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ORIGINAL PAPER

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The appropriateness of swab cultures for the release of human allograft tissue

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Abstract Surgeries utilizing human allograft tissues have increased dramatically in recent years. With this increase has come a greater reliance on the use of swab culturing to assess allograft tissues for microbial contamination prior to distribution. In contrast to the typical industrial microbiological uses for swabs, the tissue banking industry has relied on swab cultures as a sterility release method for allograft tissues. It has been reported in the literature that swabs have limitations, both in sensitivity and reproducibility, so their suitability as a final sterility release method was evaluated in this study. Two different swab-culturing systems were evaluated (COPAN, EZ Culturette) using human allograft tissues spiked with low levels of multiple bacterial and fungal microorganisms. The average microbial recoveries for all challenge microorganisms for each tissue type and each swab system were calculated. Percent recoveries for each challenge microorganism were also calculated and reported. The results indicated that both swab systems exhibited low and highly variable recoveries from the seeded allograft tissues. Further analysis indicated there was no statistical difference ($\propto = 0.05$) between the two swab systems. It is the recommendation of the authors that swab culturing not be used to assess relatively low levels of microbial contamination on allografts. Instead, alternative validated microbial detection methods with improved sensitivity and reproducibility should be employed and validated for this critical task.

Keywords Allograft tissue · Colony-forming unit · Microbial contamination · Percent recovery · Swab culture system

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Introduction

Over the past several years, the number of donors for musculoskeletal tissue has risen steadily, from 18,021 in 2000 to just over 21,000 in 2003 [18]. Not surprisingly, the number of bone allografts distributed has also increased from 675,370 allografts used in 2000 to almost 1.3 million used in 2003 [18]. With the increase in allograft use has come an increased reliance on the results from the microbial testing prior to allograft distribution. For years, swabs have been used to ascertain microbial bioburden levels on equipment and surfaces [15, 16, 17, 21, 22] or diagnose clinical infections from a patient [2, 11, 12, 27]. In contrast to the typical uses for swabs, the tissue banking industry has adopted them as a release method for allograft tissues [13] instead of a tool used to evaluate the quantity and/or type of microbial contamination indirectly related to allograft tissue processing. The significance of this application is underscored by the fact that, as far back as 1974, data were generated suggesting that swabs were not suitable for the recovery of anaerobic bacteria [4, 11], and more recently, scientific studies have demonstrated the inadequacies of swabbing allografts using a dry swab [25, 19].

The ability of the swab to recover contaminant microorganisms is dependent on two events: the first is its ability to "pick-up" viable microorganisms from the surface of the article being swabbed. The second event is the "release" of any microbial contaminants from the swab into an appropriate environment (e.g., solid agar medium, isotonic solution, growth broth).

The main objective of this study was to compare two dry-tipped swab-culturing systems and determine which system resulted in the most accurate recovery from seeded human allografts. The two swab systems evaluated were the rayon-tipped EZ Culturette (EZ; Becton Dickenson, Sparks, Md.) and the dissolvable, calcium alginate tipped COPAN (CP; Copan Diagnostics, Corona, Calif.). Several different types of human allograft tissues were individually seeded with multiple challenge

microorganisms and swabbed using either the EZ or the CP swab system and examined for microbial growth.

Materials and methods

Allograft selection

A total of 168 human allograft tissues were terminally sterilized via ^{60}Co (gamma) radiation using a validated range of 16–25 kGy (Isomedix, Morton Grove, Ill.) to eliminate any microbial contamination which would complicate enumeration of the test articles and controls [1]. The allograft tissues were categorized into two allograft families: cut tissue and soft tissue. The cut-tissue allografts consisted of allograft products which were used primarily for load-bearing applications (e.g., tricortical wedges, cortical dowels, fibular rings, femoral heads, etc.). The soft tissue allografts consisted of those commonly used for ligament and tendon reconstructions and/or bladder slings (e.g., fascia, achilles tendon, patellar ligament, etc.). The allografts were randomly split into two main groups of cut tissue ($n = 84$) and soft tissue ($n = 84$). Each group was further divided into two sub-groups of 42, one group for evaluation using the EZ swab system and the other group for testing with the CP swab system. The 42 allografts in each swab group were split evenly between the six challenge microorganisms, so each challenge microorganism was tested with seven individual allografts per swab system ($n = 7$ per challenge microorganism).

Inoculation of allograft test articles

All allograft test articles were inoculated with the six challenge microorganisms as defined in the United States Pharmacopoeia to comply with common industrial microbiological validation techniques [23]. These six challenge microorganisms covered a wide variety of microbial genera and consisted of *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404) and *Clostridium sporogenes* (ATCC 11437). A target concentration of less than 100 colony-forming units (CFUs) per challenge microorganism per allograft was achieved by performing serial dilutions from the stock cultures. Justification for using the low spiking concentration (< 100 CFU/100 μL) was derived from unpublished bioburden data, where a representative number of allografts were evaluated for natural microbial contamination (bioburden) following AlloSource's allograft cleaning and disinfection process. The bioburden data indicated that these allografts had less than 100 CFU present per allograft. Therefore in practice, the swabs must be capable of detecting these low levels of microbial contamination.

The allograft test articles were paired with a corresponding allograft (i.e., fascia with fascia, fibular ring with fibular ring, etc.) to facilitate a direct comparison between the two swab systems. Once the allograft test articles were inoculated with the challenge microorganisms, they were left to dry under aseptic conditions in a class II biological safety cabinet (LabConco, Kansas City, Mo.) for ca. 60 min or until the surface was visibly dry. The procedure was slightly modified for the soft tissue test articles, since these tissues are never allowed to completely dry during processing. To simulate this, all test articles and appropriate controls were kept moist with sterile water (B. Braun, Bethlehem, Pa.) by misting the tissues throughout the inoculation and drying steps.

Controls

For each challenge microorganism tested, there were positive and negative controls run concurrently with the test articles. An inoculum check was performed prior to any treatment to determine the starting concentration of each challenge microorganism. The swab positive controls were prepared by directly inoculating one swab from each swab system (EZ, CP) with 100 μL of the challenge microorganism (total of 12 swabs, six swabs per swab system). Drying controls ($n = 3$) were also used to ensure that the challenge microorganisms spiked onto the allograft test articles would remain viable for the duration of the study. The negative controls consisted of a total of six swabs (three swabs per swab system) that were not inoculated with the challenge microorganisms but were processed in the same fashion as all of the test articles.

Culturing methods

Following the drying step, the inoculated test articles were swabbed in a "zigzag" pattern to ensure that the greatest surface area of the allograft was swabbed. Following swabbing, the EZ swabs were streaked onto the appropriate solid agar medium, as defined by each challenge microorganism. The agar plates were streaked three times while turning the plate 120° clockwise each time, for a total of 360°. In addition to rotating the agar plate, the swab tip was turned 120° each time the plate was turned, to ensure that the entire surface of the swab was in contact with the agar plate, thus improving the likelihood of recovering the challenge microorganism.

The CP swabs were placed into the isotonic transport medium provided with the swabs and allowed to dissolve for 1 h at room temperature (ca. 22°C). Throughout and upon completion of the dissolving step, the solution was vigorously vortexed (ca. 15–30 s) and filtered using a sterile 0.22-μm filter membrane (PALL, Ann Arbor, Mich.) and a sterile filter manifold (Millipore, Bedford, Mass.). The entire solution including swab was emptied into the filter housing. Upon completion of the filtration step, the swab was aseptically removed using sterile forceps and discarded, while the membrane was aseptically transferred to the appropriate solid agar plate.

All controls and test article plates were incubated at the appropriate temperature and atmosphere as required for each challenge microorganism. *S. aureus*, *P. aeruginosa* and *B. subtilis* were incubated aerobically on tryptic soy agar (Remel, Lenexa, Kan.) at 32.5 ± 2.5°C, *Can. albicans* and *A. niger* were incubated on Saboraud dextrose agar (Remel) aerobically at 22.5 ± 2.5°C and *Clo. sporogenes* was incubated anaerobically on anaerobic blood agar (Remel) at 32.5 ± 2.5°C. Initial plate reads were performed at 24–72 h and a final read was performed at 7 days, to allow for the growth of any slow-growing and/or the resuscitation of any injured microorganisms. All data presented in this study were taken from the 7-day reads.

Statistical analysis

The average microbial recoveries were calculated for all control plates and test articles. Swab efficacy was calculated as percent recovery, where the average CFU recovered from the seven test articles per tissue category was divided by the total CFU recovered from the positive control for each challenge microorganism. A Students' *t*-test using a 95% confidence level of ($\propto = 0.05$) was used to compare the recoveries from each of the swab systems, to determine whether one swab system was superior to the other.

Results and discussion

Swab system comparison on soft tissue

Both swabs systems had extremely low recoveries with high variability from the seeded soft tissue test articles. Out of the six challenge microorganisms, *Can. albicans* had the greatest recovery, with 5 CFU for both CP and EZ swab systems. *Can. albicans* also had the greatest inoculum check (36 CFU) and positive control count (16 CFU). The challenge microorganisms evaluated with the CP swab system (*B. subtilis*, *Clo. sporogenes*, *S. aureus*, *A. niger*) had average recoveries of < 1 CFU. There were no colonies recovered from the seven *P. aeruginosa* test articles when using the CP swab system. In contrast, the EZ swab system did recover *P. aeruginosa*, albeit only 0.6 CFU from the seven test articles. However, the EZ swab did not recover *Clo. sporogenes* on any of the seven soft tissue test articles. The remaining four challenge microorganisms all had average recoveries of < 1 CFU when using the EZ swab (refer to Table 1).

Statistically, there was no difference between the microbial recoveries from the CP and EZ swab systems on seeded soft tissue ($P = 0.8923$). A significant observation from this study was the large number of test articles where no recoveries were observed for either swab system. Out of the 84 seeded soft tissue test articles for both swab systems, 52 of them (62%) were negative for growth, where growth is defined as > 1 CFU. The challenge microorganism *P. aeruginosa* did not survive the drying step, as indicated by the negative recoveries from the drying controls even though they were kept moist throughout the testing procedure.

Swab system comparison on cut tissue

A similar trend of low and highly variable recoveries was also observed with the seeded cut-tissue test articles. The CP swab system failed to recover any colonies (0 CFU) from the *B. subtilis* and the *P. aeruginosa* test articles; and the *Clo. sporogenes* and *Can. albicans* challenge microorganisms both had average recoveries of 0.1 CFU from the seven test articles. The greatest recoveries using the CP swab system were observed for the *A. niger* and *S. aureus* test articles, which had average colony counts of 1.1 CFU and 0.4 CFU, respectively. The EZ swab system also performed poorly, in terms of recovering the seeded challenge microorganisms, since four challenge microorganisms (*Clo. sporogenes*, *B. subtilis*, *S. aureus*, *P. aeruginosa*) were not recovered on any of the test articles. *A. niger* again had the greatest recovery, followed by *Can. albicans*, with recoveries of 2.0 CFU and 0.4 CFU, respectively (refer to Table 2).

Statistical analysis of the cut-tissue data for each swab system indicated that there was no statistical difference between either swab system ($P = 0.4045$). Of the 84 combined seeded cut-tissue test articles, 72 of them (86%) were negative for growth, underscoring the inability of either the CP or the EZ swab system to capture and release the challenge microorganisms from the allograft test articles. As also observed in the soft tissue study, the ca. 60-min drying step inhibited the growth of the *P. aeruginosa* challenge microorganism.

Table 1 Summary of Colony Forming Unit (CFU) Counts for Soft Tissue

Challenge	Inoculum Check		Swab Positive Control		Drying Positive Control		CP Test Article		EZ Test Article	
	Avg.	SD	CP	EZ	Avg	SD	Avg.	SD	Avg.	SD
B. subtilis	6	0.6	0	5	5	4	0.1	0.38	0.6	0.79
C. sporogenes	23	12.8	0	4	7	2	0.6	0.53	0.0	0.00
S. aureus	18	7.2	2	2	4	0	0.4	0.79	0.4	0.79
P. aeruginosa	21	10.6	3	5	0	0	0.0	0.00	0.6	0.98
C. albicans	36	8.1	9	22	4	2	5.0	10.1	5.0	3.9
A. niger	20	3.5	9	3	7	7	0.9	0.69	0.6	0.79

Table 2 Summary of Colony Forming Unit (CFU) Counts for Cut Tissue

Challenge	Inoculum Check		Swab Positive Control		Drying Positive Control		CP Test Article		EZ Test Article	
	Avg.	SD	CP	EZ	Avg.	SD	Avg.	SD	Avg.	SD
B. subtilis	6	0.6	0	5	7	2	0	0.00	0	0.00
C. sporogenes	23	12.8	0	4	7	2	0.1	0.38	0.1	0.38
S. aureus	18	7.2	2	2	6	2	0.4	0.79	0	0.00
P. aeruginosa	21	10.6	3	5	0	0	0	0.00	0	0.00
C. albicans	36	8.1	9	22	0	0	0.1	0.38	0.4	1.13
A. niger	20	3.5	9	3	1	0.3	1.1	2.61	2.0	2.83

Percent recoveries

Percent recoveries were calculated for each challenge microorganism for each tissue category as described in Table 3. For soft tissue, percent recoveries varied between 0% and 31% for either swab system. The average recovery for the CP and EZ swabs for soft tissue was 17% and 16%, respectively. The percent recoveries from the cut-tissue test articles ranged from 0% to 33%, but overall the average recoveries for both swab systems were lower (7%) than their soft tissue counterparts.

Table 4 describes the percentage of test articles that had no growth recovered by either swab system. For all swab culture systems, the number of test articles was 42. The only exception to this was the soft tissue CP test articles, where the sample size was slightly lower ($n = 35$). The *P. aeruginosa* control swabs were negative for growth and therefore were not included in this calculation since it was unclear as to whether the "no growth" results were due to the inability of the swab system to capture and release the bacteria or because the bacterial challenge was not initially present.

The American Association Tissue Banks (AATB), the recognized industry trade organization, has standards [13] for the release of allograft tissues, but they do not describe in detail the appropriate methods to be used to evaluate microbial contamination on tissue allografts. Swabs are identified as being one of the preferred methods to release human allograft tissues [13], but the data generated in this study suggests that swabs may not have the sensitivity or reproducibility necessary to be used for the release of final allograft tissues. Historically, swab studies have been performed as in-process evaluations of the natural microbial contamination (i.e., bioburden) present on allograft tissue following a cleaning and inactivation process rather than as seeded challenge studies [19, 25, 26]. The advantage of seeding with known concentrations of challenge microorganisms is that an accurate assessment of the true sensitivity and reproducibility of the swab-culturing system can be determined, in contrast to using in-process cultures where this is impossible because the starting concentration of microorganisms is unknown.

The superficial seeding model contained a drying step to ensure that the challenge microorganisms were firmly attached to the allograft test articles. With the exception of *P. aeruginosa*, all challenge microorganisms were resilient to this drying step. *P. aeruginosa* is a waterborne microorganism and as such is highly sensitive to drying, as demonstrated by this study. Excluding *P. aeruginosa*, neither swab system was able to recover *Clo. sporogenes* from the soft tissue test articles and neither swab system was able to recover *B. subtilis* or *S. aureus* on the cut-tissue test articles. This is cause for concern as the microorganism *Clo. sporogenes* has been identified as the infectious agent in several soft tissue allograft-associated adverse events [7, 10, 14]. Likewise, *S. aureus* is another adventitious agent which has been linked with allograft-associated human infection [8]. The *Bacillus* species are common environmental microorganisms commonly associated with soil and environmental contamination. However, *B. anthracis* [5, 6, 9] among other *Bacillus* species [3, 9, 28] can cause significant clinical infections if not detected.

Table 3 Summary of Percent Recoveries for Soft and Cut Tissue

Challenge Microorganisms	Soft Tissue (% Recovery)		Cut Tissue (% Recovery)	
	COPAN	EZ	COPAN	EZ
B. subtilis	3	20	0	0
C. sporogenes	30	0	5	5
S. aureus	20	20	20	0
*P. aeruginosa**	0	15	0	0
C. albicans	31	31	1	3
A. niger	15	10	18	33
AVERAGE	17%	16%	7%	7%
Std. Deviation (±)	13.1%	10.5%	9.2%	13.0%

*: The negative and low percent recoveries for *P. aeruginosa* can be partially attributed to the inability of the challenge microorganism to survive the drying step as demonstrated by the drying positive controls

Table 4 Percentage of No Growth on Test Articles

Swab System	Percent No Growth
CT-EZ	83%
CT-CP	83%
ST-EZ	50%
ST-CP*	54%

* = n = 35 not 42

Regarding swab performance, acceptable percent recovery on allograft tissues is not clearly defined. The recovery benchmark used in the medical device and pharmaceutical industries is greater than 70% recovery [24]. However, the complexity of the product must be taken into account in order for this acceptance criterion to be applied. Using this as a guideline, all recoveries from both swab systems from both tissue categories were considerably lower than this value.

Although the percent recoveries were low, an interesting trend was observed in the data. The soft tissue test articles had more positives as compared to the cut-tissue test articles. The hypothesis behind this phenomenon is that the inoculum, and conceivably any potential microbial contamination, resides on the surface of the soft tissue test articles, which facilitates the recovery of the microorganisms. In contrast, the porous cortical/cancellous matrix of the cut-tissue test articles allows absorption of the inocula into the interior of the allograft test article, making recovery more difficult. Another factor which may adversely affect swab recovery of contaminant microorganisms on allograft tissues is that the swabs are not pre-moistened. Moist swab fibers are more likely to capture and retain microorganisms than dry fibers on dry surfaces such as the freeze-dried cut-tissue test articles. It has been demonstrated that dry fibers have a desiccating effect on adherent bacteria and as such are prone to very low recoveries (3–5% [20, 29]). With respect to the overall swab performance for all challenge microorganisms, dissolving the CP swab did not dramatically increase the recovery rates when compared to the solid agar method used by the EZ swab system. This is contrary to what was anticipated, since it was thought that dissolving the swab to liberate the microorganisms from the fibrils would enhance recovery when compared to the solid agar plating method. A qualitative growth broth culture method was not evaluated in this study since actual colonies were needed to make a quantitative assessment of each swabs' recovery capabilities.

The results from this study indicate that swabs consistently demonstrated no colony formation when in fact viable bacteria were present on the seeded soft and cut-tissue test articles. When positive results were observed, they were low and highly variable. The investigators suggest that swab culturing is not an appropriate method by which to release allograft tissues and should not be relied upon as the sole method for assessing allograft tissues for microbial contamination prior to release. Instead, alternative validated microbial detection methods with improved sensitivity and reproducibility should be developed and employed to assess microbial contamination on allograft tissues prior to release.

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