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Culture methods of allograft musculoskeletal tissue samples in Australian bacteriology laboratories

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Abstract Samples of allograft musculoskeletal tissue are cultured by bacteriology laboratories to determine the presence of bacteria and fungi. In Australia, this testing is performed by 6 TGA-licensed clinical bacteriology laboratories with samples received from 10 tissue banks. Culture methods of swab and tissue samples employ a combination of solid agar and/or broth media to enhance micro-organism growth and maximise recovery. All six Australian laboratories receive Amies transport swabs and, except for one laboratory, a corresponding biopsy sample for testing. Three of the 6 laboratories culture at least one allograft sample directly onto solid agar. Only one laboratory did not use a broth culture for any sample received. An international literature review found that a similar combination of musculoskeletal tissue samples were cultured onto solid agar and/or broth media. Although variations of allograft musculoskeletal tissue samples, culture media and methods are used in Australian and international bacteriology laboratories, validation studies and method evaluations have challenged and supported their use in recovering fungi and aerobic and anaerobic bacteria.

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Introduction

Samples of musculoskeletal tissue from allografts are sent to bacteriology laboratories for determination of the bacterial and fungal bioburden. The aseptic technique of retrieving musculoskeletal tissue from living and cadaveric donors in operating theatres and morgues is performed to minimise the risk of contamination from external sources (Schubert et al. 2012). It does not reduce the microbial bioburden that may already be present in the tissue.

In Australia, there are six clinical bacteriology laboratories licensed by the Therapeutic Goods Administration (TGA 2000) to provide bioburden assessment of samples of allograft musculoskeletal tissue sent from ten tissue banks (Health Outcomes International Pty Ltd. October 2009; Varettas 2012). The bacterial and fungal culture methods used by Australian bacteriology laboratories have not been previously described. This paper summarises the current culture methods in use in TGA-licensed clinical bacteriology laboratories in Australia as well as a literature review of international methods.

Bacteriological media used in culture methods

Traditionally, culture methods for musculoskeletal allograft samples received in the bacteriology

laboratory have employed a selection of solid agar and/or broth media to initially enhance micro-organism growth and maximise recovery. Methods, media and conditions must be able to recover not only commonly encountered bacteria and fungi but also those that are fastidious, slow growing and in low numbers.

Agar culture

Swab inoculation onto solid agar plates involves rotating the swab on the agar surface to ensure maximum removal of organisms. Biopsy samples may be ground or vortexed after immersing in a small volume of fluid and a drop of this suspension inoculated onto the agar surface (Baron and Thomson 2011). Fluid samples may first be centrifuged or filtered to concentrate any organisms present.

The inoculum is streaked over 4 quadrants of the plate. The purpose of streaking is to dilute the inoculum across the agar so that isolated colonies of organisms can be obtained. Microbial growth may be inhibited on the agar surface where the residual inoculum is found, with better growth in other quadrants (Winn et al. 2006). After incubation, a semi-quantitative estimation of colony growth can be made.

Broth culture

Broth cultures are a liquid nutritional medium used for the isolation of micro-organisms and have been in use for a long time, especially for enhancing the isolation of anaerobic micro-organisms (Holman 1919). Broth cultures may be used with or without the parallel inoculation of solid agar media but are not a quantitative method and do not reflect the bioburden on the sample tested. There are various reasons to support the use of broth media however these have been the subject of much debate (Miles et al. 1985; Cartwright et al. 1994; Morris et al. 1995; Silletti et al. 1997; Gibb 1999). Fastidious organisms that are unable to grow on solid agar media are thought to be enhanced by broth media. Clinical patients are often treated for infections with antimicrobial agents and living femoral head donors receive prophylactic antibiotic treatment pre-operatively. Broth culture of samples exposed to antibiotics is thought to provide a dilution effect of the antimicrobial agents, reducing their effect and allowing organisms to be isolated. Small numbers of organisms may be present in samples, below detectable levels of agar plates, but enhanced by broth culture to detectable levels when sub-cultured.

Broth culture is generally recommended for samples such as tissue and blood (Winn et al. 2006). However, a study by Morris et al. (1995) presented data that the majority of isolates recovered only from broth cultures were not clinically significant and were an additional cost to the laboratory. This was further supported by a study by Silletti et al. (1997) where primary broth cultures were found to be unnecessary where a good swab collection was taken. Broth cultures were considered unnecessary and expensive in a study by Dietz et al. (1991) although their use in isolating low numbers of organisms was considered beneficial. Morris et al. (1995) and Derby et al. (1997) concluded that broth cultures provided no clinical value, were expensive and time consuming.

In contrast, an evaluation of 10 broth media by Scythes et al. (1996) supported the belief that very low numbers of organisms can be recovered from broth cultures, although not all broth media performed well in the study. Reinhold et al. (1988) found that <10 colony forming units (CFU)/ml could be detected by broth culture using a range of organisms. Saegeman et al. (2007) compared two culture methods of allograft tissue and concluded that broth culture using Wilkins Chalgren broth was able to recover a greater number of isolates compared to the use of a blood agar plate. A study by Veen et al. (1994) compared three different culture protocols using musculoskeletal allograft samples and concluded that inoculation of a bone sample directly into brain heart infusion broth medium with subsequent sub-culture after incubation was the better method.

Culture methods used by Australian laboratories

A summary of musculoskeletal tissue samples received and the methods used for bioburden assessment in six TGA-licensed clinical microbiology laboratories in Australia is provided in Table 1 (personal communication—confidential survey). Differences between the six laboratories include the types of samples received for testing, types of solid agar and/or broth media used and incubation periods of media until a final report is issued. Five of the laboratories receive Amies transport swabs without charcoal

Table 1 Summary of culture methods of allograft musculoskeletal tissue samples by six TGA-licenced clinical microbiology laboratories in Australia

Laboratory[a]	Sample tested	Direct inoculation of agar media	Broth media	Subculture of broth	Maximum incubation period (days)[b]
A	Swab	CH BAA	CMM	Yes	5
	Biopsy	Nil	CMM	Yes	5
B	Swab	Nil	BCB	Signal positive only	5
	Biopsy	Nil	BCB	Signal positive only	5
C	Swab	Nil	TSB-RT THIO	Yes	7
	Milled bone	Nil	TSB-RT THIO	Yes	7
D	Swab	BA BAA SAB	Nil	–	28
	Biopsy	SAB	THIO	Turbid only	28
	Sponge	Nil	THIO	Turbid only	14
E	Swab	BA BAA	Nil	–	2
	Biopsy	BA BAA	Nil	–	2
F	Swab	Nil	TSB THIO	Turbid only	14
	Biopsy	Nil	TSB THIO	Turbid only	14

[a] Information obtained via a personal confidential laboratory survey

[b] The maximum incubation period is based on the longest incubation period stated for any type of media for bacteria and/or yeast culture until the culture is complete

BA blood agar, *BAA* blood agar anaerobic, *BCB* aerobic & anaerobic blood culture bottles, *CH* chocolate agar, *CMM* cooked meat medium, 35 °C, *SAB* Sabouraud agar with chloramphenicol & Gentamicin, 28 °C, *THIO* thioglycollate broth 35 °C, *TSB* tryptone soy broth 35 °C, *TSB-RT* tryptone soy broth 25 °C

(COPAN, Italy) and one receives Amies transport swabs with charcoal (Copan). All laboratories receive at least one swab as a sample for testing and only one laboratory does not receive a corresponding biopsy sample. Half of the laboratories surveyed inoculated at least one musculoskeletal sample directly onto solid agar media. Only one laboratory did not use a broth culture for any sample received.

All of these laboratories receive other non-donor related clinical samples. The sample inoculation and culture interpretation processes of allograft musculoskeletal tissue samples are integrated within the workflow of the clinical samples. Tissue bank samples are not inoculated in separate areas with separate staff and equipment, although different methods, media and incubation periods may be used (personal communication—confidential survey).

International culture methods

Table 2 provides an international literature review of musculoskeletal tissue samples collected and of methods used to detect bioburden, highlighting the broad range of swab types, agar media, broth media and incubation periods. As in Australia, the types of swabs used to sample musculoskeletal tissue ranged from Amies transport medium with charcoal to without charcoal. Many studies used a swab for sampling but did not specify the type of swab used while others did not use a swab at all. The majority of studies used at least one broth medium, the two most common being thioglycollate and brain heart infusion broth, although many studies did not specify the type of broth used. Blood and chocolate were the most common agar plates used and incubation periods ranged from a 48 hour period to a maximum of 12 days.

Method validation

Although there are differences in the types of samples received, culture media and culture methods used in Australian and international laboratories, all have been validated as required by the regulating authority. In

Table 2 International literature review of samples and culture methods of allograft musculoskeletal tissue

Author (year)	Country of study	Swab sample	Tissue sample	Broth culture	Agar plates	Maximum incubation period[a] (days)
Tomford et al. (1990)	USA	✔	×	Thio	BRU MAC	NS
Ivory and Thomas (1993)	England	✔	×	✔	✔	NS
Barrios et al. (1994)	Spain	ATM	✔	Thio	BA	2
Deijkers et al. (1997)	Netherlands	TM	×	BHI	BA CH	7
Sutherland et al. (1997)	Scotland	ATMC	✔	RMB Thio TSB	BA MAC	NS
Aho et al. (1998)	Finland	✔	✔	BHI FAB	CH FAA	7
Farrington et al. (1998)	England	×	✔	BHI	BA SAB	3
Vehmeyer et al. (1999)	Netherlands	TM	×	BHI	BA CH	48
Segur et al. (2000)	Spain	✔	×	Thio	×	7
Liu et al. (2002)	Taiwan	✔	✔	Thio	BA EMB	5
James and Gower (2002)	England	✔	✔	✔	✔	NS
Vehmeyer et al. (2002)	Netherlands	TM	×	BHI	BA CH	7
Chiu et al. (2004)	Hong Kong	×	✔	×	BA MAC NEO	NS
Ibrahim et al. (2004)	England	✔	×	✔	✔	3
James et al. (2004)	England	✔	✔	✔	✔	4
Hou et al. (2005	Taiwan	✔	×	✔	BA	7
Van de Pol et al. (2007)	Netherlands	×	✔	Thio LSAB	BA FAA	12
Guelich et al. (2007)	USA	✔	×	Thio	BA CH MAC BRU KVL	7
Meermans et al. (2007)	Belgium	✔	×	Thio	BA CH	NS
Schubert et al. (2012)	Belgium	×	✔	Thio	BA CH	7

[a] The maximum incubation period is based on the longest incubation period stated for any type of media for bacteria and/or yeast culture until the culture is complete

✔ In use but details not specified, × Not tested, *ATM* Amies transport medium, *ATMC* Amies transport medium with charcoal, *BA* blood agar, *BHI* brain heart infusion broth, *BRU* Brucella agar, *CH* chocolate agar, *CMM* cooked meat medium (broth), *EMB* eosin-methylene blue *FAA* fastidious anaerobe agar, *FAB* fastidious anaerobe broth, *KVL* kanamycin-vancomycin laked agar, *LSAB* liquid sabouraud medium, *MAC* MacConkey agar, *NEO* neomycin blood agar, *NS* not specified, *RMB* Robertson's meat broth; *SAB* Sabouraud agar; *STM* Stuarts transport medium, *Thio* thioglycollate broth, *TM* 15 cm polyester tipped applicator into transport medium, *TSB* tryptone soya broth

Australia, the TGA recommends validation studies follow the guidelines of the British Pharmacopoeia Commission (2012) and the TGA Guidelines for Sterility Testing of Therapeutic Goods Administration (2006). In Europe, the United Kingdom and the United States of America, the relevant Pharmacopoeia and guidelines are also followed.

Validation protocols must mimic the bioburden assessment method in use with a micro-organism inoculum size of $\leq$100 CFU, using reference strains of, at least, the following micro-organisms: *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Clostridium sporogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These organisms are used to challenge the ability of the media to support their growth and the ability of the culture method to recover fungi and aerobic and anaerobic micro-organisms.

Validation outcomes provide the basis for determining optimal sampling requirements, culture media and methods. The challenge is to harmonise protocols between laboratories when variations are able to fulfill validation requirements.

Conclusion

In Australia, ten tissue banks send samples of allograft musculoskeletal tissue to 6 TGA-licensed clinical bacteriology laboratories for bioburden assessment. Worldwide, the samples received, culture media and

culture methods may vary from laboratory to laboratory. The harmonisation of bioburden assessment protocols presents a challenge as validations support the variations in use to isolate aerobic and anaerobic bacteria and fungi from allograft musculoskeletal tissue samples.

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