J Ind Microbiol Biotechnol (2011) 38:1127–1132
DOI 10.1007/s10295-011-0982-z

SHORT COMMUNICATION

Sponge swabs increase sensitivity of sterility testing of processed bone and tendon allografts

Huynh Nguyen · David A. F. Morgan · Sharon Cull · Morris Benkovich · Mark R. Forwood

Received: 29 April 2011 / Accepted: 4 May 2011 / Published online: 21 May 2011

Abstract Sterility testing is the final, and critical, step in quality control of tissue banking. It informs the decision whether to release the tissue allografts for clinical use, or not. The most common method for sterility testing of structural bone and tendon allografts is to swab using cotton tip streaks. This method provides low recovery efficiency; and therefore may pass allografts with low bioburden, providing false negatives. Our pilot data revealed organism recovery efficiencies of 60, 30 and 100% from cotton swab, membrane filtration and sponge swaps, respectively. Our aim was to develop a high sensitivity sterility test for structural bone and tendon allografts using a sponge sampling method. Eighty-one bone and tendon allograft samples were inoculated with organism suspensions (10^2 or less organisms per 0.1 mL) of *Clostridium sporogenes*, *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Bacillus subtilis, Aspergillus niger, Staphylococcus epidermidis* and *Micrococcus* spp. Nasco sponges (4 × 8 cm) were used to aseptically sample the whole surface of allograft samples. The sponges were cut in half and cultured in either tryptone soya or fluid thioglycollate broths for 14 days. Positive culture samples were further examined for microbial morphology. The results showed that the sensitivity of the method, and negative predictive value, is 100% for all inoculated organisms incubated with thioglycollate. We conclude that this sponge sampling method should be applied as the standard for sterility testing of structural bone and tendon allografts.

Keywords Sterility test · Tissue allograft · Sponge sampling method · Tissue banking safety

Introduction

Transplantation of tissue allografts is always accompanied by the risk of allograft-related infection in recipients. Therefore, tissue banks must strictly follow regulations and standards to provide allografts with acceptable sterility assurance levels (SAL) of 10^{-6}. Following donor screening and allograft processing, sterility testing is a critical final step to decide if the allografts are suitable for clinical use. For quality assurance, the test method must be highly sensitive to enable recovery of any bioburden in the processed allografts.

Swabbing with a cotton bud is common for bioburden sampling of bone allografts, but has low recovery efficiency [11]. In tissue banking, the recovery rate is from 9 to 36% at procurement [27], 17 ± 13.1% in soft tissue allografts and 7 ± 13% in bone allografts at processing [20]. The swab technique is particularly prone to errors due to variation in swab manipulation [11]; and, the cotton tip is relatively small compared to the surface area of large bone allografts. If the bioburden was particularly low, it is unlikely that the swab would collect all microorganisms on the surface. In Australia, the Therapeutic Goods Administration (TGA)

H. Nguyen · D. A. F. Morgan · S. Cull · M. Benkovich
The Queensland Bone Bank, Brisbane, Queensland, Australia

H. Nguyen · M. R. Forwood (✉)
School of Medical Science, Griffith University,
Brisbane, Queensland 4222, Australia
e-mail: m.forwood@griffith.edu.au

H. Nguyen · M. R. Forwood
Musculoskeletal Research Program, Griffith Health Institute,
Griffith University, Brisbane, Queensland 4222, Australia

D. A. F. Morgan
Brisbane Private Hospital, Brisbane, Queensland, Australia

guidelines [24] and US Pharmacopoeia (USP) [25] recommend membrane filtration and direct transfer as the preferred technique for sterility testing of therapeutic and pharmaceutical goods. In our experience, and that of others [5, 14, 21], there are several difficulties applying those methods in tissue banking.

We hypothesised that use of a sponge swab, with a broad surface area, would increase the sensitivity of sterility testing. Our specific aim was to develop a sterility testing method that is highly sensitive and applicable for bone and tendon allografts, considered to be types of medical product. The testing method was designed to assess the efficacy of organism removal on low bioburden grafts, and the support of culture media for various organism species.

Materials and methods

Selection of the organism removal technique

In a preliminary study, we compared the efficiency of three removal methods: cotton streak swab, membrane filtration, and sponge swab. Femoral heads ($n = 3$ per method) were inoculated with 100 µL of spore suspension of *Bacillus subtilis* at 30 spores/100 µL. Dual cotton tip streaks were used to swab the surface of femoral heads. Sponges were moistened before swabbing the femoral heads. For membrane filtration, femoral heads were rinsed in 60 mL of saline and the solution was transferred through the membrane chamber using a 60-mL sterile syringe. The samples were bisected aseptically and transferred into either tryptone soya broth (TSB) or fluid thioglycollate broth (ThioB) and incubated for up to 14 days. Cotton streak swabs recovered 2 positives out of 3, membrane filtration 1 out of 3; and, Nasco sponge swabs detected 3 out of 3. Therefore, the Nasco sponge method was selected for validation.

Bone materials

With consent of the donor, or donor's next of kin, original tissues donated to the Queensland Bone Bank (QBB) were transferred to research, reviewed and approved by the Queensland Health, Forensic and Scientific Services Human Ethics Committee.

Femoral heads from living donors, cortical bone portions and tendon allograft specimens from cadaveric donors were processed and packaged in accordance with the QBB standard operating procedures (SOPs) [18, 19]. Femoral heads and cortical portions had all soft tissues and surface cartilage removed, soaked in 70% ethanol and dried before packaging. Tendon allografts were processed and sectioned into smaller pieces (4 sections per tendon) and placed in sterile containers. Before inoculation with selective organisms, allograft specimens were sterilized using gamma irradiation at 50 kGy (Steritech, Australia). Twenty-four of each type of allograft were allocated to 3 replicates ($n = 8$ each), a total of 72 allograft samples. Two irradiated, non-inoculated femoral heads were sampled, and sponges cut and incubated in either TSB or ThioB as negative controls. To verify efficacy of irradiation, 2 irradiated femoral heads were directly immersed in either TSB or ThioB as irradiation controls.

Sampling materials

Sponge: Nasco Whirl–Pak Speci-Sponge Sampling Bag (Nasco, USA) contains 8×4 cm pale yellow sterile sponge. Sterile NaCl Injection BP 0.9% (Pfizer), 10 mL used to moisten the sponge.

Organisms

The microorganisms used in this study were selected as defined in the USP and TGA guidelines [24, 25] (Table 1). In addition, *Micrococcus* spp and *Staphylococcus epidermidis* were also selected because they are the most common contaminants in tissue banking [2, 4, 9, 10, 13, 17, 22, 26, 29]. Organism suspensions were prepared and supplied by Biotest (Australia) in accordance with TGA guidelines [24].

Test media

Following guidance from TGA [24] and ISO 11737-1 [12], two media were selected to assess microbial contamination: ThioB which is primarily intended for the culture of anaerobic bacteria, but will also detect aerobic bacteria; and TSB which is suitable for the culture of fungi and aerobic bacteria. Biotest (Australia) supplied the broth media.

Method

Processed and irradiated allograft specimens were thawed at room temperature for 1 h. Individual allograft specimens were inoculated with a corresponding organism suspension in a volume of 100 µL. Nasco sponges were moistened with 20 mL of sterile saline and then used to swab the inoculated allograft specimens (Fig. 1). The sponge was cut in two halves. Each half was immersed in a vessel containing either TSB or ThioB. Specimens in TSB were incubated at 22.5 ± 2.5°C and those in ThioB at 32.5 ± 2.5°C; they were then examined after 3, 5, 7 and 14 days' incubation. When growth was detected, indicated by the presence of turbidity of the medium, or after 14 days' incubation, subculture tests of the medium were transferred onto nutrient agar. TSB subcultures were incubated aerobically and

Table 1 Organism suspensions inoculated onto the allograft samples

Organism species	Strain	Features	CFUs/0.1 mL
Clostridium perfringens	ATCC 13124	Anaerobic bacterium, Gram-positive bacilli. Pathogenic, highly virulent microorganism	54
Micrococcus spp	Environmental isolate	Gram-positive cocci	82
Staphylococcus aureus	ATCC 6538	Aerobic bacterium, Gram-positive cocci. Pathogenic, highly virulent microorganism	96
Pseudomonas aeruginosa	ATCC 9027	Aerobic bacterium, Gram-negative bacilli	76
Candida albicans	ATCC 10231	Yeast	76
Staphylococcus epidermidis	Environmental isolate	Gram-positive cocci, coagulase negative Staphylococci	74
Bacillus subtilis	ATCC 6633	Aerobic bacterium, Gram-positive bacilli	80
Aspergillus niger	ATCC 16404	Mould	44

ThioB anaerobically, for 48–72 h. Gram staining was used to confirm microbial morphology. All experiments were carried out in Class II BioSafety cabinets with controlled environmental conditions, and in triplicate.

Organism growth support control: All selective organism suspensions were either inoculated with the sponge and then placed in the medium vessels containing TSB/ThioB or directly into the medium vessels.

Interpretation of results

For the test to be valid, clearly visible growth of microorganisms in both the test and positive control incubated broths must have been obtained within 14 days of incubation in at least one of the test media. Specificity of the organisms was assessed using the Gram stain, oxidase and catalase reaction to identify the inoculated culture. All environmental, irradiation and negative controls must be reported as negative.

The following acceptable recovery rates are specified [12, 25]: Good, greater than 95% recovery of all microorganisms inoculated; reasonable, at least 70% recovery of all microorganisms inoculated; fail, less than 70% recovery of all microorganisms inoculated.

Results

After incubation for 14 days in TSB, 7 out of 8 inoculated organism species showed 100% growth. *C. perfringens* was positive in only 4/9 replicates in TSB because the medium does not support anaerobic bacteria. In ThioB, the recovery rate for all organism species in all types of allograft specimens was 100%. The samples testing positive provided typical Gram stain, oxidase and catalase reaction to the reference culture inoculated.

Similar to the test incubation, positive control groups confirmed that TSB did not support anaerobic growth (C. *perfringens* negative in 5/12 replicates), whereas ThioB supported the growth of all selected inoculated organisms in all replicates. Negative controls returned 100% negative tests, indicating that the processes of gamma irradiation, sample handling and media preparation were carried out under aseptic conditions. In addition, sensitivity analysis confirmed that the sampling method has sensitivity and negative predictive value of 100%. Sensitivity: true positive/(true positive + false negative) = 71/(72 + 0) = 1. Negative predictive value: true negative/(true negative + false negative) = 2/(2 + 0) = 1.

Discussion

When donated tissue satisfies all screening tests, and is processed under aseptic procedures, a final sterility test determines the acceptability of the tissue for clinical use. If that test fails, the allograft will not be transplanted. For many years, tissue banks have adopted standard swab procedures using cotton tips for testing of their allograft sterility [20]. Unfortunately, the swab technique is acknowledged to have low sensitivity and recovery efficiency [5, 20, 27–29]. Sensitivity is the capacity of the test method to detect organisms presenting on the allografts, or its bioburden. In contrast, the negative predictive value determines how true the negative result is, or the chance of accepting a false negative. For example, the sensitivity can be as low as 10% if swabs are transferred to culture on agar plates using the streak technique, and only 39% if incubated in broth medium [27], or as low as 20% [5].

Many guidelines [1, 23] recommend membrane filtration and direct transfer methods for sterility testing. Yet, it is argued [5, 19] that tissue residues, such as soft tissue,

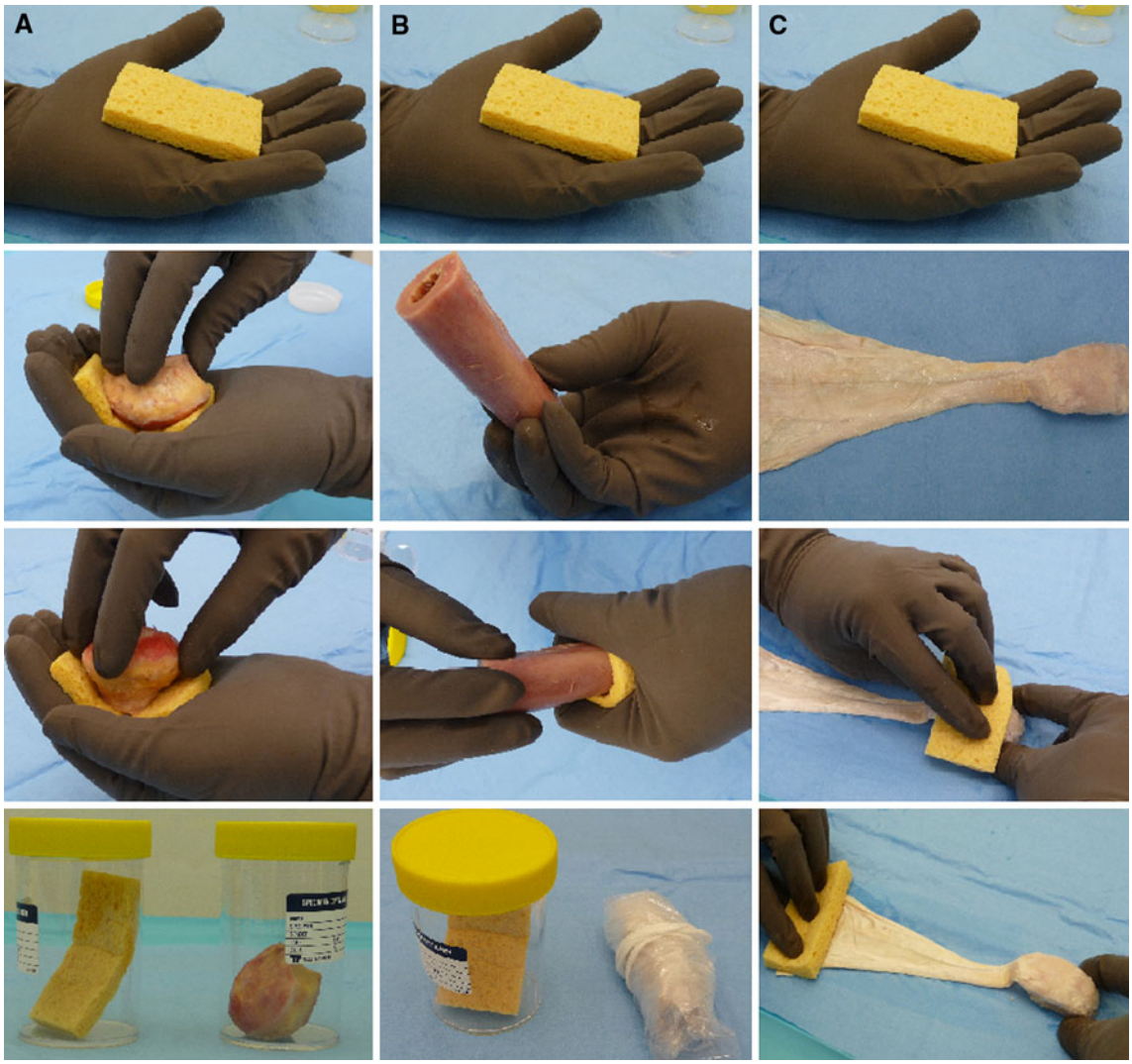

Fig. 1 Procedures for sampling processed bone (**a** and **b**) and tendon (**c**) allografts. Nasco sponge was moistened with 20 mL of sterile saline. Defined swab techniques were applied to ensure that the entire surface of the allografts was swabbed. The sponges were placed in sterile containers and immediately transferred for microbiological analysis

blood, marrow, fat and periosteal tissue on allograft surfaces may also be removed during removal applications such as vortex, shaking and sonication. As a result, it increases the turbidity of the washed solution and occludes the pores of the membrane, preventing effective filtering. In addition, direct transfer methods are not always practical for tissue banking because the batch numbers are usually low, reducing the number available for testing.

These problems suggested the use of sponge swabs and broth incubation, as applied in the meat industry, as an efficacious solution [3, 6–8, 15, 16]. This method employs moistened sponges 3 × 4 cm [7] or 8 × 10 cm [16] to swab the surface of meat products. Polyurethane sponges can detect 93% of bovine, ovine and porcine carcasses contaminated with *Enterobacteriaceae* compared with 55% of excision sampling methods. The high rate of organism recovery of sponge techniques is due to the fact that the total surface of the sponge is about ten times larger than the surface of cotton tips. Moreover, a greater contact pressure between sponge and sample surfaces may have better removal efficiency. Our data confirm that sponge sampling provides high sensitivity and negative predictive value for sterility tests of tissue allografts.

In this study, the sensitivity of sterility testing using the sponge removal technique and fluid thioglycollate broth medium was 100% for all inoculated organisms. The calculated sensitivity was based on a published formula from previous work [27]. The negative predictive value of the method can be calculated as the ratio between true negative and total (true and false) negatives [27]. In our study, all non-inoculated samples returned with negative results. Hence, the negative predictive value of the method is 100%. In other words, when a test is negative, it is highly confident that it is a true negative.

The culture medium can also affect the outcome of sterility tests. Direct immersion of the sponge into broth media was applied in this study because organisms trapped in the sponge matrix can also be cultured [28, 29]. Fluid thioglycollate medium and soybean-casein digest medium are recommended for tests of sterility [1, 23, 25]. The latter medium does not support the growth of anaerobic organisms; therefore, it is recommended that fluid thioglycollate broth should be selected. *C. perfringens* is less sensitive to oxygen than many other clostridia that could avoid detection by the proposed method. However, we included a broad range of organisms, including those most common to tissue allografts. Furthermore, guidelines pertaining to validation recommend fluid thioglycollate medium primarily for the culture of anaerobic bacteria [1, 23, 25], and it also detects aerobic bacteria. The application of other media would require additional validation to demonstrate that the medium "is capable of supporting the growth of a wide range of microorganisms in the presence of the product".

Conclusion

With a sensitivity and negative predictive value of 100%, we confirm the hypothesis that sponge sampling and direct culture of the sponge in fluid thioglycollate broth for 14 days has significantly higher sensitivity than cotton swabs. This technique should be applied as the standard sterility test of structural bone and tendon allografts.

Acknowledgments This study was supported by the Queensland Bone Bank, NH & MRC Project Grant 453624, Arthritis Australia Project Grant and Griffith University Encouragement Grant. We thank Naomi Ross and Biotest Laboratories Pty Ltd, Queensland, Australia for technical expertise in microbiological assays.

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