* spp., *Enterococcus* spp., Others (8 genera)	Paolin et al.,[12]

Table 4. Experimental model of the antimicrobial efficacy of solutions in vitro.

Composition of antibiotic cocktail	Concentration (μg/mL)	Time/ temperature	Test panel of microorganism	% contamination rate after antimicrobial intervention	Reference
Cefmetazole Lincomycin Vancomycin Polymyxin B	240 120 50 10	24 h/4°C	*S. aureus* ATCC29213, Clinical MRSA 2008-57, *Staphylococcus epidermidis* ATCC14990, *Enterococcus faecalis* ATCC29212, *Enterococcus faecium* ATCC19434,	$\leq 10^2$ CFU/mL of *S. aureus* and MRSA, $\leq 10^4$ CFU/mL of *S. epidermidis* and *P. aeruginosa*, $\leq 10^6$ CFU/mL of *A. baumannii*. $\leq 10^8$ CFU/mL of *E. coli*, MDRP. Could not eradicate *E. fecalis*, *K. pneumoniae* and *B. cepacia*	Nakaminami et al.[54]
Gentamicin Sitafloxacin Vancomycin Polymyxin B	50 50 50 10		*Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC700603, *P. aeruginosa* ATCC27853, Clinical MDRP TP1254, *Burkholderia cepacia* JCM5506, *Acinetobacter baumannii* ATCC19606	$\leq 10^4$ CFU/mL of *S. aureus* and MRSA, $\leq 10^8$ CFU/mL of *S. epidermidis* and $\leq 10^2$ CFU/mL of *E. faecalis*. $\leq 10^8$ CFU/mL of *E. coli, P. aeruginosa*, MDRP, *B. cepacia* and *A. baumannii* as well as $\leq 10^4$ CFU/mL of *K. pneumoniae*	
Gentamicin Sitafloxacin Clindamycin Polymyxin B	50 50 100 10			$\leq 10^4$ CFU/mL of *S. aureus* and MRSA, $\leq 10^8$ CFU/mL of *S. epidermidis* and $\leq 10^2$ CFU/mL of *E. faecalis*. $\leq 10^8$ CFU/mL of *E. coli, P. aeruginosa*, MDRP, *B. cepacia* and *A. baumannii* as well as $\leq 10^4$ CFU/mL of *K. pneumoniae*	
Gentamicin Sitafloxacin Vancomycin Clindamycin Polymyxin B	50 50 50 100 10			$\leq 10^4$ CFU/mL of *S. aureus* and MRSA, $\leq 10^8$ CFU/mL of *S. epidermidis* and $\leq 10^2$ CFU/mL of *E. faecalis* $\leq 10^8$ CFU/mL of *E. coli, P. aeruginosa*, MDRP, *B. cepacia* and *A. baumannii* as well as $\leq 10^4$ CFU/mL of *K. pneumoniae*	
Daptomycin Gentamicin Levofloxacin	100 100 100			Eradicate $\leq 10^5$ CFU/mL of *S. epidermidis* and *E. coli* as well as $\leq 10^4$ CFU/mL of *S. aureus*, MRSA and *E. faecalis*	
Daptomycin Gentamicin Levofloxacin	200 200 200			$\leq 10^5$ CFU/mL of *S. aureus, S. epidermidis* and *E. coli* as well as $\leq 10^4$ CFU/mL of MRSA and *E. faecalis*	
Daptomycin Gentamicin Levofloxacin	200 200 2000			$\leq 10^2$ CFU/mL of *E. faecium*	
Ceftazidime Lincomycin Polymyxin B Vancomycin	240 120 100 50	6, 24 or 48 h/4 or 22°C	*Achromobacter xilosoxidans denitrificans* CIP 77.15T, *Acinetobacter baumannii*, *Aerococcus viridans*, *Aeromonas hydrophyla*, *Bacteroides fragilis*, *Corynebacterium striatum*, *Enterococcus faecalis ATCC29212*, *Enterococcus faecalis ATCC51299*, *Escherichia coli ATCC25922*,	15 Strains and >95% kill rate, 3 Strains and 90–94.99% kill rate, 6 Strains and 80–89.99% kill rate, 10 Strains and <79% kill rate at 4°C for 48 h, 21 Strains and >95% kill rate, 2 Strains and 90–94.99% kill rate, 5 Strains and 80–89.99% kill rate, 6 Strains and <79% kill rate at 22°C for 6 h	Serafini et al.[55]

(continued)

Table 4. Continued

Composition of antibiotic cocktail	Concentration (μg/mL)	Time/ temperature	Test panel of microorganism	% contamination rate after antimicrobial intervention	Reference
Ciprofloxacin	200		*Gemella morbillorum* CIP 81.10T,	Lower activity than the others against	
Gentamicin	200		*Granulicatella adiacens,*	*Staphylococcus* strains	
Meropenem	200		*Haemophilus parainfluenzae,*		
Vancomycin	100		*Klebsiella pneumoniae, ATCC700603,*		
Ciprofloxacin	200		*Klebsiella pneumoniae, ATCC1706*	Lower activity than the others against	
Meropenem	200		*Kocuria kristinae* CIP 81.69T,	*Staphylococcus* strains	
Vancomycin	100		*Lactobacillus salivarius,*		
Gentamicin	200		*Leuconostoc mesenteroides* CIP 102388,	21 Strains and >95% kill rate,	
Meropenem	200		*Micrococcus luteus,*	2 Strains and 90–94.99% kill rate,	
Vancomycin	100		*Moraxella osloensis* CIP 100025,	5 Strains and 80–89.99% kill rate,	
			Peptostreptococcus anaerobius CIP 104411T,	6 Strains and <79% kill rate at 4°C for 48 h,	
			Cutibacterium acnes,		
			Proteus mirabilis,	28 Strains and >95% kill rate,	
				1 Strain and 90–94.99% kill rate,	
			Staphylococcus aureus (MRSA),	3 Strains and 80–89.99% kill rate,	
			Staphylococcus aureus ATCC2913 (MSSA),	2 Strains and <79% kill rate at 22°C for 6 h	
			Staphylococcus epidermidis,		
Ciprofloxacin	200		*Staphylococcus hominis,*	23 Strains and >95% kill rate,	
Gentamicin	200		*Sphingomonas paucimobilis* CIP 100752T,	5 Strains and 90–94.99% kill rate,	
Vancomycin	100		*Streptococcus agalactiae,*	5 Strains and 80–89.99% kill rate,	
			Streptococcus mitis,	1 Strain and <79% kill rate at 4°C for 48 h,	
			Streptococcus salivarius		
				29 Strains and >95% kill rate,	
				5 Strains and <79% kill rate at 22°C for 6 h	
Vancomycin	160	24h/4°, 22°	*S. epidermidis CN 1984,*	Cocktail A using 24 strains at	Pitt et al.[59]
Gentamicin	180	and 37°C	*S. epidermidis CN 2015,*	4°C and showed 25% kill rate,	
Ceftazidime	320		EMRSA 15 99/1729,	at 22°C showed 50% kill rate	
			EMRSA 15 99/1758,	and at 37°C showed 83% kill rate.	
Vancomycin	50		EMRSA 16 104,	Cocktail B using 27 strains at 4°C	
Gentamicin	4,000/2,000		EMRSA 16 159,	showed 51% kill rate, at 22°C	
Imipenem	200		EMRSA 17 36122,	showed 74% kill rate and at	
Polymyxin B	200		EMRSA 17 20960,	37°C showed 85% kill rate	
Nystatin	250		*M. luteus CN 2805,*		
			M. luteus CN 3169,		
Vancomycin	50		*E. faecium 2974,*	Cocktail C using 27 strains at 4°C	
Gentamicin	4000		*E. faecalis 2975,*	showed 74% kill rate, at 22°C	
Ciprofloxacin	200		*S. pyogenesGpA 668,*	showed 81% kill rate and at	
Amphotericin	50		*S. pyogenesGpA 679,*	37°C showed 85% kill rate	
			S. viridans 592,		
Vancomycin	500		*S. viridans 596,*	Cocktail D using 27 strains at 4°C	
Gentamicin	80		*S. viridans 610,*	showed 18% kill rate, at 22°C	
Polymyxin B	80		*C. jeikeium NCTC11913,*	showed 59% kill rate and at	
Ciprofloxacin	200		*P. acnes NCTC737,*	37°C showed 85% kill rate.	
			Cl. Perfringens NCTC8237,		

(continued)

Table 4. Continued

Composition of antibiotic cocktail	Concentration (μg/mL)	Time/ temperature	Test panel of microorganism	% contamination rate after antimicrobial intervention	Reference
Cefuroxime Amphotericin	250 50		*K. pneumoniae NCTC9633,* *P.aeruginosa NCTC10332,* *E. coli* (control) NCTC10418, *S. aureus* (control) NCTC6571, *C. albicans NCTC10231,* *C. glabrata NCPF8018,* *C. lusitaniae NCPF8481*		
Vancomycin Gentamicin Cefoxitin	50 80 240	24h/4°, 22° and 37°C	*Cutibacterium acnes* ATCC 11827, *Staphylococcus aureus* ATCC 27217, *Staphylococcus epidermidis* ATCC 49134, *Streptococcus salivarius* ATCC 25975, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 9372, *Klebsiella pneumoniae* (blood sample), *Streptococcus agalactiae* (tissue sample), *Lactococcus lactis lactis* (tissue sample), *Bacillus* spp. #7-025170 (tissue sample), *Bacillus* spp. #2-020729 (tissue sample)	All strains survived when incubated at 4°C in DMEM even in the presence of antibiotics and they remained detectable after 24 h of decontamination. After a 24 h treatment with antibiotics at 22°C, the number of CFUs in bacterial suspensions was decreased by factors of 1–6 Log, and all bacteria but *S. aureus* and *L. lactis lactis* remained detectable at the end of the incubation period. Bacterial suspensions were treated at 37°C with antibiotics, the number of CFUs dropped rapidly during incubation and viable bacteria became undetectable after 12 h of incubation except for *B. subtilis* that remained detectable at a density of 2 CFU/mL after 24 h of treatment.	Germain et al.[64]

CFU: colony forming unit.

Other forms of disinfection

Due to the frequency of bacterial resistance to antibiotics, other forms of disinfection have received increasing attention. One-hundred percent sterilization would only be achieved by applying rigorous methods such as radiation or heat sterilization, both of which would damage the valve tissue and render cardiovascular grafts unfit for implantation.[6]

Supercritical carbon dioxide ($scCO_2$), electrolyzed water, gamma radiation, ethanol-peracetic acid, and hydrogen peroxide techniques have been compared for impact on sterility and mechanical integrity of porcine decellularized aortic valves.[75] Gamma irradiation with the required sterilization dose damaged the valve cusps. Electrolyzed water and hydrogen peroxide were inadequate as a terminal sterilization technique for our valve samples due to microbial remnants. Ethanol was effective for sterilization, but the cellular remodeling potential was inhibited with fixation and cross-linking. The $scCO_2$ sterilization technique proved superior to the other methods evaluated, maintaining sound mechanical properties and structural integrity during the sterilization process. This $scCO_2$ sterilization technique could be useful in other soft, decellularized tissue constructs. Paolin et al. proposed a first-step decontamination with 0.1% sodium hypochlorite for 3 min in an adjuvant treatment to reduce bacterial bioburden in tissues retrieved from cadaveric donors; however, this technique yielded lower decontamination efficiency in cardiovascular tissues.[56]

Commercial rapid sterility testing method

The use of rapid microbiological methods (RMMs) for sterility testing relies on their ability to recover and detect microorganisms occasionally present in pharmaceuticals and on microbial viability by multiplication in liquid culture media, with performance equivalent to the compendial methods.[76]

Considering the long incubation period required to obtain an analytical result, the interest of the pharmaceutical industries in evaluating and validating rapid technologies suitable for sterility testing and for the isolation and detection of microorganisms has increased.[77,78]

Many RMM technologies provide more sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based techniques. These methods have also been shown to detect slow-growing and/or viable but non-culturable microorganisms more effectively compared to standard methods used today. Most importantly, a firm that implements an RMM in support of sterile or non-sterile manufacturing processes may realize significant operational efficiencies during the monitoring and control of critical process parameters, reducing or eliminating process variability, and reducing risk to patients. Additional benefits may include the elimination of offline assays and a reduction in laboratory overhead and headcount, lower inventories (raw material, in-process material, and finished product), a reduction in warehousing space, and a decrease in repeat testing, deviations, out-of-specification investigations, reprocessing, and/or lot rejection.

Current rapid method technologies can detect the presence of diverse types of microorganisms or a specific microbial species (qualitative), enumerate the number of microorganisms present in a sample (quantitative), and/or identify microbial cultures at the genus, species, and sub-species levels (identification). There are multiple RMM technologies available commercially. Before purchasing an RMM, there are a number of technical, quality control, business, and regulatory due-diligence activities that should be considered.

Molecular biology methods

The use of two medium cultures to determine the suitability of the tissue is standard. However, many tissue banks are reluctant to rely solely upon the two media/two incubation conditions sterility test to determine suitability of the tissue for release or the effectiveness of a disinfection and/or treatment process. In many cases, additional types of media may be added to the test to strive for the detection of slightly more fastidious organisms. These additional media will not only increase the cost of testing but may also complicate the interpretation of results, as well as raise questions regarding the extent of additional media that should be used.[79] Moreover, final sterility testing can be unreliable, especially when antibiotics remain on tissues. Given the importance of avoiding tissue donors suspected of carrying infectious diseases, the processing of donor tissues contaminated with virulent bacteria, and the occurrence of final false-negative sterility tests, methods based on molecular biology surpass conventional culture-based techniques for quantification of the members of a microbiota community in terms of sensitivity, reproducibility, and reliability.[80]

Molecular biology techniques are increasingly used for microbiological diagnosis and have been established as methodologies for the detection of infectious agents (bacteria, fungi, and viruses)[81,82]; they also add value to the diagnosis of infectious diseases caused by fastidious or intracellular microorganisms.[83–85]

Real-time polymerase chain reaction (qPCR) is used for the detection of pathogenic bacteria by targeting the 16S rDNA gene (a component of the 30S small subunit of prokaryotic ribosomes, common to all pathogenic bacteria).[86] It has been used successfully to study human valves affected by endocarditis, demonstrating that this technique can detect microorganisms more efficiently than traditional culture methods[87–89] and it could be applied for a tissue bank.[90] Moreover, positive results were observed at low concentration levels of microorganisms[81,83,91] as well as in patients undergoing treatment with antibiotics who exhibited negative results using traditional culture methods.[84]

Despite these advantages, qPCR cannot easily distinguish between viable and non-viable microorganisms,[92] which is a drawback of broad-range application of DNA-based molecular method.[93,94] Other limitations of this technique involve incorrect sampling, inappropriate storage, low microbial yield, and DNA purity

concerns,[79,90,91] as well as amplification inhibitors and contamination, all of which contribute to the requirement that results be verified with alternate tests.[92–94] Moreover, studies using qPCR for the detection of microorganisms in tissue banks are rare; generally, well-established methodologies are used, both conventional and automated.

Methods such as focusing on the presence of rapidly degrading RNA instead of DNA are the most used strategies to minimize this difficulty.[95,96] Moreover, methods using ethidium monoazide bromide combined with the use of a live-dead staining dye and with qPCR advantages (i.e. speed and sensitivity) to differentiate viable and non-viable bacterial cells showed positive results, overcoming the points such as viability and direct PCR detection of DNA targets when they are not pre-enriched.[97–99]

Aellen et al. were able to correlate bacterial viability and the copy numbers of 16S rRNA using reverse transcription polymerase chain reaction (RT-PCR) followed by qPCR in an experiment using bacterial growth (*Streptococcus gordonii*) and antibiotic-induced killing. Additionally, 16S rRNA could be used as a marker of antibiotic-induced killing; however, the differentiation between live and dead bacteria is affected by the size of the amplified fragment.[100]

Problems such as samples from infected humans and animals' host DNA present in vast excess to microbial DNA were highlighted. Furthermore, the sensitivity and specificity of molecular analysis of the microbial DNA could decrease when unspecific primer binding to host DNA occurs. A method for selective isolation of bacterial DNA from human samples was demonstrated using a commercially available kit MolYsis™ (Molzym GmbH & Co. KG, Bremen, Germany).[101]

Among molecular diagnostic methods, RT-PCR enables diagnoses to be obtained in less than 5 h, whereas traditional microbiological methods, including conventional molecular diagnostics such as broad PCR followed by sequencing can last for more than one day. RT-PCR also enables the quantification of pathogens[102] and is commonly used to quantify viral loads.[103]

An application of mass spectrometry that enables bacterial identification following PCR amplification of species-specific DNA fragments (PCR-ESI-QTOF MS) was reported.[104] This kind of technology is able to identify pathogenic bacteria. However, further studies are necessary to validate the PCR-ESI-QTOF MS methodology in the microbiology routine.[103]

Based on the results of our experimental study, it was possible to use qPCR as part of the cardiovascular tissue bank routine with success, suggesting it might be an alternative for conventional methods. However, its use should be extended to other groups of microorganisms and its results must be validated. Cut-off values and limits of detection must also be established.[105] The qPCR technique can be used as an alternative method or in conjunction with conventional and automated cultures, possibly reducing diagnosis time as well as the occurrence of false-negative or positive results. It might also increase diagnostic capacity at tissue banks, although more studies are necessary.

In addition, for an effective validation and routine control of molecular detection process, further studies are necessary to ensure quality in microbiology laboratory and tissue banks.

Conclusion

Among the wide range of available options, high-quality, safe, and efficacious procedures are essential to assess the quality and safety of tissues for human application. When the effectiveness of the methods used for decontamination and microbiological examination differs, their suitability must be demonstrated with respect to specificity, sensitivity, and robustness.

Authors' contributions

All authors were involved in writing the article. All authors reviewed, edited, and approved the final submission.

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REFERENCES

1. Lipworth W, Forsyth R, Kerridge I. Tissue donation to biobanks: a review of sociological studies. *Sociol Health Illn* 2011;**33**:792–811
2. Wale J, Arthur A, Faull C. An analysis of knowledge and attitudes of hospice staff towards organ and tissue donation. *BMJ Support Palliat Care* 2014;**4**:98–103
3. Eastlund T. Bacterial infection transmitted by human tissue allograft transplantation. *Cell Tissue Banking* 2006;**7**:147–66
4. Ibrahim T, Stafford H, Esler CN, Power RA. Cadaveric allograft microbiology. *Int Orthop* 2004;**28**:315–8
5. Tomford WW, Thongphasuk J, Mankin HJ, Ferraro MJ. Frozen musculoskeletal allografts. A study of the clinical incidence and causes of infection associated with their use. *J Bone Joint Surg Am* 1990;**72**:1137–43
6. de By T, McDonald C, Sussner S, Davies J, Heng WL, Jashari R, Bogers A, Petit P. Validation of microbiological testing in cardiovascular tissue banks: results of a quality round trial. *Eur J Cardiothorac Surg* 2017;**52**:895–900
7. Kuehnert MJ, Clark E, Lockhart SR, Soll DR, Chia J, Jarvis WR. *Candida albicans* endocarditis associated with a contaminated aortic valve allograft: implications for regulation of allograft processing. *Clin Infect Dis* 1998;**27**:688–91
8. McNeil MM, Brown JM, Carvalho ME, Hollis DG, Morey RE, Reller LB. Molecular epidemiologic evaluation of endocarditis due to *Oerskovia turbata* and CDC group A-3 associated with contaminated homograft valves. *J Clin Microbiol* 2004;**42**:2495–500
9. Fishman JA, Greenwald MA, Grossi PA. Transmission of infection with human allografts: essential considerations in donor screening. *Clin Infect Dis* 2012;**55**:720–7
10. Ross DN. Homograft replacement of the aortic valve. *Lancet* 1962;**2**:487
11. Cebotari S, Tudorache I, Ciubotaru A, Boethig D, Sarikouch S, Goerler A, Lichtenberg A, Cheptanaru E, Barnaciuc S, Cazacu A, Maliga O,

Repin O, Maniuc L, Breymann T, Haverich A. Use of fresh decellularized allografts for pulmonary valve replacement may reduce the reoperation rate in children and young adults: early report. *Circulation* 2011;**124**:S115–23
12. Paolin A, Trojan D, Petit P, Coato P, Rigoli R. Evaluation of allograft contamination and decontamination at the Treviso Tissue Bank Foundation: a retrospective study of 11,129 tissues. *PLoS One* 2017;**12**:e0173154
13. Germain M, Strong DM, Dowling G, Mohr J, Duong A, Garibaldi A, Simunovic N, Ayeni OR, Bioburden Steering C.; Cardiac Tissue Working. Disinfection of human cardiac valve allografts in tissue banking: systematic review report. *Cell Tissue Bank* 2016;**17**:593–601
14. Gonzalez-Lavin L, McGrath LB, Amini S, Graf D. Homograft valve preparation and predicting viability at implantation. *J Card Surg* 1988;**3**:309–12
15. Barratt-Boyes BG, Roche AH, Brandt PW, Smith JC, Lowe JB. Aortic homograft valve replacement. A long-term follow-up of an initial series of 101 patient. *Circulation* 1969;**40**:763–75.
16. Angell WW, Shumway NE, Kosek JC. A five-year study of viable aortic valve homografts. *J Thorac Cardiovasc Surg* 1972;**64**:329–39
17. Longmore DB, Lockey E, Ross DN, Pickering BN. The preparation of aortic-valve homografts. *Lancet* 1966;**2**:463–4
18. Beach PM Jr, Bowman FO Jr, Kaiser GA, Malm JR. Frozen irradiated aortic valve homografts. Long-term evaluation. *N Y State J Med* 1973;**73**:651–4
19. Beach PM Jr, Bowman FO Jr, Kaiser GA, Parodi E, Malm JR. Aortic valve replacement with frozen irradiated homografts. Long-term evaluation. *Circulation* 1972;**45**:I29–35
20. Heimbecker RO, Aldrige HE, Lemire G. The durability and fate of aortic valve grafts. An experimental study with a long term follow-up of clinical patients. *J Cardiovasc Surg (Torino)* 1968;**9**:511–7
21. Malm JR, Bowman FO Jr, Harris PD, Kowalik AT. An evaluation of aortic valve homografts sterilized by electron beam energy. *J Thorac Cardiovasc Surg* 1967;**54**:471–7
22. Hudson RE. Pathology of the human aortic valve homograft. *Br Heart J* 1966;**28**:291–301
23. Barratt-Boyes BG. Homograft aortic valve replacement. *N Z Med J* 1965;**64**:Suppl:41–3
24. Wain WH, Greco R, Bodnar E, Ross DN. Aortic valve replacement with homograft and autograft valves. Performance of 615 valves over 15 years. *Acta Med Port* 1980;**Suppl 1**:109–16.
25. Barratt-Boyes BG, Roche AH, Whitlock RM. Six year review of the results of freehand aortic valve replacement using an antibiotic sterilized homograft valve. *Circulation* 1977;**55**:353–61
26. Watts LK, Duffy P, Field RB, Stafford EG, O'Brien MF. Establishment of a viable homograft cardiac valve bank: a rapid method of determining homograft viability. *Ann Thorac Surg* 1976;**21**:230–6
27. Kowalski JB, Mosley GA, Merritt K, Osborne J. Assessment of bioburden on human and animal tissues: part 1 – results of method development and validation studies. *Cell Tissue Bank* 2012;**13**:129–38
28. Kowalski JB, Merritt K, Gocke D, Osborne J. Assessment of bioburden on human and animal tissues: part 2 – results of testing of human tissue and qualification of a composite sample for routine bioburden determination. *Cell Tissue Bank* 2012;**13**:431–9
29. Suessner S, Hennerbichler S, Schreiberhuber S, Stuebl D, Gabriel C. Validation of an alternative microbiological method for tissue products. *Cell Tissue Bank* 2014;**15**:277–86
30. Dashti-Khavidaki S, Dabardani F, Mahdavi-Mazdeh M, Ravanasa E, Hosseini SK. Efficacy of decontamination protocol by antimicrobial treatment in Iranian Tissue Bank (ITB). *Cell Tissue Bank* 2015;**16**:381–8
31. Buzzi M, Guarino A, Gatto C, Manara S, Dainese L, Polvani G, Tothova JD. Residual antibiotics in decontaminated human cardiovascular tissues intended for transplantation and risk of falsely negative microbiological analyses. *PLoS One* 2014;**9**:e112679
32. AATB. *Microbiological process validation & surveillance program*, No. 5, version 2. McLean, VA: Author, 2016.
33. Brubaker S, Lotherington K, Zhao J, Hamilton B, Rockl G, Duong A, Garibaldi A, Simunovic N, Alsop D, Dao D, Bessemer R, Ayeni OR, Bioburden Steering C, Tissue Recovery Working Group. Tissue recovery practices and bioburden: a systematic review. *Cell Tissue Bank* 2016;**17**:561–71
34. Gall K, Smith S, Willmette C, Wong M, O'Brien M. Allograft heart valve sterilization: a six-year in-depth analysis of a twenty-five-year experience with low-dose antibiotics. *J Thorac Cardiovasc Surg* 1995;**110**:680–7
35. Journeaux SF, Johnson N, Bryce SL, Friedman SJ, Sommerville SM, Morgan DA. Bacterial contamination rates during bone allograft retrieval. *J Arthroplasty* 1999;**14**:677–81
36. Schubert T, Bigare E, Van Isacker T, Gigi J, Delloye C, Cornu O. Analysis of predisposing factors for contamination of bone and tendon allografts. *Cell Tissue Bank* 2012;**13**:421–9
37. van Kats JP, van Tricht C, van Dijk A, van der Schans M, van den Bogaerdt A, Petit PL, Bogers AJ. Microbiological examination of donated human cardiac tissue in heart valve banking. *Eur J Cardiothorac Surg* 2010;**37**:163–9
38. Paolin A, Romualdi C, Romagnoli L, Trojan D. Analysis of potential factors affecting allografts contamination at retrieval. *Cell Tissue Bank* 2017;**18**:539–45
39. Bettin D, Harms C, Polster J, Niemeyer T. High incidence of pathogenic microorganisms in bone allografts explanted in the morgue. *Acta Orthop Scand* 1998;**69**:311–4
40. European Association of Tissue Banks (EATB) Musculoskeletal Council. Standards for musculoskeletal tissue banking, Chairman: Loty, B 1996.
41. Morris JA, Harrison LM, Partridge SM. Postmortem bacteriology: a re-evaluation. *J Clin Pathol* 2006;**59**:1–9
42. Canadian Blood Services (CBS). *Leading Evidence based practice guidelines for: tissue recovery, microbial sampling, processing of musculoskeletal tissue, processing of cardiac tissue, processing of skin tissue*. Final Report. Ontario, Canada. 2016.
43. EDQM. European Directorate for the Quality of Me–dicines & HealthCare. Strasbourg, France 2018;1–30.
44. Shintani H. Validation study of rapid assays of bioburden, endotoxins and other contamination. *Biocontrol Sci* 2016;**21**:63–72
45. Heng WL, Seck T, Tay CP, Chua A, Song C, Lim CH, Lim YP. Homograft banking in Singapore: two years of cardiovascular tissue banking in Southeast Asia. *Cell Tissue Bank* 2013;**14**:187–94
46. Villalba R, Solis F, Fornes G, Jimenez A, Eisman M, Gonzalez AI, Linares MJ, Casal M, Gomez VJL. In vitro susceptibility of high virulence microorganisms isolated in heart valve banking. *Cell Tissue Bank* 2012;**13**:441–5
47. Council of Europe European Pharmacopoe 9th Edition, 2017
48. 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;**39**:387–90
49. Bugno A, Lira RS, Oliveira WA, Almodovar AA, Saes DP, Pinto Tde J. Application of the BacT/ALERTR 3D system for sterility testing of injectable products. *Braz J Microbiol* 2015;**46**:743–7
50. Cetin ES, Kaya S, Demirci M, Aridogan BC. Comparison of the BACTEC blood culture system versus conventional methods for culture of normally sterile body fluids. *Adv Therapy* 2007;**24**:1271–7
51. Kraft L, Tuon FF, Ribeiro VST, Wollmann LC, Suss PH, Pilonetto M, da Costa FDA. Comparison of automated and conventional microbiological examination of donated human cardiac tissue in heart valve banking. *Cell Tissue Bank* 2018;**19**(4):499–505
52. Kraft L, Ribeiro VST, Wollmann LC, Suss PH, Tuon FF. Bioburden in transport solutions of human cardiovascular tissues: a comparative evaluation of direct inoculation and membrane filter technique. *Cell Tissue Bank* 2018;**19**(3):447–454
53. ISO. *Sterilisation of healthcare products – radiation – part 2: establishing the sterilisation dose*. Geneva: Author, 2006.
54. Nakaminami H, Tajima M, Koishikawa K, Shiratori Y, Shioura M, Sasatsu M, Noguchi N. Development of effective antimicrobial cocktails to prevent bacterial contamination of allograft tissues under low temperature conditions. *Interact Cardiovasc Thorac Surg* 2018;**26**:1–9
55. Serafini A, Riello E, Trojan D, Cogliati E, Palu G, Manganelli R, Paolin A. Evaluation of new antibiotic cocktails against contaminating bacteria found in allograft tissues. *Cell Tissue Bank* 2016;**17**:619–28

56. Paolin A, Trojan D, Carniato A, Tasca F, Massarin E, Tugnoli A, Cogliati E. Analysis of the effectiveness of sodium hypochlorite decontamination of cadaveric human tissues at retrieval. *Cell Tissue Bank* 2016;**17**:611–8
57. Ireland L, Spelman D. Bacterial contamination of tissue allografts – experiences of the donor tissue bank of Victoria. *Cell Tissue Banking* 2005;**6**:181–9
58. de By TM, Parker R, Delmo Walter EM, Hetzer R. Cardiovascular tissue banking in Europe. *HSR Proc Intensive Care Cardiovasc Anesth* 2012;**4**:251–60
59. Pitt TL, Tidey K, Roy A, Ancliff S, Lomas R, McDonald CP. Activity of four antimicrobial cocktails for tissue allograft decontamination against bacteria and Candida spp. of known susceptibility at different temperatures. *Cell Tissue Bank* 2014;**15**:119–25
60. Fan YD, Van Hoeck B, Holovska V, Jashari R. Evaluation of decontamination process of heart valve and artery tissues in European Homograft Bank (EHB): a retrospective study of 1,055 cases. *Cell Tissue Bank* 2012;**13**:297–304
61. Jashari R, Tabaku M, Van Hoeck B, Cochez C, Callant M, Vanderkelen A. Decontamination of heart valve and arterial allografts in the European Homograft Bank (EHB): comparison of two different antibiotic cocktails in low temperature conditions. *Cell Tissue Bank* 2007;**8**:247–55
62. Tabaku M, Jashari R, Carton HF, Du Verger A, Van Hoeck B, Vanderkelen A. Processing of cardiovascular allografts: effectiveness of European Homograft Bank (EHB) antimicrobial treatment (cool decontamination protocol with low concentration of antibiotics). *Cell Tissue Bank* 2004;**5**:261–6
63. Stolinski J, Marek G, Marcinkowska Z, Jaskier M, Barecka D, Bartus K, Kapelak B, Sadowski J, Dziatkowiak A. Allogenic heart valve bank in the Department of Cardiovascular Surgery and Transplantology of Jagiellonian University in Cracow – 23 years experience in the treatment of aortic valve or aortic root diseases. *Cell Tissue Bank* 2006;**7**:175–82
64. Germain M, Thibault L, Jacques A, Tremblay J, Bourgeois R. Heart valve allograft decontamination with antibiotics: impact of the temperature of incubation on efficacy. *Cell Tissue Bank* 2010;**11**:197–204
65. Lange PL, Hopkins RA. Allograft valve banking; techniques and technology. In: RA Hopkins (ed.) *Cardiac reconstruction with allograft valves*. New York: Springer-Verhg, 1989, pp.37–63.
66. O'Brien MF, Stafford G, Gardner M, Pohlner P, McGiffin D, Johnston N, Brosnan A, Duffy P. The viable cryopreserved allograft aortic valve. *J Card Surg* 1987;**2**:153–67
67. United States Pharmacopeia (USP). *Medicare prescription drug benet model guidelines source information.* United States do America: 2015.
68. ISO. *Sterilization of health care products – microbiological methods – part 1: Determination of a population of microorganisms on products*. Geneva: Author, 2018.
69. Soo A, Healy DG, El-Bashier H, Shaw S, Wood AE. Quality control in homograft valve processing: when to screen for microbiological contamination? *Cell Tissue Bank* 2011;**12**:185–90
70. Leeming JP, Lovering AM, Hunt CJ. Residual antibiotics in allograft heart valve tissue samples following antibiotic disinfection. *J Hosp Infect* 2005;**60**:231–4
71. Gatto C, Dainese L, Buzzi M, Terzi A, Guarino A, Pagliaro PP, Polvani G, D'Amato Tothova J. Establishing a procedure for dimethyl sulfoxide removal from cardiovascular allografts: a quantitative study. *Cell Tissue Bank* 2013;**14**:205–12
72. Jashari R, Faucon F, Hoeck BV, Gelas SD, Fan Y, Vandenbulcke S. Determination of residual antibiotics in cryopreserved heart valve allografts. *Transfus Med Hemother* 2011;**38**:379–86
73. Thong BY. Update on the management of antibiotic allergy. *Allergy Asthma Immunol Res* 2010;**2**:77–86
74. EDQM. *Guide to the quality and safety of tissues and cells for human application*. France: European Committee (Partial Agreement) on Organ Transplantation (CD-P-TO), 2017.
75. Hennessy RS, Jana S, Tefft BJ, Helder MR, Young MD, Hennessy RR, Stoyles NJ, Lerman A. Supercritical carbon dioxide-based sterilization of decellularized heart valves. *JACC Basic Transl Sci* 2017;**2**:71–84
76. 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**:27–35
77. Peris-Vicente J, Carda-Broch S, Esteve-Romero J. Validation of rapid microbiological methods. *J Lab Autom* 2015;**20**:259–64
78. USP. Validation of alternative microbiological methods. In: *United States Pharmacopeia*. 40th ed. Rockville: The United States Pharmacopeial Convention, Inc., 2017.
79. Alexander K, Bryans T. Evaluation of the sterility test for detection of microbial contaminants of allografts. *Cell Tissue Bank* 2006;**7**:23–8
80. Nagarajan K, Loh KC. Molecular biology-based methods for quantification of bacteria in mixed culture: perspectives and limitations. *Appl Microbiol Biotechnol* 2014;**98**:6907–19
81. Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR 3rd, Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006;**19**:165–256
82. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. *Nucleic Acids Res* 2002;**30**:1292–305
83. Millar BC, Xu J, Moore JE. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J Clin Microbiol* 2002;**40**:1575–80
84. Rampini SK, Bloemberg GV, Keller PM, Buchler AC, Dollenmaier G, Speck RF, Bottger EC. Broad-range 16S rRNA gene polymerase chain reaction for diagnosis of culture-negative bacterial infections. *Clin Infect Dis* 2011;**53**:1245–51
85. Morel AS, Dubourg G, Prudent E, Edouard S, Gouriet F, Casalta JP, Fenollar F, Fournier PE, Drancourt M, Raoult D. Complementarity between targeted real-time specific PCR and conventional broad-range 16S rDNA PCR in the syndrome-driven diagnosis of infectious diseases. *Eur J Clin Microbiol Infect Dis* 2015;**34**:561–70
86. Wimberly BT, Brodersen DE, Clemons WM Jr, Morgan-Warren RJ, Carter AP, Vonrhein C, Hartsch T, Ramakrishnan V. Structure of the 30S ribosomal subunit. *Nature* 2000;**407**:327–39
87. Fournier PE, Thuny F, Richet H, Lepidi H, Casalta JP, Arzouni JP, Maurin M, Celard M, Mainardi JL, Caus T, Collart F, Habib G, Raoult D. Comprehensive diagnostic strategy for blood culture-negative endocarditis: a prospective study of 819 new cases. *Clin Infect Dis* 2010;**51**:131–40
88. Boussier R, Rogez S, Francois B, Denes E, Ploy MC, Garnier F. Two-step bacterial broad-range polymerase chain reaction analysis of heart valve tissue improves bacteriological diagnosis of infective endocarditis. *Diagn Microbiol Infect Dis* 2013;**75**:240–4
89. Harris KA, Yam T, Jalili S, Williams OM, Alshafi K, Gouliouris T, Munthali P, NiRiain U, Hartley JC. Service evaluation to establish the sensitivity, specificity and additional value of broad-range 16S rDNA PCR for the diagnosis of infective endocarditis from resected endocardial material in patients from eight UK and Ireland hospitals. *Eur J Clin Microbiol Infect Dis* 2014;**33**:2061–6
90. Tasca Ribeiro VS, Tuon FF, Kraft L, Suss PH, Wollmann LC, Roderjan JG, Brito DA, Alexandrino F, Malgarin JS, Morello LG, da Costa FDA, Pillonetto M. Conventional culture method and qPCR using 16S rDNA for tissue bank: a comparison using a model of cardiac tissue contamination. *J Med Microbiol* 2018;**67**:1571–1575
91. Cherkaoui A, Emonet S, Ceroni D, Candolfi B, Hibbs J, Francois P, Schrenzel J. Development and validation of a modified broad-range 16S rDNA PCR for diagnostic purposes in clinical microbiology. *J Microbiol Methods* 2009;**79**:227–31
92. Vollmer T, Piper C, Horstkotte D, Korfer R, Kleesiek K, Dreier J. 23S rDNA real-time polymerase chain reaction of heart valves: a decisive tool in the diagnosis of infective endocarditis. *Eur Heart J* 2010;**31**:1105–13
93. Josephson KL, Gerba CP, Pepper IL. Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl Environ Microbiol* 1993;**59**:3513–5
94. Lemarchand K, Masson L, Brousseau R. Molecular biology and DNA microarray technology for microbial quality monitoring of water. *Crit Rev Microbiol* 2004;**30**:145–72

95. Sheridan GE, Masters CI, Shallcross JA, MacKey BM. Detection of mRNA by reverse transcription-PCR as an indicator of viability in Escherichia coli cells. *Appl Environ Microbiol* 1998;**64**:1313–8
96. Vesey G, Ashbolt N, Fricker EJ, Deere D, Williams KL, Veal DA, Dorsch M. The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. *J Appl Microbiol* 1998;**85**:429–40
97. Nogva HK, Dromtorp SM, Nissen H, Rudi K. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. *Biotechniques* 2003;**34**:804–8–10 12–3
98. Rudi K, Moen B, Dromtorp SM, Holck AL. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. *Appl Environ Microbiol* 2005;**71**:1018–24
99. Nocker A, Camper AK. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Appl Environ Microbiol* 2006;**72**:1997–2004
100. Aellen S, Que YA, Guignard B, Haenni M, Moreillon P. Detection of live and antibiotic-killed bacteria by quantitative real-time PCR of specific fragments of rRNA. *Antimicrob Agents Chemother* 2006;**50**:1913–20
101. Horz HP, Scheer S, Vianna ME, Conrads G. New methods for selective isolation of bacterial DNA from human clinical specimens. *Anaerobe* 2010;**16**:47–53
102. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;**6**:986–94
103. Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D. Modern clinical microbiology: new challenges and solutions. *Nat Rev Microbiol* 2013;**11**:574–85
104. Ecker DJ, Sampath R, Massire C, Blyn LB, Hall TA, Eshoo MW, Hofstadler SA. Ibis T5000: a universal biosensor approach for microbiology. *Nat Rev Microbiol* 2008;**6**:553–8
105. Gubbe K, Scharnagl Y, Grosch S, Tonn T, Schmidt M, Hourfar KM, Karl A, Seifried E, Wilkemeyer I, Kalus U. Validation of Virus NAT for HIV, HCV, HBV and HAV using post-mortal blood samples. *Transfus Med Hemother* 2012;**39**:381–5