The Microbial Bioburden Assessment of Allograft Musculoskeletal Tissue

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Tissue and Organ Transplantation

Introduction

The World Health Organisation (WHO) defines transplantation as the ‘transfer (engraftment) of human cells, tissues or organs from a donor to a recipient’ (WHO 2009). In this definition, blood and stem cell transfusions are grouped under the category of human cells and tissues and includes the transplant of tissue, eyes (corneas), heart valves and skin. Transplants are termed allografts when transplanted from one person to another and autografts when transplanted from one part of a person's body to another part.

The value of organ transplantation and the life-saving properties of a new heart, liver or kidney are widely recognised. However, the transplant of tissues such as bone, cartilage, tendons and ligaments is not. These tissues are grouped as musculoskeletal tissues as they form part of the skeletal and/or muscular system of the body, vital in providing support and mobility.

There are some significant differences between human cell, organ and tissue transplants. Unlike organs, where the time to transplant is critical, bone can be stored for indefinite periods of time. Cells and organs must be matched according to blood group and other compatibility criteria before being transplanted otherwise acute rejection by the donor will occur. Musculoskeletal tissues however do not need to be matched, an advantage of tissue transplantation for donors and recipients, allowing anyone to donate or receive a musculoskeletal tissue transplant.

Bone is the second most transplanted allograft, blood being the first (Australian Organ & Tissue Authority Feb 2010). Musculoskeletal tissue transplants outnumber all other organ and tissue transplants however the demand for musculoskeletal tissue for transplant is not met in Australia (Australian Organ & Tissue Authority n.d., Health Outcomes International 2009).

History of Musculoskeletal Tissue Transplant

The transplant of musculoskeletal tissue from one person to another has been going on for many centuries. Bone transplants have been documented since the 1800’s, many involving animal bones grafted to humans. In 1865, Ollier unsuccessfully transplanted a portion of
human periosteum to another man (Macewan 1881). In November 1879, Macewan transplanted two wedges of bone from a 6 year old patient into a three year old child, the first of several bone transplants for the child (Macewan 1881). An entire knee joint transplant together with menisci and crucial ligaments was performed by Lexer in 1907 (May 1942) and the first reported use of preserved bone in orthopedic surgery was in 1942 (Inclan 1942).

Along with research in transplantation came research in the preservation and storage of musculoskeletal tissue. Tissue has been boiled prior to transplant (Gallie 1914), stored immersed in Ringers solution or plasma under cold storage (Wilson 1947), treated with chemicals (Wilson 1947), freeze-dried (Carr & Hyatt 1955) and frozen (Wilson 1947). The preservation and storage of musculoskeletal tissue plays an important part in maintaining the important physical and cellular properties of bone, crucial to a successful transplant.

Properties of Bone

Autograft bones possess both properties of osteoinduction and osteogenesis, allograft bone does not. Osteoinduction is the ability to stimulate new bone formation by recruitment of mesenchymal stem cells from the host bed. Osteogenesis is the ability to regenerate by the production of new bone. A graft that supports the attachment and migration of osteogenic cells, either osteoblasts or osteoprogenitor cells, at the site of the bone graft, is termed to have osteoconductivity. Osteoconductivity allows osteoprogenitor cells, or non-differentiated stem cells, to differentiate into osteoblasts (Galea & Kearney 2005).

Fresh allografts contain osteogenic cells which, once implanted, elicit an acute allograft rejection response in the recipient against the cellular components of the allograft, resulting in damage to the graft (Galea & Kearney 2005). Bone is inherently osteogenic in that it contains cells known as osteoblasts which have the capacity to ensure graft binding at the implant site. To prevent acute rejection responses, the cellular component of allograft bone is destroyed by freezing or removed during processing. Freezing of allograft bone at -80°C diminishes immunogenicity and degradation by enzymes such as proteinase and collagenase (Aro & Aho 1993).

Musculoskeletal allografts are still immunogenic in that they can elicit an immune response in the recipient (Horowitz et al 1990). This response is antigen non-specific and is characterised by a cellular infiltrate at the graft site, starting with neutrophils and macrophages and then T and B lymphocytes. The removal of necrotic cells and tissues thereby creating spaces and channels for blood vessels from surrounding tissues to ‘creep’ in along
with osteoclasts and osteoblasts – this process is termed creeping substitution and ultimately the allograft bone becomes incorporated as a new bone (Galea & Kearney 2005). In other words, allografts only function as an osteoconductive scaffold for bone ingrowth (Zhang 2008).

**Reasons for Musculoskeletal Tissue Transplant**

Musculoskeletal conditions include over 150 diseases and syndromes such as arthritis, osteoporosis and back pain and, although generally not life-threatening, have the greatest impact on morbidity, influencing health and quality of life (Australian Institute of Health and Welfare 2008). More than 6 million Australians (31% of the population) are affected by musculoskeletal conditions, affecting both adults and children. These conditions can cause long-term disability, acute and chronic pain and limit activity and mobility at home and at work, leading to psychological distress.

In Australia in 1996, osteoarthritis accounted for 5.7% of total years of life lost to disability in females and 3.9% in males. In 2002, arthritis and musculoskeletal disorders were Australia’s 7th national health priority (Hazes & Woolf 2000). Many musculoskeletal conditions can be treated surgically to provide pain relief, joint function and improve quality of life. In Australia in 2005–2006, 44,446 total knee and hip joint replacements were performed with knee replacements (25,897 procedures) more common than hip replacements (18,549 procedures) - this figure has increased since the periods 2000-01 and 2003-04 (Australian Institute of Health and Welfare 2008). Osteoporotic fractures increase with age and, with an aging population, the musculoskeletal disease burden will continue to increase (Brooks 2004).

**Source of Allograft Musculoskeletal Tissue for Transplant**

Bone can be obtained from living or cadaveric donors. Bone is obtained from living donors as femoral heads from total hip replacement surgery, the donors receiving pre-operative prophylactic antibiotics. The femoral head is routinely removed during surgery and would be discarded if not donated. Femoral heads from living donors represent the majority of bone donated however the amount of bone retrieved is small.

Cadaveric donors have the potential to donate a greater quantity and variety of musculoskeletal tissue. Donors who are brain dead, caused by a severe head injury or brain haemorrhage, or where death has occurred as a result of the heart stopping, are termed cadaveric donors. Cadaveric bones that can be donated include the tibia, femur, humerus,
iliac crest, fibula, ulna, radius, rib, acetabulum, hemipelvis and patella. Other musculoskeletal tissues include the meniscus (fibrocartilage in the knee joint), fascia lata (connective tissue from the side of the leg) and tendons/ligaments such as the medial ligament, Achilles tendon and the patella tendon.

Clinical Use of Allograft Musculoskeletal Tissue

Orthopaedic surgeons use musculoskeletal allografts, including bone, cartilage, tendons and ligaments, in reconstructive orthopaedic surgery, as treatment for bone tumours, failed joint replacements and bone loss from trauma and injury. The clinical indication of the patient will determine the size, shape and type of bone allograft required. Bone loss due to tumours can be replaced with a whole bone from the same site as required. Allograft musculoskeletal tissue donations can be processed to produce a variety of graft materials for surgical procedures. This allows the surgeon to choose allograft bone for transplant of the same anatomical location. The length of the bone may be varied to suit the graft site. The grafted bone will also then be similar in regards to mechanical and osteoconductive properties as the recipient site. Dentists also use bone for periodontal therapy.

Fractures require correction and bone tissue is used here to increase patient recovery. Joints may be destabilised due to a broken ligament and the ligament can be replaced by a donor tendon. The surgical repair of knee joints may require a donor meniscus transplant, bone wedges may be used to modify the bone angles, or for anterior cruciate ligament (ACL) repair (McGuire & Hendricks 2009). Bone allografts are used in spinal fusion and to reconstruct defects during revision hip arthroplasty (Abbas et al 2007).

Bone can be crushed or morselised and is used as a paste or a ‘filler’ during surgery. Hip or knee surgery requires a bone filling material, such as morselised or ground bone, to be used before a new prosthesis is inserted.

Fascia lata is a dense tissue which runs down the lateral side of the upper part of the leg. It can be used in orthopedic, ophthalmic and urogyneacological conditions. Torn or damaged tendons and ligaments can be replaced by donated tendons and ligaments. Usually donor tendons and ligaments are transplanted to the same anatomical position or can be modified by the surgeon to replace ligaments/tendons in other anatomical sites or to reinforce revised joints.
Musculoskeletal Tissue Banks

Introduction

A tissue bank is a facility which is involved with the process of donor assessment, tissue retrieval, processing, storage and distribution of musculoskeletal tissue. The term ‘bank’ refers to the storage or ‘banking’ of tissue until required for transplant. The use of fresh allograft bone from a cadaver or a living donor was normal practice before the development of tissue banks and was associated with complications due to bacterial contamination and acute allograft rejection, leading to poor transplant results. This was a process performed by individual surgeons at individual hospitals before the establishment of tissue banks.

Dr G.W. Hyatt is credited as establishing the first tissue bank in 1949, known as the United States Navy Tissue Bank (Strong 2000). Post-mortem bone was retrieved under sterile conditions and facilities were developed for the processing, chilling, freezing and freeze-drying for bone storage and distribution of bone to all Navy medical facilities, with research and development a major part of the tissue bank. Other tissues were procured such as tendons, fascia lata, skin and cardiovascular tissue with organ retrieval later becoming incorporated. Donor screening was introduced and exclusion and acceptance criteria were established, as well as gaining permission from the next of kin. This tissue bank initiated not only research on tissue storage methods but also investigation and development of tissue sterilizing methods, immunology and cryobiology with the subsequent development of immunosuppressive therapies. Services such as a graft registry and training programs were integrated. During the 1950’s tissue banks were established in Europe in Czechoslovakia, Poland and the United Kingdom (Galea & Kearney 2005). These tissue banks developed methodologies that led to the improvement of allograft musculoskeletal transplants. Smaller tissue banks began to develop in hospitals performing orthopaedic surgery, servicing only the hospital they were associated with, and generally only retrieving femoral heads from living donors.

Tissue banks may deal with only one type of musculoskeletal tissue, such as bone, or may be generalised and deal with all types of musculoskeletal tissue – this will be determined by their musculoskeletal tissue source (Vangsness et al 2003). For example many tissue banks deal with only living donors and donated femoral heads and are referred to as bone banks.
Musculoskeletal Tissue Banks in Australia

As in other countries, tissue banking in Australia developed according to the needs of orthopaedic surgeons, starting as small establishments within a hospital. Femoral heads were removed from living donors during surgery, stored in a refrigerator or freezer, to be used later in another patient. The surgeon would make alterations to the bone while in theatre, grinding or cutting to size as required. In Australia, there are currently 10 musculoskeletal tissue banks with at least one tissue bank in New South Wales, the Australian Capital Territory, Victoria, South Australia, Western Australia and Queensland. There is currently no musculoskeletal tissue bank in Tasmania and the Northern Territory.

In NSW, the Rachel Forster Bone Bank, is located at the Rachel Forster Hospital in Redfern Sydney and was established in 1984. Barwon Health Bone Bank is located within Geelong Hospital in Victoria and has retrieved femoral heads from living donors in the Geelong region for use in orthopaedic surgery since 1986 (Health Outcomes International 2009). The Queensland Bone Bank, located at the Princess Alexandra Hospital Brisbane, was established in 1987 and retrieves musculoskeletal tissue from living and cadaveric donors (Australian Organ & Tissue Authority n.d.). The South Australia Tissue Bank is located at the Royal Adelaide Hospital in Adelaide South Australia, was established in 1988 and retrieves only femoral heads from living donors for allograft transplant. In 1989 the Victorian Institute of Forensic Medicine established the Donor Tissue Bank of Victoria (DTBV). The retrieval, processing, storing and distribution of corneas, cardiac and musculoskeletal tissue was performed and it was the first to retrieve cadaveric tissue (Ireland & McKelvie 2003). The Hunter New England Bone Bank, located in Newcastle NSW, retrieves only femoral heads from living donors and was established in 1992. The Perth Bone and Tissue Bank is the only tissue bank in Western Australia and was established in March 1992, retrieving musculoskeletal tissue from living and cadaveric donors (Winter et al 2005). In New South Wales, the NSW Bone Bank, located in Sydney, retrieves tissue from living and cadaveric donors and was established in 1994 (Mellor 2008). The first privately owned bone and tissue facility in Australia, Australian Biotechnologies, was founded in 2000 and is located at Frenchs Forest in Sydney NSW (Australian Biotechnologies/Introduction 2011). At Australian Biotechnologies bone is received, processed and distributed from living donors retrieved from the NSW Bone Bank. Donor consent, identification and retrieval remain with the NSW Bone Bank (Health Outcomes International 2009). The ACT Bone Bank is located in Canberra and was established in 2003 (Gallagher 2011).

In Australia, tissue banks are involved in all or some of the different stages required prior to the availability of tissue for use. These stages include donor assessment, tissue retrieval,
tissue processing, tissue storage and tissue distribution. All government-funded tissue banks are non-profit organisations and operate on a cost-recovery basis via health funds. Musculoskeletal tissue allografts are classified as prostheses and are listed on the Medical Benefit Schedule. The fee incorporates the cost of donor assessment, retrieval, processing, testing, re-testing, storage and distribution of musculoskeletal allografts.

Orthopaedic surgeons and theatre staff are involved in the recovery and packaging of allografts but are funded via their health employer and not the tissue bank. Many healthcare professionals provide unpaid support of their local tissue bank, including Microbiology laboratories who are involved in meetings, consultations, licence-related activities, validations and research funded by their organisations and not part of any contractual agreement.

National Transplantation Reforms in Australia

It was recognised many years ago that the donation rate in Australia was very low based on figures of donations per million population. In 1987 the Australian Health Ministers Advisory Council’s Donor Organ Working Party was established to identify the key issues affecting donation in Australia. This generated the formation of the National Coordinating Committee on Organ Transplantation in 1989, which later become the Australian Coordinating Committee of Organ Registries and Donation (ACCORD), and is now known as the Australian and New Zealand Donation Registry (ANZOD). In 2002 the Commonwealth Government established a national body, known as Australians Donate, in an effort to increase organ and tissue donation through various initiatives. In October 2006 the Howard Government and the then minister for Health and Ageing, Tony Abbott, established the National Clinical Taskforce on Organ and Tissue Donation. In July 2006 the framework of the National Reform Agenda on Organ and Tissue Donation was agreed to by all State Health Ministers. The Taskforce submitted its final report in January 2008 with 51 recommendations and 6 critical action areas identified, after which it was disbanded. A review of Australians Donate in 2007 found it ineffective and it was disbanded on the 1st April 2008 (Thomas & Klapdor 2008).

On the 2nd July 2008 the Rudd government proposed a $151.1 million national funding package to boost organ and tissue donation in Australia (Office of the Prime Minister July 2008). This new reform package titled ‘A World’s Best Practice Approach to Organ and Tissue Donation for Transplantation’ is primarily aimed at increasing the number of organ and tissue donations in Australia, incorporating many of the recommendations made by the Howard Government in 2006. The focus of the July media release was only on organ
donation but in a subsequent media release on the 18th September 2008 tissue donation was also included (Office of the Prime Minister September 2008). As part of the national reform package, and under the Australian Organ and Tissue Donation and Transplantation Authority Act 2008, an independent statutory authority, known as the Australia Organ and Tissue Donation and Transplantation Authority, was established. Legislation was passed in the Senate on the 13th November 2008 and the Australian Organ and Tissue Donation Authority began on the 1st January 2009. The media release on this day again omitted reference to ‘tissue’ transplantation. In February 2009, Health Outcomes International, a healthcare management consultancy firm, was engaged by the Department of Health and Ageing to “evaluate supply and demand trends for eye and tissue donation and transplantation in Australia, together with current arrangements to support these activities, and provide recommendations for the implementation of the National Eye and Tissue Network” (Health Outcomes International 2009). On the 24th February 2009 a 15-member Advisory Council was named, along with the newly appointed CEO Karen Murphy, this media release retained tissue donation in its title (Office of the Prime Minister 2009). The Advisory Council represents a cross-section of Australian individuals involved in organ and tissue donation.

The DonateLife Network was officially launched on the 1st November 2009 by the then Prime Minister Kevin Rudd (Australian Organ & Tissue Authority n.d.). This is a national network of organ and tissue donation agencies, managed by a medical director from each state or territory and is aimed at raising community awareness and promoting family discussion regarding organ and tissue donation. The DonateLife Network comprises 76 major Australian hospitals with over 160 staff. In an effort to raise community awareness and family discussion, the DonateLife Network launched a Family Discussion Kit on the 23rd February 2010 as part of the Australian Organ and Tissue Donor Awareness Week. This media release quoted a figure of 799 Australians receiving organ donations from 217 organ donors. No figures for musculoskeletal donations are quoted, impossible to do so as a national or state register of musculoskeletal donations does not exist. Donation figures are kept by individual tissue banks and have been collated by the Australasian Tissue Biotherapeutics Forum (ATBF) although these figures are not available on their website.
Donor Assessment

Introduction

The suitability of musculoskeletal donors needs to be assessed to ensure the safety of the recipients. The risk of transmission of infectious diseases via the allograft to the recipient must be minimised. This is achieved by various means and includes a donor questionnaire, physical appearance of cadaveric donors, microbiological (including virology and serology) and histopathological assessment of donor tissue samples.

Donor Questionnaire

Donor assessment via a questionnaire is one of the first steps in reducing the potential risk of transmission of infectious agents by assessing the medical and social history of the donor. A donor is not considered suitable unless all selection and exclusion criteria have been determined. The questionnaire is a very important part of the process and may provide information leading to the early exclusion of the suitability of the donor tissue. For example, a donor that suffers from a degenerative neurological disease such as Alzheimer’s, dementia, Creutzfeldt-Jacob disease, motor neurone disease or multiple sclerosis will be excluded from donation. These diseases have an unknown aetiology and may also compromise the accuracy of the donor’s answers to the questionnaire. Social criteria such as drug use and tattoos are also included in the questionnaire. In Australia, donor questionnaire guidelines and rationale have been ratified by the ATBF and are available on the ATBF website (ATBF 2011).

Living donors are assessed via a questionnaire prior to orthopaedic surgery. Donor questionnaire information for cadaveric donors is supplied by the next of kin or another person close to the donor. The next of kin is not always the best person for donor information if they have not lived with or seen the donor for a long time.

Physical Appearance of Donor

Prior to the removal of cadaveric musculoskeletal tissue allografts, the physical appearance of the donor is examined for signs of high-risk behaviour, such as needle-stick marks, to determine if the infectious risk of the donor is high. A guidance document published by the American Association of Tissue Banks (AATB) recommends assessment of the cadaveric donor for tattoos, body piercing, skin lesions, trauma to a potential retrieval site, jaundice, enlarged lymph nodes and other indications (AATB 2005).
For cadaveric donors, an autopsy is an important tool in determining suitability of musculoskeletal tissue donation. Van Wijk et al (2008) found that in 26.1% of cases a contraindication for musculoskeletal tissue donation was discovered because of an autopsy. On the other hand, in those patients where a cause of death was unknown, an autopsy allowed almost 70% of these donors to be eligible for musculoskeletal tissue donation.

**Microbiology Testing**

Microbiology results are a vital part of determining the suitability of the donor tissue for transplant. Musculoskeletal tissue samples are collected to determine the bacterial load of the sample - the bioburden assessment of all musculoskeletal allografts is a mandatory test prior to transplantation. Donor blood is also collected for serology and virology testing. Mandatory blood testing is performed on all donors for hepatitis, syphilis, human immunodeficiency virus and human T cell lymphotropic virus (Table 1). Living donors must be retested at 180 days post-allograft donation to ensure that the initial blood collection was not during a ‘window period’ where false-negative results can occur.

Optional blood tests may be performed in addition to the mandatory tests, at the discretion of the tissue bank, and include cytomegalovirus (CMV), toxoplasmosis and Epstein Barr virus (EBV). These tests may be performed routinely on cadaveric donors who are also organ donors.
Table 1: Mandatory Blood Tests for Musculoskeletal Tissue Donors

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV 1 and 2 antibody (anti-HIV-1 &amp; anti-HIV-2)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C antibody (anti-HCV)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B surface antigen (HBsAg)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B core antibody (anti-HBc)</td>
<td></td>
</tr>
<tr>
<td>Human T cell lymphotropic virus I and II antibody (anti-HTLV-I &amp; anti-HTLV-II)</td>
<td></td>
</tr>
<tr>
<td>Syphilis antibody</td>
<td></td>
</tr>
<tr>
<td>HIV-1 RNA* nucleic acid amplification</td>
<td></td>
</tr>
<tr>
<td>HCV RNA* nucleic acid amplification</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleic acid amplification testing for HIV and HCV is not considered to be required where 180 day post-donation re-testing as identified below is conducted prior to the release of tissue.

180 days post donation:
- HIV 1 and 2 antibody (anti-HIV-1 & anti-HIV-2)
- Hepatitis C antibody (anti-HCV)
- Hepatitis B surface antigen (HBsAg)
- Hepatitis B core antibody (anti-HBc)

Other Tests

Donor assessment criteria also include the histopathological assessment of the donor. Samples are sent to Histopathology for malignancy investigation.

It is possible for a Rhesus (Rh) negative person to form antibodies against an Rh-positive allograft. Therefore, it is recommended that the Rh status of the donor be identified to avoid transplanting an Rh-positive tissue to an Rh-negative female of child-bearing age or to female children.

Allograft Musculoskeletal Tissue Retrieval

Tissue retrieval is the actual bodily removal of tissues from donors. In living donors undergoing orthopaedic surgery, the retrieval of tissue is performed by the surgeon under standard sterile operating room procedures. In Australia, theatre staff who have been trained by tissue banks, label, sample and package tissue samples before transporting them to the tissue bank. Tissue collection and packaging kits are supplied by the tissue banks. The ACT Bone Bank has a co-coordinator present at all retrievals to facilitate this process.

Cadaveric tissue retrieval in Australia is generally performed in a mortuary which has autopsy facilities. Guidelines for the recommended timeframe for the retrieval of musculoskeletal tissue have been documented by the ATBF, however practices may vary between individual tissue banks (ATBF 2011). Musculoskeletal tissue retrieval from cadaveric donors must be performed within 24 hours of death if the body has been
refrigerated (1–10°C) within 15 hours of death, or within 15 hours of death if the body has not been refrigerated. At the Perth Bone & Tissue Bank (PBTB) tissues are retrieved within a 24-hour period from the time of death, providing the body has been refrigerated within 6 hours after death (Winter et al 2005). The retrieval of musculoskeletal tissue is the last retrieval performed on the cadaveric donor. Organs and eyes are removed first and on some cadavers an autopsy may have been performed as well (Eastlund 2000). These physical manipulations of the donor may allow bacteria to cross intestinal or mucosal barriers to reach other tissues.

Cadaveric tissue is retrieved in a specific order to minimize contamination. Australian tissue banks train their retrievalists to remove lower bones in the order of tibia first, then fibula, femur and hemipelvis last. When taking tendons the following order is followed: anterior tibialis, tibia/patella tendon, posterior tibialis, fibula, peroneus longus, Achilles tendon, femur, hamstrings and hemipelvis. Upper limbs are retrieved concurrently. Allograft tissue should be stored for no longer than 72 hours at 1–10°C or colder while in transport to the tissue bank.

**Allograft Musculoskeletal Tissue Processing**

Processing of bone involves the removal of bone marrow, blood and tissue from the bone after the tissue retrieval and/or resizing the tissue, ready for use by surgeons. The reasoning is that blood borne infections can be transmitted via the blood and marrow on the bone and this is an important step in reducing the risk of transmission of micro-organisms. Processing of allograft musculoskeletal tissue is not performed by most of the tissue banks in Australia. Processing may also involve the washing of the bone by antiseptics and/or antibiotic solutions in order to inactivate or remove surface contaminants and may not penetrate the tissue (Tomford 2000). In Australia, Australian Biotechnologies processes femoral heads and cancellous bone pieces by milling or grinding them into a filling material (Australian Biotechnologies/Products 2011).

**Allograft Musculoskeletal Tissue Storage and Distribution**

Musculoskeletal tissue allografts require storage prior to transplant. The term ‘tissue bank’ refers to storage or ‘banking’ of tissues. In Australia, musculoskeletal tissue is stored frozen at temperatures ranging from -20°C to -80°C for periods of 6 months to a maximum of 5 years. Freezing results in the removal of free water in the tissue, a requirement for tissue spoilage (Galea & Kearney 2005). Deep freezing at -80°C creates water crystals within the tissue, causing cell destruction and reducing immunogenic reactions. Once all criteria have
been met and the musculoskeletal tissue has been cleared for transplant, the tissue banks will distribute the tissue to surgeons for transplant within their own health facility or transport to another if required.
Therapeutic Goods Act 1989

In Australia, musculoskeletal tissue is considered a ‘therapeutic good’ as it is produced, or manufactured, for therapeutic use in humans. The manufacture of musculoskeletal tissue as a therapeutic good includes any stage of the retrieval, processing, storage, testing and distribution of the tissue. A facility involved with any of these stages is part of the manufacturing process. The Therapeutic Goods Act 1989 was introduced in Australia as a national licensing scheme and in September 1995 the Australian Code of Good Manufacturing Practice for Human Tissues was published. This was revised to combine blood and tissues and a harmonisation with the European Union, and was released in August 2000 as the Australian Code of Good Manufacturing Practice – Human Blood and Tissues (GMP) (Ireland & McKelvie 2003).

All facilities involved in the manufacture of musculoskeletal tissue must be audited for compliance of the GMP by the Therapeutic Goods Administration (TGA) before a licence is issued. To maintain their manufacturing licence, facilities must have protocols in place that meet the regulatory standards outlined in the GMP and which subsequently ensures the quality of tissue before transplant. A site audit by the TGA ensures that the manufacturing facility complies with the GMP by having trained personnel, calibrated and maintained equipment, donor assessment criteria, procedures and records all monitored by a good quality system (TGA 2000).

The TGA began a review of the national regulatory framework for human tissues and biological therapies under the recommendation of the Australian Health Ministers Conference in July 2002. The Therapeutic Goods Act 1989 received Royal Assent on the 31st May 2010 to create a new regulatory framework for a type of therapeutic good known as a biological which became effective on the 31st May 2011 with a 12 month transition period (TGA Dec 2010). Banked human musculoskeletal tissue and processed musculoskeletal tissue are within the scope of the biologicals framework.

An amended GMP has been drafted and the title has been expanded to ‘Australian Code of Good Manufacturing Practice Human Blood and Blood Components, human tissues and
human cellular therapies’ (TGA Nov 2010). After extensive public consultation the release of this code has been delayed.

There are four therapeutic goods orders (TGO) that set product-specific standards and specify the minimum technical requirements required for the specified biological and another TGO which sets the standard for the labeling of biologicals. These TGOs have been authorised by a delegate of the Minister of Health and Ageing under Section 10 of the Therapeutic Goods Act 1989 and are listed below:

- TGO 83 - Standards for human musculoskeletal tissue (TGA 2011)
- TGO 84 - Standards for human cardiovascular tissue (TGA 2011)
- TGO 85 - Standards for human ocular tissue (TGA 2011)
- TGO 86 - Standards for human skin (TGA 2011)
- TGO 87 – Standards for the labelling of biologicals (TGA 2011)

A new TGO is still in draft which will provide the standards for minimisation of infectious disease transmission for human blood, blood components, human tissues and human cellular therapy products.

Microbiology laboratories are part of the manufacturing process of musculoskeletal allograft tissue as they assess the bioburden of tissue and the virology and serological testing of donors. However, the history of the GMP is based in the pharmaceutical or production industry and not in a clinical microbiology laboratory. Subsequently, clauses of the GMP are subject to interpretation by laboratory staff and assessors. A major issue that has confronted several tissue banks has been finding a TGA-licensed microbiology laboratory nearby. One tissue bank has had to close and two others send their samples to a TGA-licensed microbiology laboratory in another state. Non-TGA licensed laboratories perform the same type of testing on other clinical samples on a daily basis. Furthermore, local microbiology laboratories can provide benefits associated with proximity such as decreased transport costs, decreased specimen transit time and ultimately decreased time to processing of the sample. Microbiology laboratories based within a clinical setting such as a hospital do not consider tissue bank associated work as their core business. Therefore, the financial, staff and workload burden required to maintain a TGA licence is not a necessity for most microbiology laboratories servicing these tissue banks.

There are many challenges faced by tissue banks and laboratories in meeting the regulatory requirements of the TGA. The staff and budget requirements required to maintain their TGA
licence impacts on the core business of the facility. A well-known fall-out of any TGA audit is the differences in interpretation of the GMP among TGA auditors. Laboratories and tissue banks alike spend a predominant amount of time responding to deficiencies raised after an audit, sometimes to substantiate an interpretation accepted at a previous audit with another auditor, an issue also raised by Health Outcomes International (2009).

**AS ISO 15189 – 2009 Medical Laboratories – Particular Requirements for Quality and Competence**

The National Association of Testing Authorities (NATA), Australia is recognised by the Australian Commonwealth Government, by way of a Memorandum of Understanding, as the only national accreditation body for establishing and maintaining competent laboratory practice. Laboratories must demonstrate compliance against the criteria in AS ISO 15189-2009 Medical Laboratories – Particular requirements for quality and competence. Accreditation by NATA is based on a peer-review process with mandatory laboratory assessments involving a NATA lead assessor and selected volunteer assessors from other medical laboratories. This is a technical audit as well as assessing quality systems and processes. Compliance to AS ISO 15189 is mandatory for all medical laboratories in Australia.


NCS International (NCSI) is a subsidiary of NATA and is a certification body for independent recognition against national and international management system standards. This certification is not mandatory for Australian laboratories but has been attained by some. Laboratories may undergo a voluntary certification audit by NCSI against the standard AS/NZS ISO 9001:2008 Quality management systems – Requirements. This audit is not based on technical competence but rather on an organisation’s compliance of their quality management system to the standard and is not a peer review audit.
Musculoskeletal Tissue Infection

Introduction

Infections arising from orthopaedic surgery can lead to repeat surgery, antibiotic treatment, disability, work and lifestyle loss, pain and the potential subsequent effects such as anxiety and depression. Orthopaedic surgery has traditionally employed an extensive use of implantable biomaterial devices, such as orthopaedic plates, rods and total joints. These devices may be made of metals, alloys and polymers and are considered biologically inert. However, infection is still common with the use of these devices as bacteria have the ability for adhesion onto theses devices. Problems with allograft tissue transplant are often due to the lack of successful tissue integration and bacterial adhesion which may lead to biofilm formation with resulting organism resistance to host defense mechanisms and antimicrobial therapy (Esterhai 1990, Katsikogianni & Missirlis 2004).

Total joint replacement patients will almost all have an underlying bone disease of inflammatory or ischemic origin, the others will have fractures. Blood supply and host defenses can be impaired by inflammation, ischaemia and fractures, placing these patients at risk of infection (Merritt 1990). Bone infection will ultimately lead to bone loss and mechanical failure if unable to be resolved and will almost always result in failure of the graft. Host-mediated events are initiated by invading bacteria which produce enzymes, exotoxins and endotoxins (Horowitz 1990).

Basic Concepts of Infectious Disease

An infection implies that micro-organisms are present in a living patient. The micro-organisms causing the infection are termed pathogens, the virulence of the pathogen may vary and will be reflected in the patient’s symptoms. A local inflammatory response generally occurs at the site of tissue injury or destruction by micro-organisms.

Micro-organisms that are not commonly associated with infection can become pathogens if the ‘opportunity’ arises, therefore termed opportunistic infections. These organisms tend to be normal inhabitants of human sites, such as Staphylococcus epidermidis of the skin. Whether the organism becomes a pathogen depends on a series of factors such as the resistance of the patient, virulence properties of the organism and prophylactic therapy which may have killed or reduced numbers of other organisms. Opportunistic pathogens
may reach sites of potential infection by different entry points such as inhalation, ingestion, direct contact or inoculation. Inoculation via tissue allografts to the patient is the risk undertaken with allograft transplantation.

**Sources of Bacteria and Fungi in Allograft Musculoskeletal Tissue**

Musculoskeletal allografts have been described as life-enhancing rather than life saving procedures however their use presents a risk of transmission of infection. Microbial presence in musculoskeletal allografts may be present within the tissue at the time it was harvested. Cadaveric donor tissue can be contaminated if the retrieval of tissue occurs after the allowed time of retrieval from the time of death. Bacteria and fungi multiply and invade the bloodstream or mucosal/bowel barriers that are breaking down post-mortem, favouring those organisms that are able to grow rapidly in tissue with a low oxygen concentration (Patel & Trampuz 2004). Trauma is another factor contributing to the passage of bacteria and fungi into the bloodstream and therefore to other body sites. Refrigerating the body after death slows down the decomposition of the body.

The physical appearance of the cadaver is taken into account prior to retrieval. Obvious areas of skin infections should be avoided. In my laboratory, tissue was contaminated with Group B streptococcus when retrieved from an area with skin ulcers.

Contamination of musculoskeletal allografts may occur at the time of retrieval, processing or packaging with microorganisms from an external source – from personnel, contaminated reagents and equipment, environmental surfaces and by aerosols. The Centre for Disease Control (CDC) (1996) reported the contamination of electrolyte solutions and contaminated deionised water was reported by Farrington et al (1996) which subsequently contaminated allograft tissue.

Femoral head samples from living donors are collected under operating room conditions. The number of people in an operating room influences the number of bacteria present in the air. Although aseptic technique is used, bacteria cannot be totally excluded from the operating room setting. Blomgren et al (1983) measured a decrease in the number of airborne bacteria in operating rooms performing total hip replacements when the surgical team wore total body exhaust suits as compared to conventional theatre clothing. Howarth (1985) found that in a busy operating theatre each person present can release 50000 viable microorganisms per minute and the movement of air is very important in an operating theatre in determining where the microorganisms settle.
The aseptic technique of retrieving musculoskeletal tissue from living and cadaveric donors in operating theatres and morgues is performed to minimize the risk of contamination from external sources. It does not reduce the microbial bioburden already present in the tissue.

Forsell and Liesman (2000) investigated seven independent variables in an effort to determine which have the greatest effect on the bioburden of cadaveric tissue. The physical recovery environment, recovery before or after an autopsy, the length of time to complete the recovery, the number of staff involved in the recovery of tissue and the impact of organ and skin recovery were found to be statistically significant in the number of positive cultures obtained from a retrospective review of data obtained from seven tissue banks. However there were differences among the participating bone banks and it was concluded that no one factor was common to all. The cause of death and the length of time from death to recovery of tissue were not identified as being statistically significant variables.

**Clinical Presentation of Bacterial and Fungal Joint Infections**

Prosthetic joint infection can be classified into three categories depending on the appearance of infection after surgery: early (<3 months), delayed (3 – 24 months) or late (>24 months) (Zimmerli et al 2004). Early infections become evident less than 3 months after surgery and patients typically show symptoms of fever, joint pain, a purulent drainage and redness of the skin surrounding the prosthesis site. The indications for infection are not as obvious for patients with delayed infection and may include allograft loosening and joint pain with the causative organism typically of the less virulent type such as coagulase-negative staphylococci and *Propionibacterium acnes*. Early and delayed infections are usually acquired during implantation of the allograft. Late infections are generally as a result of haematogenous seeding with the most likely sources of bacteraemia and fungaemia being the skin and respiratory, dental and urinary tract infections.

**Bacterial Virulence Factors and Pathogenicity**

Pathogens are organisms that are capable of causing disease and the ability of an organism to cause disease is its pathogenicity. The virulence of an organism refers to the degree of pathogenicity it demonstrates. Virulence factors are structural components or products produced by bacteria that allow the organism to become a pathogen and cause infection. Virulence may be attributed to several factors involving the host and the organism and these interactions may change under different conditions.
Coagulase-negative *staphylococcus* species, such as *Staphylococcus epidermidis*, is most frequently involved in allograft infections. Although coagulase-negative staphylococci are the most common isolate of musculoskeletal tissue infections, *S. aureus* is the most virulent of the staphylococci. *Staphylococcus* sp. possess several properties that are believed to contribute to its ability to cause disease. These virulence factors are not found in all strains of staphylococci and include capsule formation, cell wall constituents, enzymes, haemolysins and toxins.

Coagulase-negative staphylococci are considered normal flora of human skin and mucous membranes. Although generally considered an organism of low virulence, its pathogenicity in orthopaedic graft infections has been well established (Gristina et al 1990). *S.epidermidis* produces a capsular polysaccharide adhesic and a polysaccharide intracellular adhesic which aids in the adhesion of the bacteria to allograft surfaces and colonise the surface (Dunne 2002). From this initial colonising stage, the bacteria multiply to form multiple layers of bacterial cells on the allograft surface, referred to as a biofilm. The biofilm is held together by bacterial exopolysaccharides called a glycocalyx, and provides important functions. It is able to trap nutrients from the surroundings, provides a protective barrier against antibiotics and bacteriophages and stabilises the bacterial matrix to the graft surface.

Some strains of *S. aureus* also produce an exopolysaccharide that may prevent ingestion of the organism by polymorphonuclear cells. *S. aureus* cell walls contain protein A which has the ability to bind the Fc region of immunoglobulin G (IgG) molecules. Protein A functions as a virulence factor by interfering with opsonisation and ingestion of the organisms by polymorphonuclear cells activating and eliciting immediate and delayed type hypersensitivity reactions. *Staphylococci* cell walls also contain peptidoglycans and techoic acids which function in the specific adherence of Gram-positive bacteria to mucosal surfaces. Catalase is an enzyme produced by staphylococci and may function to inactive toxic hydrogen peroxide and free radicals formed by the myeloperoxidase system with phagocytic cells after ingestion of the microorganisms.

Free and bound coagulase is produced by *S. aureus* and may act to coat the bacterial cells with fibrin, making them resistant to opsonisation and phagocytosis. Fibrinolysins are produced by *S. aureus* and may break down clots and allow spread of infection to tissues. Lipases are produced by strains of *S. aureus* causing chronic furunculosis that may help to spread the organism in cutaneous and sub-cutaneous tissues.
*S. aureus* produces a variety of haemolysins that affect host cell function and/or morphology. Alpha-haemolysin has lethal effects on a wide variety of cell types, including human polymorphs and will lyse erythrocytes from several animal species. The toxin is dermonecrotic on subcutaneous injection. B-haemolysin is a sphingomyelinase that is active on a variety of cells. Y-Toxin lyses erythrocytes from many species, including humans by unknown mechanisms. Δ-toxin is strongly surface active and it disrupts biological membranes by a detergent like action. Leucodin is an exotoxin produced by *S. aureus* that exerts a direct toxic effect on human polymorph membranes, causing deregulation of the cytoplasma, cell swelling and lysis.

Group A streptococci produce a surface protein, known as the M protein, which enables infection by resistance to phagocytosis, intracellular invasion, surface adherence and lysis of polymorphonuclear leucocytes. Group A streptococci and staphylococci also produce the enzymes streptokinase and staphylokinase that can hydrolyse fibrin clots, helping the organism spread into tissues.

Gram-negative bacteria interfere with the host inflammatory response by producing lipopolysaccharide, the lipid A protein having the ability to activate complement and stimulate the release of various cytokines that clinically present as endotoxic shock. Many bacteria produce enzymes that act extracellularly. For example, *S. aureus, S. pneumoniae, Group B streptococci* and *P. acnes* produce hyaluronidase which hydrolyses the intercellular matrix of mucopolysaccharides in tissue, allowing the organisms to spread through connective tissue, and thus may act to spread the organisms to adjacent areas. Many Clostridium species, *C. perfringens, C. septicum, C. sordelli* and others, produce cytotoxins, cytolysins, collagenases, phospholipases, hyaluronidases and deoxyribonucleases that allow these organism to spread into tissues and muscle by colonising devitalised tissue and destroys the collagen matrix of muscle and connective tissue (Winn et al 2006).

Plasmids allow the transfer of genetic material between organisms. The spread of antimicrobial resistance via plasmids enables organisms to gain resistance to therapeutic interventions, maintaining pathogenesis and infection. B-lactamases, most of these are plasmid mediated, render staphylococci resistant to penicillin and ampicillin.

Culture-positive allograft samples collected from musculoskeletal donors may not signify infection. As the most common isolate is coagulase-negative staphylococci, it is more than likely that contamination occurred post-collection. Correlation between culture-positive
allograft results and post-transplant clinical infection has not been found (James & Gowan 2002, Hou et al 2005, Guelich et al 2007, Kappe et al 2009).

Fungal Virulence Factors and Pathogenicity

Fungal infections are considered systemic when the infection is located in organs and tissues. Systemic fungal infections have increased in organ transplant recipients due to various reasons such as the use of immunosuppressive therapy, prolonged or multiple courses of antibiotic therapy and extensive invasive surgery. Fungi are abundant diverse organisms readily encountered in the environment, however only a few species are clinically significant in humans. Common invasive fungi include yeasts (such as Candida and Cryptococcus), moulds (such as Aspergillus and Fusarium), dimorphic fungi (such as Blastomycetes and Coccioidoides) and others such as Pneumocystis. The most important and clinically significant fungi in humans are Aspergillus and Candida species.

As with bacterial virulence factors, fungal virulence factors must also be capable of adhesion, invasion and colonisation to become pathogens and evade host immune defences. The ability of a fungus to grow at 37°C is an important virulence factor. If the fungus is not able to grow at that temperature, systemic infection in deep tissues in humans is not possible. The ability of a fungus to grow at 37°C allows the fungus to produce virulence factors that enable its survival. Fungi that are not able to grow within the human body will not be pathogenic even if virulence factors are produced.

Members of the genus Candida are the most common cause of fungal infections in humans. The most clinically significant organisms of this genus are C. albicans, C. glabrata, C. tropicalis and C. parapsilosis. These organisms are normal inhabitants of the gastrointestinal tract and the vagina and symptomatic infection will develop when these fungi overgrow due to a diminished host defence. Host defences can be altered by antimicrobial therapy or corticosteroid treatments. Systemic infections of deeper organs may be due to intravascular devices such as catheters.

Within the Aspergillus species, Aspergillus fumigatus is the most common cause of human disease, followed by A.flavus and A.niger (Hogan et al 1996). This mould is found in the indoor and outdoor environment and its ability to cause disease is largely influenced by the immune-competence of the individual. The aerosol spread of fungal spores focuses infection of this pathogen in the respiratory system of the human host, colonising the airways and lungs and can result in systemic infection if dissemination to the blood stream and organs occurs.
Cryptococcus neoformans possess a thick polysaccharide capsule capable of inhibiting phagocytosis by host cells. C. albicans expresses a large number of adhesins that aid in adherence to host cells. Hydrolytic enzymes are also secreted which have the ability to damage host tissue. C. albicans is able to adapt to the different pH of different environments, such as the neutral pH of the bloodstream and the more acidic pH of the vagina (Brown 2002).

Dimorphic fungi undergo morphological transformations to aid in pathogenicity. Coccidioides immitis grows as a filamentous form in the environment but transforms to large spherules in the lung which release endospores in the host. Other fungi that exist in a mycelial form in the environment and a yeast-like form inside the host include Histoplasma capsulatum and Paracoccidioides brasiliensis.

Reported Cases of Bacterial Infections Post-Transplant of Allograft Musculoskeletal Tissue

In a study by Tomford & Starkweather et al (1981), 21 of 303 (6.9%) of patients reported evidence of post-operative infection. Where organism involvement could be identified, the most common bacteria were Enterobacter species, Staphylococcus epidermidis and Staphylococcus aureus. Lord & Gebhardt et al (1988) reported infections in 33 of 283 (11.7%) of patients who had a massive bone allograft, the predominant organism being Staphylococcus epidermidis. Wang & Zinderman et al (2007) reviewed FDA MedWatch Reports for the period 2001 – 2004 and found that 37 of 65 reports identified bacteria and fungi as the causative organisms (25 fungi and 42 bacteria). Aerobic bacteria were predominant in this study with S. aureus the most common isolate. Other isolates include coagulase-negative staphylococcus, Enterococcus species, Enterobacter species, Pseudomonas species and anaerobic bacteria such as Peptostreptococcus, Clostridium and Bacteroides species. Kappe & Cakir et al (2009) observed a 6.9% post-operative infection rate with the bacteria isolated reported as coagulase-negative staphylococci, Enterobacter cloacae, Enterococcus faecalis, Staphylococcus aureus and Corynebacterium xerosis. Post operative infections were reported for 4.9% of patients investigated by Winter & Cowie et al (2005) with the most common organism isolated being coagulase-negative staphylococci. Sutherland & Raafat et al (1997) reported an infection rate of 12.2% with S. aureus the most common organism isolated. In these studies, the post-operative infection organism was not always present from the samples collected at retrieval for microbiological assessment.
Coagulase-negative staphylococci are commonly isolated from pre- and post-transplant samples but bio-typing has not been performed to determine if the isolates are the same.

Knee surgery is a common orthopaedic procedure and musculoskeletal tissue allografts are frequently used for ACL injuries. There were two significant bacterial contamination events in musculoskeletal allografts that highlighted the transmission risk involved and facilitated changes to be made. The first event involved 4 patients in Florida and Louisiana, USA, in 2000 (CDC Dec 2001). In Florida on April 5th, 2000, a 16 year old female had ACL reconstruction using a bone-tendon-bone allograft. On April 21st, she presented at a local orthopaedic clinic with swelling and redness of the knee and was subsequently treated with intravenous antimicrobial therapy with removal of the allograft and screw. The fluid aspirated from the knee was cultured and *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* were isolated.

On the 7th April 2000 a 40 year old man underwent an ACL reconstruction using a bone-tendon-bone allograft (CDC Dec 2001). He developed symptoms of infection on April 24th and a culture of the drainage fluid at the site of the operation isolated *Pseudomonas aeruginosa*, the same genotypic pattern as in the case above. The bone supplied to these two patients was from the same tissue bank and from the same donor. The allografts had been irradiated prior to transplant.

In another case on October 9th, 2000, a 55 year old female had ACL reconstruction using a bone-tendon-bone allograft and developed symptoms on the 17th October (CDC Dec 2001). Culture of her knee aspirate isolated *Citrobacter werkmanii/youngae* and a beta-haemolytic *Streptococcus* species. On October 19th, 2000, a 29 year old female underwent the same procedure with a knee aspirate culture isolating *Klebsiella oxytoca* and *Hafnia alvei* (CDC Dec 2001). Both these patients received bone from the same tissue bank and from the same donor. *Serratia liquefaciens* was isolated from cultures taken from the donor allografts during tissue processing. In these cases, the bone was intended for terminal sterilisation but it did not take place due to human error.

The second event, also in the USA, involved three unexpected deaths of three patients within one week of undergoing knee surgery (CDC Nov 2001, CDC Dec 2001, CDC 2002, Kainer et al 2004). Patient 1 had a knee osteochondral allograft and Patients 2 and 3 had total knee replacements. Two local hospitals were involved in the surgery and all three patients died 36–82 hours later, following symptoms of severe abdominal pain and sudden drop in blood pressure, consistent with septic or cardiogenic shock. Blood cultures obtained
from Patient 1 before his death grew *Clostridium sordellii*, although blood cultures taken from Patients 2 and 3 were negative at the end of the incubation period. Autopsy tissues from Patients 2 and 3 did not isolate *Clostridium species*.

The allografts received by these patients were from the same cadaveric donor with no evidence indicating that the donor was septic or had risk factors for *Clostridium species*, such as drug use or abdominal trauma. An investigation was initiated by CDC and by March 2002, a total of 26 reports of bacterial infections associated with musculoskeletal tissue allografts were reported, including the previously reported cases. CDC defines an allograft-associated infection as ‘any surgical site infection (SSI) at the site of allograft implantation occurring within 12 months of allograft implantation in an otherwise healthy patient with no known risk factors for SSI’ (CDC 2002). *Clostridium species* was the causative organism for 13 of the 26 patients (50%) – *C. septicum* causing 12 infections and *C. sordellii* one. Aseptic technique was used in the processing of the allografts but there was no terminal sterilisation. In other cases, 11 patients were infected with Gram-negative bacilli, 5 with a polymicrobial infection. Eight of these patients had received allografts that were processed aseptically but there was no terminal sterilisation. Gamma irradiation was reported on allografts received by three patients.

The cases involving *Clostridium species* highlighted some very important issues in tissue processing and bioburden assessment. The tissue-processing company supplying the donor allograft bone routinely processed a ‘companion tissue’ alongside the allograft, both of which were soaked in a suspension of an antibiotic/antifungal solution. The companion tissue is removed from the solution and sent to the microbiology department for bioburden assessment. A swab culture was not taken before tissue processing. Cultures of the companion tissue of the donor by the tissue-processing company were negative although positive cultures were obtained from non-implanted tissue tested by the CDC. Two explanations were proposed by the CDC for this discrepancy. Firstly, the companion tissue cultured by the tissue-processing company only after suspension in the antibiotic / antifungal solution may be false negative due to the residual antibiotics on the tissues causing a bacteriostatic effect. Secondly, the companion tissues are much smaller representations of the allograft and may not have been representative of the entire allograft. *Clostridium* species are spore forming bacteria and antibiotic / antifungal solutions will not eliminate spores. It is thought that the source of the *Clostridium* species on the donor allograft was via haematogenous seeding by bowel flora, aided by the delay in tissue retrieval after death, trauma and delay in refrigeration (CDC 2002). Reducing the microbial load on tissue by gamma irradiation or ethylene oxide would eliminate *Clostridium* spores in allograft tissue.
but is not suitable for all tissue types. Another issue is that the isolation of an organism such as *Clostridium* species from one allograft would require a determination of the suitability of all allografts from that donor, even if all other allografts are culture negative.

Interestingly, in a study by Malinin et al (2003) of musculoskeletal tissue recovered from cadaveric donors, 64 of 795 (81%) consecutive donors had positive cultures of *Clostridium species*. These were identified by the culture of blood, bone marrow and / or tissue samples. Only 30 of the 64 (47%) Clostridium isolates were isolated from tissue samples and only 8 of these were from a tissue sample alone.

In 2003, another case of allograft-associated infection was reported in Colorado USA. A 17 year old healthy male underwent elective ACL repair with a hemi-patellar tendon allograft. Clinical symptoms six days after the procedure indicated infection and surgical intervention and antibiotic treatment was required (CDC 2003, Eastlund 2006). Group A streptococcus was isolated from the patient's blood cultures, wound drainage and tissue samples. The source of the infection was determined to be the cadaveric allograft donor who had undergone cervical spinal fusion three weeks before his death and had symptoms of toxic shock syndrome with back pain, vomiting, fever, skin rash and hypotension. Group A streptococci was isolated from the donor's blood, lungs, skin and vessels. A final false-negative culture result of the donor’s tendons was obtained which had been processed with an antibiotic solution but with no other disinfection or sterilisation steps before distribution for transplant.

**Reported Cases of Fungal Infections Post-Transplant of Allograft Musculoskeletal Tissue**

A review of 83 reports of a transplant infection, received by FDA’s Med Watch adverse event reporting system in the United States, for the period 2001–2004 identified 25 fungal infections from tendon, bone, heart valve, blood vessel, ocular tissue and skin allografts (Wang et al 2007). Tendons accounted for 20% of these fungal isolates and comprised of either a *Candida* or an *Aspergillus* species. It is unclear whether any fungi were isolated from bone allografts in this review.

A literature search on clinically indicated fungal pathogens isolated from musculoskeletal allografts yielded few results (Crawford 2005, Azam 2009, Eastland & Winters 2010). There were many reported fungal infections in solid-organ and stem cell transplant recipients but appear to be uncommon from musculoskeletal tissue transplants (Paya 1993, Kubak et al
Fungal pathogens account for 5% of infections in kidney transplant recipients and 40% in liver transplant recipients (Paya 1993).

A review of the literature providing results of contamination testing and infection rates of allograft musculoskeletal tissue is presented in Table 2. This table also includes references where infection was diagnosed post-transplant. Table 2 provides a summary of fungal isolates from musculoskeletal tissue allografts from Australian and overseas studies and overall the isolation of a fungal pathogen is very low. This is not unexpected as the majority of allograft tissue is donated from relatively healthy living donors. Factors which may influence the possibility of fungal contamination in cadaveric donations often lead to the exclusion of these allograft tissues prior to retrieval, for example, diminished immune-competency in HIV positive patients.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Positive Cultures</th>
<th>Contamination or Infection Rate (%)</th>
<th>Number of Fungal Isolates from Positive Cultures (%; Identification)</th>
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<tr>
<td>Tomford et al</td>
<td>1981</td>
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<td>1990</td>
<td>17</td>
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<td>Chapman &amp; Villar</td>
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<td>1994</td>
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<td>Journeaux et al</td>
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<td>Bettin et al</td>
<td>2000</td>
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<td>2009</td>
<td>Not indicated</td>
<td>31 (Candida sp, Aspergillus sp, Rhodotorula minuta, Aureobasidium sp)</td>
<td></td>
</tr>
<tr>
<td>Varettas &amp; Taylor</td>
<td>2011</td>
<td>4.9</td>
<td>2/170 (1.2%; Cryptococcus laurentii and fungus)</td>
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Bioburden Reduction Methods of Allograft Musculoskeletal Tissue

What is Bioburden?

In the Microbiology laboratory, the bioburden assessment of samples received from the tissue bank determines the estimated numbers of bacteria or fungi on an allograft tissue. Bioburden assessment is often mistaken as being the same as a sterility test. In contrast, sterility testing is performed on batches of products and provides an estimate of the probable sterility of a batch.

Bioburden assessment testing must take into account the type of organism and the numbers present. The method used must be able to recover a wide range of organisms that includes fastidious and non-fastidious organisms, spore-formers and non-spore formers. The method must also recover low numbers of organism that may be present. Even poor methods will recover organisms if they are present in very high numbers.

Introduction

Micro-organisms function by their metabolic activities, dependent on chemical reactions and influenced by temperature and water. Therefore, altering environmental conditions can have a detrimental effect on micro-organisms and form the basis of bioburden reduction processes. The bioburden reduction of musculoskeletal tissue before final storage can be used to inactivate or kill all microorganisms. There are several methods of bioburden reduction that have been developed and used throughout the history of tissue banking for musculoskeletal tissue, all with associated limitations. The term sterilisation is often used incorrectly when referring to bioburden reduction methods. Some methods can be referred to as sterilisation methods and others should not.

Autoclaving and Boiling

One of the most effective methods of killing micro-organisms is by using high temperature combined with high humidity. Autoclaving combines heat in the form of saturated steam under pressure, killing micro-organisms by coagulating their proteins (Pelczar et al, 1977). Most vegetative bacterial cells are killed in 5-10 minutes at temperatures of 60-70°C using moist heat. Most bacterial spores require temperatures greater than 100°C for periods ranging up to 180 minutes for Bacillus species and up to 60 minutes for Clostridium species.
Vegetative cells of fungi are usually killed in 5-10 minutes at 50-60°C using moist heat, their spores requiring a higher temperature at 70-80°C (Pelczar et al, 1977). Autoclaving is damaging to bone, cannot be used on other musculoskeletal tissue and is not able to sterilise micro-organisms within fats and oils as the steam cannot reach them.

Boiling does not reach the high temperatures attained by autoclaving. Boiling will destroy vegetative cells but some bacterial species can withstand boiling for many hours making this an unsuitable bioburden reduction process for contaminated material, not taking into account the physical destruction of some types of musculoskeletal tissue if this process is used.

Boiling and autoclaving can be performed on bone but not on other musculoskeletal tissues, however the osteoinductive properties of bone are reduced and these methods are not recommended as a bioburden reduction process.

**Antibiotic Solutions**

Immersion of allograft tissue into fluids containing a variety of antibiotics has been found to be inadequate as a bioburden reduction process and may mask bacteria that are present. This bacteriostatic effect was found to be responsible for infections via allograft tendon and cartilage which gave final false-negative culture results due to their processing with an antibiotic solution (CDC 2003; Eastlund 2006).

**Freezing**

Allograft musculoskeletal tissue can be stored frozen prior to transplant for up to 5 years. However, this cannot be considered a form of sterilisation as micro-organisms can survive freezing for an equally long time. As is regularly performed in clinical microbiology laboratories, micro-organisms are stored at low temperatures for long periods of time as culture collections (at -80°C) and for storing patient samples and culture plates (at 4-7°C). Liquid nitrogen at a temperature of -196°C is used to preserve cultures of many micro-organisms and the initial chilling will kill a small percentage of the population but not all. Frozen micro-organisms are considered dormant in that they do not perform metabolic activity but are able to be revived by sub-culture onto agar and broth media.

**Supercritical Carbon Dioxide**

Supercritical carbon dioxide is considered a sterilisation process, also known as dense CO₂. Supercritical CO₂ has the density of a liquid and expands as a gas when used at a
temperature higher than its critical temperature (31.1°C) and critical pressure (72.9atm/7.39 MPa), resulting in temporary acidification which is lethal to micro-organisms. This process has been reported as a means of inactivating bacteria since the 1950’s by Fraser (1951) and the 1960’s by Foster (1962), having been used predominantly in the food and pharmaceutical industry and has been investigated on biological material with varying success on bacterial spores, bacteria and yeast (Spilimbergo et al 2003, Dillow et al 1999). White et al (2006) achieved a terminal sterilisation level of 10^{-6} sterility assurance level (SAL) that can be used on packaged materials such as musculoskeletal allografts. A SAL of 10^{-6} is defined as the one-in-a-million probability of a living micro-organism being present after sterilisation.

Sterilisation by supercritical carbon dioxide has been found not to have any adverse affects on the biomechanical properties of musculoskeletal tissue and does not leave a toxic residue. In Australia, this method is in use at Australian Biotechnologies for the sterilisation of milled bone (Australian Biotechnologies/Products 2011).

Microwave Sterilisation
The microwave sterilisation of femoral head allografts was investigated (Dunsmuir 2003) as an alternate to the labour-intensive and expensive bioburden reduction methods. The core of the femoral heads were inoculated with \textit{S. aureus} and \textit{Bacillus subtilis} before being irradiated for 1, 2 and 6 minutes at 800W in a 2450 MHz microwave. No growth was obtained in specimens subjected to microwave irradiation for 2 minutes or longer. Further study is required on this method on different types of musculoskeletal tissue with a more extensive range of micro-organisms. This method is not in use in Australia as a bioburden reduction method on allograft tissue.

Ethylene Oxide
Ethylene oxide has been used for the bioburden reduction of musculoskeletal tissue, however it is not in use in Australia. It is used in a gaseous state and after the sterilisation process, the ethylene oxide is removed and replaced with carbon dioxide. One of the limitations in using this type of sterilisation is the potential risk of toxicity of ethylene oxide and its by-products to staff involved in sterilisation by this means, with an increased risk of malignancy with extended or intermittent exposure and a high rate of abortion in pregnant female workers who are exposed to the gas. (Vangsness et al 2003). Concerns have also been raised regarding the detrimental effect on the osteoconductive properties of bone.
(Thoren & Aspenberg 1995), its ability to penetrate large cortical bone (Prolo et al 1980) and its ability to cause of intraarticular reactions (Jackson 1990; Roberts 1991).

**Gamma irradiation**

The most common process of bioburden reduction in Australia is by gamma irradiation from a Cobalt 60 source, with its ability to penetrate through packaging around the tissue a major advantage as repacking, with the risk of contamination, is not required. A balance is required in the use of bioburden reduction by gamma irradiation to achieve a dose that confidently reduces numbers of microorganisms but does not affect tissue structure. In Poland, a dose of 35 kGy is recommended (Dziedzic-Goclaw ska et al, 2005). Baker et al (2005) validated an allograft sterilisation method with a dose of at least 9.2 kGy to achieve a sterility level of $10^{-6}$ SAL. It is important to remember that irradiation is not a sterilisation process but a bioburden reduction process.

In Australia, 25 kGy has been historically accepted, with much more research being performed to decrease this level. Nguyen et al (2007) states that the standard of 25 kGy is based on the sterilisation of non-biological products. The improvement in the standard of the tissue banking industry has resulted in a better quality tissue allograft, which should allow the radiation dose to be decreased to between 15–25 kGy, reducing the adverse effects to the tissue. In another study, Nguyen et al (2008) validated the radiation dose of 15 kGy for surgical bone allografts. The privately owned tissue bank Australian Biotechnologies are currently using the dose of 15kGy on soft tissue allografts (Australian Biotechnologies/Products 2011) as is the Perth Bone and Tissue Bank for soft tissues (Perth Bone and Tissue Bank n.d.).

**Issues Associated with Bioburden Reduction Methods of Allograft Musculoskeletal Tissue**

There has been much discussion and research into the bioburden reduction of allograft musculoskeletal tissue prior to long-term storage. Achieving the bioburden reduction or sterilisation of allograft tissue is not as simple as sterilising non-organic materials. Allograft tissue has a non-uniform and complex physical structure. The density of the tissue is also a factor in the penetration of sterilants, especially gases and liquids, in and out of the tissue. Sterility cannot be assured if using sterilants that do not penetrate the tissue wall (Vangsness et al 2003). Musculoskeletal tissue allografts from cadaver donors can be contaminated with a high bioburden. The biomechanical properties of musculoskeletal tissue
must be maintained and not damaged by any bioburden reduction procedure used, while at the same time ensuring high levels of bioburden are destroyed. (McAllisster et al 2007). Importantly, not all musculoskeletal tissue can be subject to bioburden reduction methods.

In Australia, the majority of tissue banks terminally gamma-irradiate allograft musculoskeletal tissue if bacterial and fungal culture results from bioburden assessment testing are negative. All culture-positive allograft tissue is generally discarded. Only two tissue banks do not irradiate or perform any bioburden reduction method prior to storage and transplant, also discarding all culture-positive results (personal communication via confidential tissue bank survey). The physical properties of allograft tissue can be affected to some degree by any bioburden reduction method.

Tissue banks, surgeons and recipients must be confident that the musculoskeletal tissue being transplanted has undergone microbiology bioburden assessment testing by the most sensitive and accurate methods available today, especially in those musculoskeletal tissues where bioburden reduction methods are not used. This will subsequently have an impact on those allografts which undergo terminal gamma-irradiation prior to storage and transplant. Irradiation levels can be significantly reduced if bioburden assessment methods are reliably sensitive, thereby maintaining the biomechanical properties musculoskeletal tissue which are important for a successful musculoskeletal transplant. Ideally, bone that is considered culture-negative or ‘no growth’ should not need to be irradiated, removing the deleterious biomechanical effects on tissue. Studies have shown that there is less bone production post-transplant of irradiated bone compared to non-irradiated bone (Tomford 1995). The terminal bioburden reduction process of allograft musculoskeletal tissue is optional and is not a substitute for thorough donor assessment and microbiological screening.
Principles of Sample Collection of Allograft Musculoskeletal Tissue

The collection of samples for microbiological examination is a very important part of the bioburden assessment process. If the requirements for specimen collection are not met then the final result may be a false interpretation. The samples must be collected in a manner that meets stringent guidelines and the material collected must be representative of the site. All attempts must be made to avoid contamination from other sites, the collector and the environment. Contamination with exogenous flora can mask tissue results and may make interpretation difficult.

Samples for bacteriological and fungal culture must ideally be collected before the initiation of antimicrobial therapy. This is contradictory for musculoskeletal donors where prophylactic antibiotics are prescribed to living donors prior to orthopaedic surgery. Cadaveric donors have often received treatment prior to their death although sepsis is an exclusion factor for donation.

Musculoskeletal tissues are not retrieved from patients where there is an active infection. Living donors are generally well at the time of their hip replacements and sepsis is an exclusion factor for living and cadaveric donors. Therefore, samples received from tissue banks for bioburden assessment may or may not contain microorganisms. The purpose of bacterial and fungal culture is to be able to potentially isolate any organisms present, therefore methods, media and conditions must be able to recover not only commonly encountered bacteria and fungi but also those that are fastidious and slow growing.

Sampling of musculoskeletal allografts for microbial bioburden should be taken at the time of retrieval and prior to any allograft processing (SaBTO 2011). Generally, allograft musculoskeletal tissue samples received in the Australian microbiology laboratory comprise of a piece of tissue and/or a swab of that tissue. The tissue sample size is approximately 1cm³, as recommended by standard practice (Thomson 2007). The swab is rotated globally over the tissue to maximize the surface area sampled. Swabs and/or tissue samples are collected at the time of tissue retrieval. Another tissue sample is collected and kept with the allograft. In most tissue banks, if the culture of the tissue is found to be positive, the second
tissue sample undergoes irradiation with the allograft if this is the standard practice of the bank. The second tissue sample is then re-cultured post-irradiation.

Swab Samples of Allograft Musculoskeletal Tissue

Ideally a swab must be able to collect micro-organisms from a sample and then release sufficient numbers of these micro-organisms onto agar and/or broth media for detection. Living donors are generally clinically well however still receiving pre-surgical prophylactic antibiotics. These antibiotics may produce a bacteriostatic effect on organisms, reducing the chance of organism isolation. Musculoskeletal tissue is not a uniform material and care must be taken to swab over the entire area of the tissue.

Swabs provide a fast and convenient method of sample collection. Evaluations of the best swab to use have been going on for a long time (Ellner & Ellner 1966, Bartlett & Hughes 1969). Early studies found that the type of swab used was extremely important. A low pH of the cotton-wool of a swab was found to have an antibacterial effect on bacteria (Anderson 1965). Bourbeau (2005) describes the properties that are important in selecting a swab transport device. Consideration must be taken of the transport media, the swab stick, the swab fibers, swab tip preparation, swab tip characteristics, liquid or gel based transport media and charcoal additives. Swabs are not all the same and need to be evaluated on the sampling requirements of the sample to be swabbed.

There are numerous publications on the assessment of swab transport devices. In a study by Sautter and Wilson (1988) four different swab systems were evaluated over a 48 hour time period each with a different manufacturer, two containing Amies medium without charcoal and two containing Modified Stuart’s medium. The swabs containing Amie’s medium performed best with the highest colony counts recovered and the lowest reduction of organisms over the 48 hour period. Van Horn et al (2008) evaluated three swab transport systems and found the Eswab (Copan) able to release a greater number of micro-organisms than the others tested. Ronholdt & Bogdansky (2005) concluded that swab cultures should not be solely relied on to determine allograft bioburden in their study using two dry-tipped swab culturing systems.

Nguyen et al (2011) used a sponge swabbing technique in their study to validate the use of sponges to sample structural bone and tendon allografts. Sponge swabs have been used in the meat industry for carcass sampling and are now finding an application in the clinical field (Byrne et al 2004, Doi et al 2011).
Transport time from the time of sampling to culturing in the laboratory also needs to be considered as in Australia many microbiology laboratories are off-site to the tissue bank, in some cases interstate. Delacour et al (2009) found that the Amies swab transport system by Copan maintained the survival of *S. aureus* strains for up to three weeks at room temperature. Rishmawi et al (2007) investigated the survival of a range of aerobic bacteria in three bacterial transport swab systems and emphasised the need to culture swabs as soon as possible with a maximum delay of 24 hours. Stoner et al (2004) found that Group B streptococcus could be isolated from swab transport systems containing Amies medium for up to 4 days when stored at room temperature.

**Biopsy Samples of Allograft Musculoskeletal Tissue**

A biopsy of tissue is an appropriate method for sample collection to maximize the recovery of all potential pathogens, especially anaerobic bacteria, acid-fast bacilli and fungi (Pfyffer 2007, Sutton 2007, Thomson 2007). The recommendation is that a sample of 1 cm³ of tissue is appropriate (Thomson 2007). Histopathological examination of tissue is to differentiate between infection and malignancy. Tissues must be transported in a sterile container. Conventionally, tissues are ground or minced during processing to release micro-organisms and to provide equal samples for all media and smears. Mincing can be performed using a sterile scalpel to cut the sample. Grinding can be performed with a mortar and pestle, a Griffith’s tube tissue grinder or mechanically using a stomacher or masticator. The homogenate is then used to inoculate culture media. Grinding can render fungal hyphae non-viable and a small piece of tissue should be used to inoculate fungal media. Tissues can maintain a reduced atmosphere in spite of transport under aerobic conditions.

Two tissue samples of approximately 1cm³ are collected at the time of retrieval of every femoral head and cadaveric tissue. One sample remains with the packaged bone in a sterile container while the other is transported to the microbiology laboratory for bioburden assessment. Biopsy samples of allograft tissue are a representative sample of the tissue, taken from one side of the tissue, but perhaps not a true representation of the entire tissue.

**Swab versus Biopsy Samples for Bioburden Assessment**

Studies have found that $10^5$ viable organisms per gram of tissue are required for wound infection (Levine 1976). These numbers of organisms can be detected from samples obtained by swabs and bone pieces. Many studies of failed joint replacements could not find an organism by conventional culture methods. Perhaps these numbers are too low for conventional methods and more sensitive methods are required or another non-microbial
factor may be involved. The uniqueness of allograft musculoskeletal tissue provides a challenging approach to bioburden assessment.

There are many studies based on the effectiveness of a swab culture compared to a biopsy sample. Levine et al (1976) found that there was correlation between bacterial counts from open wounds using swab and biopsy samples. A semi-quantitative bioburden assessment is performed on bone surfaces using a swab sampling technique but for biopsy samples cultured only in broth, only qualitative assessment is possible. Some studies have shown that swab culture results correlate to those of tissue biopsy culture results (Levine 1976). A study by Chua et al (2005) found tissue cultures superior to swab cultures in patients with pacemakers and implantable cardioverter defibrillators. In this study, tissue was obtained from the pocket of the implantable cardiac device for microbiological culture. In living bone donors, a bone piece is collected from only one side of the femoral head, leaving the remainder of the femoral head intact.

A swab is easier to collect, sampling a greater area of the musculoskeletal tissue. Swab transport systems are very easy to use and those that contain transport medium prevent the drying out of the sample or overgrowth of micro-organisms. Levine et al (1976) and Bornside & Bornside (1979) compared swab culture with the biopsy culture of wounds. Their studies supported the use of a swab system for sample collections as equal to culture results obtained from biopsy samples. Swabs also require minimal handling in the laboratory compared to biopsy samples which may be ground for inoculation onto agar and/or broth media or fluid suspensions which may require filtering or centrifugation prior to inoculation. The agitation of swabs in sterile saline with subsequent inoculation of an aliquot onto culture media provided improved micro-organism isolation (Collee et al 1974, Yrios et al 1975, Sautter & Wilson 1988).

**Allograft Musculoskeletal Tissue Samples for Bioburden Assessment in Australia**

Seven out of 10 tissue banks in Australia collect a biopsy sample and a swab of the allograft at the time of retrieval for bioburden assessment (personal communication via confidential tissue bank survey). Of these 7 banks, one bank also included a sponge sample of the femoral head collected at retrieval and another bank collected their swab intra-operatively during implant of the allograft. Two tissue banks collected only a swab at retrieval and another collected only a bone chip at retrieval for bioburden assessment.
The majority of tissue banks use swabs containing Amies medium without charcoal with one tissue bank using Amies medium with charcoal as their swab transport system. Gel-based transport systems such as those used by Australian tissue banks have been found to perform favourably in studies performing recovery investigations of aerobic and anaerobic micro-organisms (Perry 1997, Hindiyeh et al 2001, Stoner 2004, Morosini et al 2006). Charcoal has traditionally been used as an additive in agar and broth media for certain micro-organisms such as Bordetella species and acts by reducing the production of hydrogen peroxide and absorbing free fatty acids which are toxic to some micro-organisms (Bourbeau 2005). Charcoal is not routinely used for general non-selective culture or swab transport systems in Australia.

Allograft musculoskeletal tissue biopsy and swab samples are sent to the microbiology laboratory for bioburden assessment. In Australia, tissue banks are storing these samples at either room or fridge temperature although most banks transport them to the laboratory at fridge temperature. Human & Jones (2004) and Stoner et al (2008) concluded in their studies that the swabs held at 4-8°C provided better viability of the micro-organisms tested rather than room temperature storage before and during transport to the laboratory. The transport time and temperature of allograft samples is an important factor to consider for several tissue banks located off-site to the Microbiology laboratory and in two cases interstate transport is required.
Microbiological Examination of Allograft Musculoskeletal Tissue Samples for Bioburden Assessment

Culture-based Methods to Determine Bioburden Assessment of Allograft Musculoskeletal Tissue Samples

Introduction

Traditionally culture methods for samples received in the microbiology laboratory for bacterial and fungal culture have employed a selection of selective and non-selective solid agar and/or broth media to enhance growth and isolation and maximize recovery of bacteria and fungi. Non-selective media are designed to optimise the growth of the majority of organisms and generally contain blood. Selective agars are designed to select pathogens from samples that may contain a mixture of commensal organisms, such as would be found in respiratory or genital sites of collection.

Agar Culture Plates

Culture media is prepared and poured into petri dishes where they solidify and are used for the isolation of bacteria and fungi. Culture media solidifies at room temperature due to the addition of agar to the formulation of the culture medium. Agar is extracted from species of the red algae know as agarophytes (Gelidium, Gracilaria, Pterocladia, Acanthopeltis and Ahnfeltia species). It is extracted, decolourised, filtered, dried and milled to a powder. Agarose and agarpectin are the two major polysaccharides found in agar. Agarose is responsible for the high strength gelling properties of agar and agarpectin provides viscosity (Bridson 2006).

Different culture media are used for the isolation, selection, identification and/or differentiation of micro-organisms, determined by the formula of the medium. Carbohydrates, such as glucose, essential metals and minerals, indicator substances, selective agents and/or other components, such as cysteine, are added to culture media depending on their requirements. Buffering agents are added to culture media and are essential in maintaining the pH at the optimum necessary for growth of the desired micro-organism. The three most commonly used agar plates in the Australian microbiology laboratory for bacterial isolation
are horse blood agar, chocolate agar and MacConkey agar and for fungal isolation Sabouraud agar.

**Blood Agar**

Blood agar is a non-selective general purpose medium widely used for the growth of a broad range of pathogenic or non-pathogenic organisms. The blood in this medium is from a mammalian source, generally horse blood in Australia, typically at a concentration of 5–10%. Blood agar is an enriched, differential media used to isolate fastidious organisms and detect haemolytic activity. β-haemolytic activity will show lysis and complete digestion of red blood cell contents surrounding the colony, for example, as produced by beta-haemolytic streptococci. α-haemolysis will only partially lyse haemoglobin and will appear green, for example, by viridans streptococci. γ-haemolysis (or non-haemolytic) is the term referring to a lack of haemolytic activity.

**Chocolate Agar**

Chocolate agar is a non-selective, enriched growth medium capable of growing a range of bacteria. It is a variant of the blood agar plate. It contains red blood cells, which have been lysed by heating very slowly to 56 °C. Chocolate agar is used for growing fastidious bacteria, such as *Haemophilus influenzae*, which require growth factors such as NAD and hematin, found inside the blood cells and are released by lysis. No chocolate is actually contained in the plate and is named due to its appearance.

**MacConkey Agar**

MacConkey agar is both a selective and differential agar. It is selective because it contains bile salts and a crystal violet dye, which inhibits most Gram-positive bacteria from growing but allows the growth of Gram-negative coliforms. MacConkey agar contains a neutral red dye, lactose and peptone. Bacteria able to utilise the lactose in the media will produce acid, lowering the pH of the agar, and colonies will appear pink. Non-lactose fermenting bacteria are not able to utilise the lactose and will use peptone instead, producing ammonia which raises the pH of the agar, and colonies will appear colourless. This media is able then to easily differentiate lactose fermenters such as *Escherichia coli* and *Klebsiella sp* from non-lactose fermenting *Salmonella sp* and *Pseudomonas sp.*
Sabouraud Agar

Sabouraud agar is used for the growth and isolation of fungi, including yeast. Antibiotics such as chloramphenicol and gentamicin can be added and have the advantage of reducing the growth of commensals from samples normally inhabited by mixed populations of microorganisms.

Streaking of Agar Plates

Agar plates are inoculated with either a swab or a pipette for liquid cultures or biopsy samples can be rolled across the surface. Swabs must first inoculate non-selective then selective agar plates. Swab inoculation onto agar plates must involve rotating the swab onto the agar to ensure maximum removal of organisms. The inoculum is streaked using a sterile metal or plastic loop, streaking the initial inoculums over 4 quadrants of the plate. The purpose of streaking is to dilute the inoculums across the agar so that isolated colonies of bacteria 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). This ‘antibiotic affect’ is often seen in samples collected from hospital in-patients who have been on antibiotic therapy.

Broth Culture Methods to Determine Bioburden Assessment of Allograft Musculoskeletal Tissue Samples

Principle

Broth cultures are a liquid nutritional medium used for the isolation of bacteria and fungi and have been in use for a long time, especially for enhancing the isolation of anaerobic microorganisms (Holman 1918). Broth cultures may be used with or without the parallel inoculation of agar media. There are various reasons to support the use of broth media but 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 agar media are thought to be enhanced by broth media, especially anaerobic organisms. Clinical patients are often treated for infections with antimicrobial agents and living femoral head donors receive prophylactic antibiotic treatment pre-operatively. Furthermore, allografts may be exposed to antibiotics in the wash solution used in the post-retrieval processing stage by some tissue banks. Broth culture of allograft 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. Anaerobic organisms can be isolated from tissue samples of approximately 1cm³ or greater even if transported to the laboratory under aerobic conditions (Thomson 2007). Broth culture is generally recommended for samples such as tissue and blood (Winn et al 2006).

**Cooked Meat Medium**

Cooked meat medium (CMM) is a broth culture medium for the primary growth of aerobic and anaerobic organisms. It is prepared using heart muscle as the 'meat component' and includes peptone, sodium chloride and glucose. CMM is able to promote bacterial growth from very low numbers of organisms. The meat in the broth provides reducing substances and settles to the base of the container providing an oxygen-free environment for the growth of anaerobic organisms while aerobic bacteria prefer to grow at the top of the broth in the container. The supernatant of CMM is clear allowing early detection of turbidity and therefore the growth of bacteria (Bridson 2006). CMM has also been found to be an excellent medium for the long-term storing of organisms (Holman 919; Barr 1980).

**Tryptone Soya Broth**

Tryptone soya broth (TSB) is a nutritious clear medium for the growth of bacteria. It is commonly used for sterility testing and supports the growth of most common aerobic and facultative anaerobic bacteria. It is not recommended as a primary enrichment broth culture for clinical samples. In clinical laboratories, it is recommended for use as a suspension medium for antibiotic susceptibility testing or quality control inoculations. It is not recommended for the isolation of fastidious micro-organisms such as *Haemophilus* species or for strict anaerobic bacteria (BD 2008).

**Thioglycollate Broth Medium**

Thioglycollate broth is a clear broth medium able to support the growth of aerobic and anaerobic organisms. Aerobic organisms will grow in the top layer, anaerobic organisms in the bottom while facultative organisms will growth throughout the medium. (Bridson 2006).
Literature Review of Broth Culture Methods

Just as the value of swab collected samples has been debated, so too has the value of broth cultures for microbial isolation. The recommendation has been to use broth culture after inoculation of primary agar culture media or to place tissue samples directly into broths, depending on the type of sample being processed (Thomson 2007). Broth cultures are believed to reduce the effects of inhibitory substances such as antimicrobial agents and to enhance the recovery of low numbers of organisms. A study by Morris et al (1995) presented data that the majority of isolates from broth cultures only 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.

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. Cooked meat broth was found to be one of the broth media that recovered the greatest variety of organisms at a low inoculum. Cooked meat broth has been shown to be able to maintain anaerobic cultures for extended periods (Claros et al 1995).

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. Derby et al (1997) and Morris et al (1995) concluded that broth cultures provided no clinical value, were expensive and time consuming. Saeman 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. The first protocol inoculated a bone sample directly into brain heart infusion broth medium with subsequent sub-culture after incubation. The second protocol swabbed the bone, replaced the swab in the transport medium and transported the swab to the microbiology laboratory where it was cultured onto agar plates. The third protocol placed the swab into broth media after plating onto the agar media in protocol 2. The positive culture results of each protocol were recorded as 92% for Group 1, 9% for Group 2 and 36% for Group 3. Veen et al concluded that protocols 2 and 3 were inadequate for the bioburden assessment of musculoskeletal allograft tissue and protocol 1 which involved inoculation of the biopsy directly into broth culture only was the better method. A study by Silletti et al (1997) concluded that primary broth cultures are unnecessary if swabs are used correctly to sample wounds.
Fluid Extraction Methods to Determine Bioburden Assessment

In the fluid extraction method, a swab of the tissue, a biopsy sample or the entire femoral head is immersed in a sterile fluid such as saline. The fluid is then subsequently tested, either by filtering and placing the filter onto agar plates or by culture of an aliquot of the fluid onto agar and/or broth media. Alternatively, swab samples of allografts can be vortexed in a sterile solution prior to inoculation of an aliquot onto media.

Collee et al (1974) found that minimal numbers of anaerobic bacteria were recovered from swabs inoculated with a simulated exudate containing thousands of bacteria when plated directly onto solid agar media. Their research found that the organisms were retained within the swab and agitation into 1mL of sterile broth which was then cultured recovered a considerable greater number of bacteria. Yrios et al (1975) was able to recover 78% of bacteria inoculated onto swabs when vortexed for 20 seconds in 5mL of broth. Sautter & Wilson (1988) also found an improved recovery of Streptococci species if swabs were first rinsed in 1mL of sterile saline and then a saline aliquot cultured compared to direct culture onto agar plates, eliminating the need for a broth culture.

Dennis et al (2009) compared two different methods of culturing musculoskeletal allograft tissues using swabs and a saline wash solution used in the processing of the allograft. The swabs were placed into thioglycollate broth medium and incubated for up to 14 days unless growth was evident earlier and the broth sub-cultured onto agar plates. The saline wash solution required two 20mL aliquots to be each inoculated into two bottles of thioglycollate broth media and also incubated for up to 14 days, with sub-culture on evidence of growth. The saline wash method isolated 18/20 organisms while the swab culture method detected 4/20 organisms.

Literature Review of Media Used in the Bioburden Assessment Methods of Allograft Musculoskeletal Tissue Samples

There is a broad range of swab types, agar media, broth media and incubation periods used by different laboratories to detect bacteria and fungi in musculoskeletal tissue allografts. Table 3 provides a literature review of musculoskeletal tissue samples collected and of methods used to detect bioburden and illustrates the differences found. 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 a broth medium, the most common being thioglycollate broth and brain heart infusion broth, although many studies did not specify the type of broth used, while other studies used a combination of broth media. Blood and chocolate agar plates were the most common agar plates used and incubation periods ranged from a 48-hour period to a maximum of 12 days.
<table>
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<th>Maximum Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saies et al</td>
<td>1990</td>
<td>Australia</td>
<td>Yes - NS</td>
<td>Bone Biopsy</td>
<td>Stuarts Medium</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tomford et al</td>
<td>1990</td>
<td>USA</td>
<td>Yes - NS</td>
<td>No</td>
<td>Thio</td>
<td>Brucella; MAC</td>
<td>NS</td>
</tr>
<tr>
<td>Ivory &amp; Thomas</td>
<td>1993</td>
<td>England</td>
<td>Yes - NS</td>
<td>No</td>
<td>Yes – NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Barrios et al</td>
<td>1994</td>
<td>Spain</td>
<td>ATM</td>
<td>Bone biopsy</td>
<td>Thio</td>
<td>Blood</td>
<td>48 hrs</td>
</tr>
<tr>
<td>Campbell &amp; Oakeshott</td>
<td>1995</td>
<td>Australia</td>
<td>STM</td>
<td>No</td>
<td>Yes – NS</td>
<td>Blood</td>
<td>7 days</td>
</tr>
<tr>
<td>Ivory &amp; Thomas</td>
<td>1993</td>
<td>England</td>
<td>Yes - NS</td>
<td>No</td>
<td>Yes – NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Deijkers et al</td>
<td>1997</td>
<td>Netherlands</td>
<td>TM</td>
<td>No</td>
<td>BHI</td>
<td>Blood; Choc</td>
<td>7 days</td>
</tr>
<tr>
<td>Sutherland et al</td>
<td>1997</td>
<td>Scotland</td>
<td>ATMC</td>
<td>Bone biopsy</td>
<td>Swab – RMB</td>
<td>Blood; MAC</td>
<td>NS</td>
</tr>
<tr>
<td>Aho et al</td>
<td>1998</td>
<td>Finland</td>
<td>Yes - NS</td>
<td>Bone biopsy</td>
<td>BHI</td>
<td>Chocolate; FAA</td>
<td>7 days</td>
</tr>
<tr>
<td>Bettin et al</td>
<td>1998</td>
<td>Germany</td>
<td>No</td>
<td>Tissue pieces</td>
<td>dextrose- and Schaedler-broth and Kimmig-(yeasts)</td>
<td>Blood; Choc; Endo agar</td>
<td>10 days</td>
</tr>
<tr>
<td>Farrington et al</td>
<td>1998</td>
<td>England</td>
<td>No</td>
<td>Bone biopsy</td>
<td>BHI</td>
<td>Blood; Sabouraud</td>
<td>3 days</td>
</tr>
<tr>
<td>Journeaux et al</td>
<td>1999</td>
<td>Australia</td>
<td>Yes - NS</td>
<td>Yes</td>
<td>Thio</td>
<td>Blood; Choc</td>
<td>7 days</td>
</tr>
<tr>
<td>Vehmeyer et al</td>
<td>1999</td>
<td>Netherlands</td>
<td>TM</td>
<td>No</td>
<td>BHI</td>
<td>Blood; Choc</td>
<td>48 hrs</td>
</tr>
<tr>
<td>Sommerville et al</td>
<td>2000</td>
<td>Australia</td>
<td>Yes - NS</td>
<td>Yes</td>
<td>Brewers Liquid culture medium</td>
<td>Blood; Choc</td>
<td>7 days</td>
</tr>
<tr>
<td>Segur et al</td>
<td>2000</td>
<td>Spain</td>
<td>Yes - NS</td>
<td>No</td>
<td>Thio</td>
<td>Blood; EMB</td>
<td>5 days</td>
</tr>
<tr>
<td>Liu et al</td>
<td>2002</td>
<td>Taiwan</td>
<td>Yes - NS</td>
<td>Bone biopsy</td>
<td>Thio</td>
<td>Blood; EMB</td>
<td>7 days</td>
</tr>
<tr>
<td>James &amp; Gower</td>
<td>2002</td>
<td>Australia</td>
<td>Yes - NS</td>
<td>Bone biopsy</td>
<td>Yes – NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Vehmeyer et al</td>
<td>2002</td>
<td>Netherlands</td>
<td>TM</td>
<td>No</td>
<td>BHI</td>
<td>Blood; Choc</td>
<td>7 days</td>
</tr>
<tr>
<td>Chiu et al</td>
<td>2004</td>
<td>Hong Kong</td>
<td>No</td>
<td>Bone biopsy</td>
<td>No</td>
<td>Blood; MAC; Neo</td>
<td>NS</td>
</tr>
<tr>
<td>Ibrahim et al</td>
<td>2004</td>
<td>England</td>
<td>Yes - NS</td>
<td>No</td>
<td>Yes – NS</td>
<td>Yes – NS</td>
<td>3 days</td>
</tr>
<tr>
<td>James et al</td>
<td>2004</td>
<td>England</td>
<td>Yes - NS</td>
<td>Bone biopsy</td>
<td>Yes – NS</td>
<td>Yes – NS</td>
<td>4 days</td>
</tr>
<tr>
<td>Hou et al</td>
<td>2005</td>
<td>Taiwan</td>
<td>Yes - NS</td>
<td>No</td>
<td>Semi-solid broth tube</td>
<td>Blood</td>
<td>7 days</td>
</tr>
<tr>
<td>Ireland &amp; Spelman</td>
<td>2005</td>
<td>Australia</td>
<td>Yes - NS</td>
<td>No</td>
<td>Thio</td>
<td>Blood</td>
<td>9 days</td>
</tr>
<tr>
<td>Winter et al</td>
<td>2005</td>
<td>Australia</td>
<td>Transtube swab</td>
<td>Bone biopsy</td>
<td>Yes – NS</td>
<td>Blood</td>
<td>9 days</td>
</tr>
<tr>
<td>Author</td>
<td>Year</td>
<td>Country of Study</td>
<td>Swab Sample</td>
<td>Tissue Sample</td>
<td>Broth Culture</td>
<td>Agar Plates</td>
<td>Maximum Incubation period</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------</td>
<td>------------------</td>
<td>-------------</td>
<td>---------------</td>
<td>-----------------------</td>
<td>----------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>van de Pol et al</td>
<td>2007</td>
<td>Netherlands</td>
<td>No</td>
<td>Bone biopsy</td>
<td>Thio Liquid Sabouraud medium</td>
<td>Blood; FAA;</td>
<td>12 days</td>
</tr>
<tr>
<td>Guelich et al</td>
<td>2007</td>
<td>USA</td>
<td>Yes - NS</td>
<td>No</td>
<td>Thio</td>
<td>Blood; Choc; MAC; Brucella; KVL</td>
<td>7 days</td>
</tr>
<tr>
<td>Varettas &amp; Taylor</td>
<td>2011</td>
<td>Australia</td>
<td>ATM</td>
<td>Bone biopsy</td>
<td>CMM</td>
<td>Blood; Choc</td>
<td>5 days</td>
</tr>
</tbody>
</table>

**NOTE:**

1. 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.

**KEY:**

ATM: Amies Transport Medium; ATMC: Amies Transport Medium with Charcoal; BHI: Brain heart infusion broth, Choc: Chocolate agar; CMM: Cooked meat medium (broth); EMB: Eosin-methylene blue; FAA: Fastidious anaerobe agar; FAB: Fastidious anaerobe broth; KVL: kanamycin-vancomycin laked agar; MAC: MacConkey agar; NEO: Neomycin blood agar; NS: Not specified; RMB: Robertson’s Meat Broth; STM: Stuarts Transport Medium; THIO: Thioglycollate broth; TM: 15cm polyester tipped applicator into transport medium; TSB: Tryptone Soya broth
Bioburden Assessment Methods of Allograft Musculoskeletal Tissue Samples in Australia

The number of microbiology laboratories providing bioburden assessment services to tissue banks is relatively few with laboratories performing testing to more than one tissue bank. For example, in Australia, 6 tissue banks send their samples to just two microbiology laboratories in Sydney for bioburden assessment. Tissue banks are limited in their choice of a microbiology laboratory based on whether the laboratory holds a TGA-licence. This has required two tissue banks to send their samples interstate for bioburden assessment.

Table 4 summarises the methods and media used by five Australian clinical microbiology laboratories to determine the bioburden assessment of allograft musculoskeletal tissue samples. The information in this table was obtained via a confidential survey of microbiology laboratories involved in the bioburden assessment of allograft musculoskeletal samples. Although only five laboratories are represented in this table, the differences in samples received and bioburden assessment methods regarding broth media, agar media and incubation periods between each laboratory is apparent. All of these laboratories are clinical laboratories and are receiving other non-donor related clinical samples from patients. At four of these laboratories, the sample inoculation and culture interpretation processes of tissue bank samples are integrated within the workflow of the clinical samples; tissue bank samples are not inoculated in separate areas with separate staff and equipment. Only one laboratory represented in Table 4 has a dedicated laboratory area and staff for allograft sample testing.

Although there is a wide range of sampling methods and agar and broth media used in Australian laboratories, all bioburden assessment methods have been validated as required by the GMP (TGA 2000). Bioburden assessment method validation is performed to determine that the method is able to detect micro-organisms in the presence of musculoskeletal allograft samples. In Australia, the TGA recommends validation studies follow the guidelines of the British Pharmacopoeia (2009), the TGA Guidelines for Sterility Testing of Therapeutic Goods (2006) and other relevant standards such as ISO 11737-1:2006. Validation protocols must mimic the bioburden assessment method in use with a micro-organism inoculum size of <100 colony forming units (CFU), using reference strains of, at least, the following micro-organisms: *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Clostridium sporogenes*, *Candida albicans* and *Aspergillus niger* and the inclusion of negative controls. These organisms are used to challenge the ability of the media to support their growth and the ability of the method to recover fungi and aerobic and anaerobic micro-
organisms. The validation process must be documented with expected outcomes determined. A final report will summarise the results of the validation and conclude on the suitability of the bioburden assessment method or if corrective action is required.
Table 4: A Summary of the Bioburden Assessment Methods and Media used for Allograft Musculoskeletal Tissue Samples by Five Clinical Microbiology Laboratories

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Allograft Musculoskeletal Sample Tested</th>
<th>Direct Inoculation Of Agar Media</th>
<th>Broth Media (O₂)</th>
<th>Broth Subculture Agar Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Swab – collected at retrieval</td>
<td>Nil</td>
<td>Thio 35°C 48hrs</td>
<td>HB &amp; HBAN 35°C 7 days</td>
</tr>
<tr>
<td></td>
<td>Living donor Pre-processing swabs – duplicate swabs</td>
<td>Nil</td>
<td>1ˢᵗ swab: TSB 25°C 7 days 2ⁿᵈ swab: Thio 35°C 7 days</td>
<td>1ˢᵗ swab: TSA 25°C 7 days &amp; HBA 35°C 7 days 2ⁿᵈ swab: RCA ANO₂ 35°C 7 days; HBAN 35°C 7 days; (If Thio is turbid add HBO₂ 35°C 7 days)</td>
</tr>
<tr>
<td></td>
<td>Living donor Post-processing swabs – duplicate swabs</td>
<td>Nil</td>
<td>1ˢᵗ swab: TSB 25°C 7 days 2ⁿᵈ swab: Thio 35°C 7 days</td>
<td>1ˢᵗ swab: TSA O₂ 25°C 7 days &amp; HB O₂ 35°C 7 days 2ⁿᵈ swab: RCA ANO₂ 35°C 7 days; HBAN 35°C 7 days; (If Thio is turbid add HBO₂ 35°C 7 days)</td>
</tr>
<tr>
<td></td>
<td>Cadaveric retrieval swabs</td>
<td>Nil</td>
<td>1ˢᵗ swab: TSB 25°C 7 days 2ⁿᵈ swab: Thio 35°C 7 days</td>
<td>1ˢᵗ swab: TSA 25°C 7 days &amp; HBO₂ 35°C 7 days 2ⁿᵈ swab: RCA ANO₂ 35°C 7 days; HBAN 35°C 7 days; (If Thio is turbid add HBO₂ 35°C 7 days)</td>
</tr>
<tr>
<td></td>
<td>Milled Bone sample</td>
<td>Nil</td>
<td>TSB 25°C 7 days</td>
<td>TSA 25°C 7 days &amp; HBO₂ 35°C 7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thio 35°C 7 days</td>
<td>RCA ANO₂ 35°C 7 days; HBAN 35°C 7 days; (If Thio is turbid add HBO₂ 35°C 7 days)</td>
</tr>
<tr>
<td>Laboratory</td>
<td>Allograft Musculoskeletal Sample Tested</td>
<td>Direct Inoculation Of Agar Media</td>
<td>Broth Media ((O_2))</td>
<td>Broth Subculture Agar Media</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------</td>
<td>-------------------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>Swab</td>
<td>HB (O_2) 35°C 2 days</td>
<td>Nil</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HBAN 35°C 2 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SABCG (O_2) 28°C 4 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>SABCG (O_2) 28°C 4 weeks</td>
<td>Brew 35°C 7 days</td>
<td>If turbid: Choc (CO_2) 35°C; HBAN 35°C</td>
<td>If not turbid, reported as negative</td>
</tr>
<tr>
<td>Sponge</td>
<td>Nil</td>
<td>Brew 35°C 14 days</td>
<td>If turbid: Choc (CO_2) 35°C; HBAN 35°C</td>
<td>If not turbid, reported as negative</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>Swab</td>
<td>Choc (CO_2) 35°C 5 days;</td>
<td>CMM 35°C 48hrs</td>
<td>Choc (CO_2) 35°C 72hrs;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HBAN 35°C 5 days;</td>
<td></td>
<td>HBAN 35°C 72hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAB (O_2) 30°C 4 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone chip</td>
<td>Nil</td>
<td>CMM 35°C 48hrs</td>
<td>Choc (CO_2) 35°C 72hrs;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HBAN 35°C 72hrs</td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>Swab &amp; bone chip</td>
<td>Nil</td>
<td>Sample vortexed in TSB then TSB aliquot inoculated into (O_2) &amp; (ANO_2) blood culture bottles, incubated in a continuous-monitoring instrument for 5-days</td>
<td>Bottles that signal positive are unloaded, sub-cultured and isolates are identified.</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>Swab &amp; bone chip</td>
<td>After agitation in TSB, HB 2-days</td>
<td>Nil</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Key:** \(ANO_2\): Incubated in anaerobic atmosphere; **BREW**: Brewers broth (Thioglycollate); **Choc**: Chocolate agar; **CMM**: Cooked meat medium (broth); **HB**: Horse blood agar; **HBAN**: Horse blood agar, incubated anaerobically; **N/A**: Not applicable; \(O_2\): Incubated in aerobic atmosphere; **RCA**: Reinforced clostridial agar; **SAB**: Sabouraud agar; slope; **SABCG**: Sabouraud agar slope with Chloramphenicol & Gentamicin; **sec**: seconds; **Thio**: Thioglycollate broth; **TSA**: Tryptone soy agar; **TSB**: Tryptone soy broth
Culture-based Identification Methods for Micro-Organisms
Commonly Isolated from Allograft Musculoskeletal Tissue

Preliminary Identification Characteristics of Bacteria

Gram stain

The Gram stain differentiates bacteria into two classes, the Gram-positive and the Gram-negative, based on the differences in the chemical structure of their cell wall (Pelczar et al 1977). The Gram stain forms the basis for the initial identification of bacteria and is the most widely used differential staining technique in the clinical microbiology laboratory. A bacterial smear is prepared and heat-fixed on a glass slide which is then subject to 4 stains in the following order: crystal violet, iodine solution, alcohol (or other decolourising agent) and carbol fuchsin (or other counterstain). Under microscopic examination, bacteria that retain the crystal violet / iodine complex and resist decolourising will appear purple and are termed Gram-positive. Bacteria that are decolourised and lose the crystal violet / iodine complex appear pink and are Gram-negative. Fungi are not classified by their Gram-stain reaction however yeasts will appear purple on Gram-stained smears making them more visible in smears of clinical samples. Other fungi and hyphal elements may appear purple, pink or ‘speckled’.

Cultural Appearances

Colonies will appear on solid agar surfaces after incubation and their appearance is important in their identification. Different types of bacteria produce large mucoid colonies, such as Klebsiella species, whereas others such as the Streptococci species are much smaller. Characteristics such as haemolysis, pigment production, shape, texture and colony margin are apparent on some agar plates and all contribute to the identification of the organism. Different types of media contain different nutrients and this can affect the cultural appearance of some micro-organisms. Incubation temperature can affect colony morphology; some bacteria will be quite small at room temperature but much larger at body temperature. The incubation atmosphere can also have the same affect.
Atmospheric Requirements for Growth

Organisms can be divided into three general groups based on their oxygen requirements. Strict aerobes will only grow in the presence of oxygen, strict anaerobes will only grow in the absence of oxygen and facultative organisms will grow under both aerobic and anaerobic conditions. Fungi are slower growing than bacterial and fungal cultures should have extended incubation periods of up to 4 weeks. In a clinical laboratory, fungi cultures are incubated optimally at 25°C-30°C (Sutton 2007). Most yeast grow well on the common mycological and bacteriological media producing white colonies generally within 48 to 72 hours at 35°C-37°C.

Indole Test

The indole test detects the ability of an organism to split indole from the tryptophan molecule. The indole can be detected by a reagent which involves a chemical combination producing a distinct colour. The presence or absence of indole formation is an easy test used for bacterial identification. For example, *Escherichia coli* is indole positive whereas *Klebsiella* species is indole negative and *Proteus mirabilis* is indole negative whereas other *Proteus* species are indole positive (MacFaddin 1979).

Oxidase Test

The oxidase test is based on the production of an oxidase enzyme, present as part of the cytochrome system in aerobic, micro-aerophilic and facultative anaerobic organisms. Cytochrome oxidase participates in the electron transport chain by transferring electrons (hydrogen) to oxygen with the formation of water. The oxidase reagent contains a compound that changes colour when oxidised. This test is helpful in differentiating the *Enterobacteriaceae*, which are negative, from the positive genera such as *Aeromonas, Pseudomonas, Neisseria, Campylobacter* and *Pasteurella* which are all oxidase positive (MacFaddin 1979; Winn et al 2006).

Catalase Activity

The catalase test detects the presence of the enzyme catalase demonstrated by the breakdown of hydrogen peroxide. Hydrogen peroxide is produced as one of the oxidative end products of aerobic carbohydrate metabolism. Catalase converts hydrogen peroxidase...
into oxygen and water, ridding the cell of this lethal by-product. This test is used primarily to differentiate between catalase-negative *Streptococcus* species and catalase-positive *Staphylococcus* species (MacFaddin 1979; Winn et al 2006).

**Bacterial Growth Quantification**

A bacterial cell will grow by increasing in size and doubling in mass, until it divides into two identical cells. Viable cell count methods are used to determine the number of organisms present. The two most common methods are the pour plate method and the spread plate method. The pour plate method involves dilutions of organisms mixed into tubes of molten agar medium. The tubes are then poured into petri dishes, incubated, and the number of colonies within the media are counted. The spread plate method is an easier method with a known quantity of an organism dilution spread over the surface of a solid agar plate. Colonies are counted following incubation. These viable count methods have some limitations. These methods are really measuring colony-forming units (CFU) and not individual cells as it takes many generations of a multiplying cell to produce a colony visible to the unaided eye. A cell may divide many times and not be visible using these count methods. Also, the increase in mass of a cell is an indication of growth and this is not evident using a viable count method.
Micro-Organisms Commonly Isolated from Allograft
Musculoskeletal Tissue

\textit{Staphylococcus} species

\textit{Staphylococcal} species will easily grow on standard bacteriological media such as blood agar plates. Colonies appear as white–cream colonies and will appear as clusters of Gram-positive cocci in a Gram stain. A latex test can be performed to determine if the isolate is a \textit{Staphylococcus aureus} or a non-\textit{S. aureus}. The latex test is based on the agglutination of \textit{Staphylococcus} species that produce clumping factor and/or protein A with sensitized latex particles. Non-\textit{S. aureus} isolates are generally grouped as coagulase-negative staphylococci and generally do not require further identification when isolated from donor musculoskeletal tissue for transplant. If the latex test is unable to determine if the isolate is a \textit{S. aureus} or a coagulase-negative staphylococci, a tube coagulase test can be performed. The tube coagulase test is based on the production of extracellular coagulase which activates prothrombin and subsequent clot formation of plasma. The coagulase test detects coagulase, an enzyme produced by \textit{Staphylococcus aureus}, a protein which has prothrombin-like activity which can convert fibrinogen into fibrin. Coagulase is present in two forms, free and bound, and is detected by separate testing procedures. The slide coagulase test detects bound coagulase, also known as clumping factor, visible to the naked eye as aggregates of fibrin strands when the bacterial cells are suspended in plasma (fibrinogen). The tube coagulase test detects free coagulase by the formation of a clot in a tube suspension of the bacteria and plasma (Winn et al 2008). Microbiology laboratories are increasingly using molecular methods for the identification of indeterminate \textit{Staphylococcus} species for the presence of the fem, nuc and mecA genes.

\textbf{Gram-negative Bacilli}

Members of the Enterobacteriaceae are one of the most frequently encountered bacterial isolates from human clinical samples, such as \textit{Escherichia coli}, \textit{Klebsiella} species, \textit{Proteus} species and \textit{Enterobacter} species (Winn et al 2006). Members of this group comprise the Gram-negative bacilli which do not form spores, grow well on MacConkey agar, ferment glucose and other sugars, are catalase-positive and are oxidase-negative. These organisms will initially be identified by their morphological appearance on agar plates such as MacConkey agar, which may characterise them on their ability to ferment lactose, or blood
agar which may show evidence of swarming. Other biochemical tests will differentiate these organisms such as their ability to ferment sugars.

Non-fermentative Gram-negative bacilli are also commonly isolated from human clinical samples and include *Pseudomonas* species, *Acinetobacter baumanii* and *Stenotrophomonas maltophilia*. Members of this genus are aerobic, non-spore forming, non-lactose fermenters and are can be identified on morphological characteristics, such as pigment production, and biochemical tests.

**Streptococcus species**

*Streptococcus* species are Gram-positive cocci and are generally classified on the basis of haemolysis produced on blood agar plates. Beta-haemolytic streptococci are identified on the basis of their Lancefield group, determined by the use of a latex agglutinating kit. The isolation of Group A streptococci, also known as *S. pyogenes*, will generally determine the tissue unsuitable for transplant (AATB 2007). Alpha and non-haemolytic streptococci can be differentiated by biochemical and enzymatic testing.

**Propionibacteria**

*Propionibacterium* species are anaerobic Gram-positive, non-spore forming bacilli. *P. acnes* is the most common species encountered in clinical samples as it is found as part of the normal flora of the skin, nasopharynx, oral cavity, gastrointestinal and genitourinary tracts. Other human isolates include *P. avidum*, *P. granulosum* and *P. propionicum*. These organisms typically indicate contamination with normal flora but can be clinically significant, especially in cases where a foreign body, trauma, surgery or immuno-suppression is present. *Propionibacterium* species has been implicated in cases of late post-surgical infection after transplantation such as heart valves and bone. Identification to the genus level can be made on Gram-stain by the typical Chinese-letters appearance and aero-tolerance with further biochemical or molecular testing for species identification.

**Corynebacteria**

The genus *Corynebacterium* is composed of club-shaped Gram-positive, catalase-positive, non-motile, non-spore forming and facultative bacilli. Like the *Propionibacteria*, the *Corynebacteria* colonise many parts of the body including the skin, upper respiratory tract and mucosal membranes, confusing microbiologists and clinicians on their clinical
significance when isolated. These bacteria have been implicated in infection from samples collected from normally sterile body sites and from multiple blood culture bottle collections. The Gram-stain appearance of Chinese-letters and aero-tolerance of the *Corynebacteria* are similar identification criteria to the *Propionibacteria*, with further testing required for speciation.

**Anaerobic bacteria**

Anaerobic bacteria prefer growth in the absence of air although some are aerotolerant. Anaerobic bacteria comprise the Gram-positive anaerobes such as *Clostridium* species and the Gram-negative anaerobes such as the *Bacteroides* species. *Clostridium* species are spore-formers and are one of the few organisms which, if isolated, will lead to the automatic exclusion of the musculoskeletal tissue as unsuitable for transplant (AATB 2007). This is as a result of the death of a patient who received a transplant contaminated with *Clostridium sordelli* (CDC 2002).

**Fungi**

Fungi are ubiquitous, eukaryotic micro-organisms. The two basic forms of fungi are yeast and moulds. Systemic human infection is generally caused by direct inhalation into the lungs or by entry via a wound or trauma site. Yeast are commensal organisms of the gastrointestinal tract and skin and cause infection when conditions allow them to proliferate and spread to other parts of the body via the bloodstream (Garber 2001).

Most of the fungal species causing clinical infection are found on superficial skin and subcutaneous tissues. Systemic fungal infections can be caused by spore inhalation or by trauma leading to inoculation of contaminated soil or plant material. Fungi can be opportunistic infections in patients immunocompromised by their condition and/or their treatment, such as patients with severe burns and leukaemias.

Yeasts are unicellular fungi and are among the commonest fungal infections in the clinical setting, their incidence greatly increased with the use of antibiotics, corticosteroids and anti-tumour agents. Dissemination of yeast through the body to cause systemic infections are facilitated in patients with indwelling catheters, in organ transplant recipients and in patients with prosthetic devices. Candida infections have been associated with the implant of medical devices such as central venous catheters, peritoneal dialysis catheters, heart valves and pacemakers.
Symptoms of systemic fungal infections can be clinically similar to bacterial and viral infections (Garber 2001, Stevens 2002). The first indication may be as a result of the direct microscopic examination of clinical samples where yeast or hyphae may be evident.

Fungal pathogens as a cause of bone and joint infections have been reported since 1886 (Keating 1932). Keating reviewed 25 cases of bone and joint infections caused by fungi, however none of which were as a result of musculoskeletal allograft transplant.

**Allograft Musculoskeletal Tissue Rejection Criteria Based on Organism Recovery**

There has been a wide range of micro-organisms isolated from allograft musculoskeletal tissue samples and these have been grouped by Deijkers et al (1997) into organisms of low pathogenicity or high pathogenicity. Vehmeyer et al (2002) re-grouped these organisms as skin commensals or non-skin commensals to identify the contamination source. The low pathogenicity/skin commensals group includes coagulase-negative staphylococci, *Corynebacterium* species, *Propionibacterium acnes* and *Micrococcus* species. The high pathogenicity/non-skin commensals group include *S. aureus*, *Clostridium* species, *Bacteroides* species, beta-haemolytic streptococci, *Pseudomonas* species and members of the Enterobacteriaceae.

Tissue banks are required to document acceptance and rejection criteria of musculoskeletal allografts, including a micro-organism list (TGA 2000). Based on the documented list, the micro-organism recovered from an allograft sample will determine if the allograft is rejected for transplant or, if part of the tissue bank process, will be irradiated and re-tested. In Australia, tissue banks that do not irradiate culture-positive allografts will discard the tissue regardless of the organism isolated. Tissue banks that use irradiation as part of their processes will irradiate allografts from which skin commensals are isolated and, if culture-negative on re-testing after irradiation, will store the allograft for transplant. These tissue banks will discard allografts that are culture-positive with non-skin commensals. Currently, the ATBF does not have micro-organism acceptance or rejection criteria. The AATB Guidance Document (2007) recommend the discard of allografts from which *Clostridium* species or Group A streptococci are isolated, including the discard of other individual tissue from the same donor.
Limitations of Culture-based Methods of Allograft Musculoskeletal Tissue Samples

The debate of swab versus tissue sample culture has been evaluated in many studies (Bornside & Bornside 1979, Davies et al 2006, Gardner et al 2006, Bonham 2009). The use of swab cultures to determine microbial bioburden has been discredited by some studies (Ronholdt & Bogdansky 2005) while others have found them favourable (Levine et al 1976). Allograft musculoskeletal tissue cultures received from tissue banks try and optimise microbial detection by consisting of a swab sample and/or a tissue sample from the donor tissue. The swab is rolled over the majority of the surface of the tissue. The tissue sample is taken using a bone cruncher and a tissue fragment is sent to the laboratory as a representative sample of the donor tissue. Whereas soft tissue can be ground using a tissue grinder, bone is unable to be ground in manual grinders such as the Griffith’s tube or mortar and pestle.

Inoculation of a swab onto agar plates provides a semi-quantitative assessment of the microbial bioburden of the musculoskeletal tissue. Tissue fragments may be rolled onto agar plates before being placed into a broth medium for a defined period at 35-37°C before sub-culturing onto non-selective agar plates, providing a qualitative microbial culture result only. The growth of microorganisms on culture is considered reflective of the organisms present in a sample. False-negatives can occur if the numbers of organisms are below cultivable levels, are fastidious or affected by antimicrobial therapy as well as other issues such as transport time to the laboratory.

In allograft musculoskeletal tissue, the high incidence of isolating coagulase-negative staphylococci creates a challenge in determining its significance. Dietz et al (1991) found no difference in the types of organisms isolated from patients undergoing operative procedures who had received pre-operative antibiotics and those that had not. This study recovered positive cultures from 58% of patients who had undergone elective procedures in an operating theatre environment. Only one patient showed clinical signs of post-operative infection, however the organism isolated was different to the one isolated from samples taken during the procedure. Twenty-five percent of broth cultures were positive with coagulase-negative staphylococci as the predominant isolate. This study felt that broth cultures provided misleading information as they resulted in a high isolation of clinically unimportant isolates however could be of use for the isolation of low numbers or antibiotic affected ‘true’ pathogens such as S. aureus and Gram-negative bacilli.
A broth culture is routinely included in the microbial bioburden assessment of samples by many laboratories to facilitate the isolation of low numbers of organisms and neutralise the potential antibiotic carryover from patients receiving treatment.
Non-Culture Methods to Determine Bioburden Assessment of Allograft Musculoskeletal Tissue

Introduction

Traditionally the growth and isolation of bacteria and fungi on culture plates was the first step in the detection and identification of micro-organisms. This is no longer a requirement with the advent of nucleic acid techniques. Conventional culture-based isolation and identification methods are based on the phenotypic characteristics of organisms which may be influenced by nutritional or environmental conditions. There may also be a wide variation in a phenotypic characteristic within the one group of organisms. This may lead to a misinterpretation of results and subsequent misidentification of the organism. Nucleic-acid based methods make use of the more stable genotypic characteristics of the micro-organism. Nucleic acid techniques are becoming more and more useful to decrease laboratory turnaround times so that results can be available at an earlier time and to increase sensitivity of organism detection. Nucleic acid techniques may be used on colonies isolated after overnight incubation or directly from appropriate samples. The real advantage of nucleic acid testing (NAT) can only be obtained if the time-consuming step of obtaining colonies on agar plates can be avoided.

Nucleic Acid Testing in Microbiology

Introduction to DNA and RNA

Deoxyribonucleic acid (DNA) is composed of two strands, each strand composed of repeating nucleotide monophosphates. Nucleotides consist of a sugar (deoxyribose), a phosphate and one of four bases - adenine, guanine, thymine and cytosine. Nucleotide bases extend internally from the sugars into the double-stranded DNA molecules.

RNA is a single-stranded polymer consisting of alternate sugar (ribose) and phosphate molecules. Nucleotide bases are covalently bonded to the sugar however in RNA the nucleotide base thymine is replaced by the base uracil. DNA polymerase is the molecule whose principal function is to synthesise new strands of DNA in a 5’ - 3’ direction from a single-stranded template.
Polymerase Chain Reaction

The polymerase chain reaction (PCR) was first described by Kary Mullis in 1983 and its development has led it to become an important technique in the clinical microbiology laboratory (McPherson & Moller 2000). PCR is the most widely accepted and the most commonly used non-culture method and nucleic acid amplification technique used in clinical microbiology laboratories. PCR amplification is based on the repetitive nature of the natural DNA replication process which results in multiple copies of the target DNA.

The PCR process can be divided into three repetitive stages, the first, denaturation, requiring the separation of double-stranded DNA at temperatures above 90°C. The second step involves the annealing of oligonucleotide primers at about 50–60°C and finally followed by primer extension at 70 - 78°C where DNA synthesis occurs.

Initially the sample is combined with a master mix mixture consisting of oligonucleotide DNA primers, the four nucleotide triphosphates, thermostable DNA polymerase, magnesium chloride and water or buffers. DNA denaturation is achieved by increasing the temperature of the sample and mastermix mixture to 95°C causing the breakage of the hydrogen bonds linking the bases. The bonds between deoxyribose and phosphates are stronger covalent bonds and do not break by heating.

The thermal denaturation of DNA is reversible by cooling and is the next step of the PCR process known as primer annealing. The reaction mixture is cooled to between 40°C and 65°C allowing the oligonucleotide primers to hybridise with the single strands of the template DNA molecule. The annealing process aims to replicate a specific target sequence of approximately 100–3500 base pairs using primers that define the ends of that target sequence. Base pairs consist of two nucleotides on opposite complementary DNA or RNA strands connected by hydrogen bonds. Adenine (A) forms a base pair with thymine (T); Guanine (G) with cytosine (C) in DNA. In RNA, thymine is replaced by uracil (U). Primers are short synthetic sequences of single-stranded DNA typically consisting of 20 – 30 bases.

The next step, primer extension, requires the temperature to be raised to approximately 72°C and a thermostable polymerase (e.g. Taq DNA Polymerase) creates new double-stranded DNA identical to the original target DNA. The binding and joining of complementary nucleotides that are free in solution (dNTPs) occurs in this step.
This process or cycle is performed usually about 30–40 times and can exponentially generate up to a billion copies in a matter of hours. These processes can be performed in automated programmable thermal cycler instruments. Early researches manually transferred samples from water baths of different temperatures to allow the PCR process to take place. The use of automated closed systems for amplification and extraction have reduced hands on time, reduced contamination and increased the speed of the assay.

Real-time PCR (RT-PCR) improved the traditional PCR technique by allowing qualitative and quantitative measurements. RT-PCR allows the amplification and fluorescent detection steps to be performed by a single instrument in a single vessel with data recorded during the process. A RT-PCR instrument measures the accumulation of PCR products during amplification with fluorescent dyes. All RT-PCR systems detect a fluorescent dye which is correlated to the amount of PCR product in the reaction. Real-time PCR allows quantification of nucleic acid targets as the fluorescent signal is plotted against the number of assay cycles. A fluorescent signal is detected earlier where there is a high amount of target nucleic acid present in the sample. RT-PCR allowed PCR to enter the clinical microbiology laboratory where it has been embraced. The incorporation of automated amplification instruments has enabled a significant reduction time for assays. The incorporation of non-specific fluorescent dye into RT-PCR assays allowed monitoring of reactions from the beginning of the assay to the end.

Multiplex PCR contains two or more primer sets in the same reaction mix allowing the detection of more than one micro-organism or more than one nucleic acid targets within one micro-organism. Nested PCR uses one set of primers initially then is followed by subsequent amplification of the products produced with a second set of primers.

PCR can be an organism specific molecular assay or can be a broad-range assay based on ribosomal genes (rDNA). All bacterial species contain rDNA consisting of highly conserved nucleotide sequences. This allows the design of broad-range PCR assays that uses PCR primers that are targeted at these conserved regions of rDNA. PCR based methods have been evaluated for the detection of bacteria and fungi in blood cultures, joint fluids and cerebral spinal fluid (CSF) but not from allograft musculoskeletal tissue samples.
Clinical Applications of Specific-Target RT-PCR Techniques

Specific molecular assays targeting specific organisms are widely used in the diagnosis of bloodstream infections. A positive-signaled blood culture bottle is Gram-stained and then specific molecular testing can be performed. The identification of *S. aureus* and MRSA from blood culture bottles has been investigated (Shrestha et al 2002, Hallin et al 2003, Eigner et al 2005, Ruimy et al 2008, Stamper et al 2007). The detection of organisms causing vaginitis, *Gardnerella vaginalis, Trichomonas vaginalis* and *C. albicans*, can be performed using nucleic acid testing rather than the traditional culture methods (Speers 2006).

Non-culture methods such as RT-PCR have been adopted for chlamydial and gonococcal infections (Speers 2006). The identification of *Mycobacterium* species is rapidly and accurately performed by RT-PCR with differentiation of *M. tuberculosis* complex from atypical mycobacterium such as *M. avium/intracellulare* (Lachnik et al 2002, Espy et al 2006). The detection of Group A streptococci from throat swabs and *Clostridium difficile* using RT-PCR techniques has been found to be as sensitive as the gold standard culture method and immunoassay methods, providing results on the same day (Espy et al 2006).

RT-PCR techniques are especially useful for slow-growing or poorly culturable bacteria. The laboratory culture of *Bordetella pertussis* has relied on the isolation of the organism from selective agar plates. This organism is slow growing and extended incubation often results in an overgrowth of fungal contaminants on the plates or failure of the organism to grow although clinically indicated. RT-PCR has replaced the culture method in clinical laboratories with a much greater sensitivity and detection rate. Other slow growing organisms such as *Bartonella henselae, Legionella sp, Mycoplasma pneumonia* and *Tropheryma whipplei* have benefited with improved detection using molecular methods (Espy et al 2006, Sails 2009). Invasive fungal disease can be difficult to diagnose and are associated with a high morbidity and mortality especially in immunocompromised patients. The most common fungal pathogens involved in these infections are *Candida* and *Aspergillus* species, both of which can be rapidly identified by PCR (Klingsport & Jalal 2006).

The causative organisms commonly associated with meningitis can be rapidly detected using RT-PCR (Espy et al 2006, Sails 2009). Organisms such as *Haemophilus influenza, Neisseria meningitidis* and *Streptococcus pneumoniae* are associated with a high mortality and morbidity and rapid diagnosis is essential. In cases of meningitis, antibiotics are provided very early before cultures are obtained with subsequent culture-negative results – RT-PCR provides the advantage of being positive in these cases (Corless 2001, Sails 2009).

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Bacterial antibiotic resistance genes, such as methicillin (oxacillin)-resistance in *S. aureus* (MRSA) and vancomycin resistance in *Enterococcus* species (VRE) show improved sensitivity using RT-PCR techniques compared to culture methods, with the availability of results on the same day. Culture and broth-based screening of VRE and MRSA in clinical laboratories is a time-consuming and laborious task with a high volume of samples. Nucleic acid testing can also be beneficial for epidemiological applications such as in outbreaks of *Listeria monocytogenes* cases (Espy et al 2006).

**Broad-Range RT-PCR**

Many RT-PCR methods have been described to identify specific organisms. The use of broad-range RT-PCR techniques is generally applied to those samples where an organism is anticipated but culture results are generally negative, such as heart tissue from cases of suspected infective endocarditis and CSF samples (Schabereiter-Gurtner et al 2007, Sontakke et al 2009). Broad-range RT-PCR may also be used in cases where a microorganism has been cultured but phenotypic identification has been unsuccessful.

In broad-range RT-PCR techniques, primers complementary to a conserved region of a gene are used, such as the 16S rRNA bacterial gene or the 18S rRNA gene of fungi. Any amplified product is sequenced and compared to existing sequences in public databases, accessed via the internet, to determine organism identification. One of the most commonly used databases is GenBank (www.ncbi.nlm.nih.gov/Genbank).

**Clinical Application of Broad-Range RT-PCR Techniques**

**Infective Endocarditis**

Infective endocarditis (IE) is an infection involving the endothelial lining of the heart, primarily affecting the valves (Lang et al 2004). Blood cultures are routinely collected in the diagnosis of IE which often remain negative after incubation. This may be as a result of antimicrobial treatment received by the patient prior to blood collection or the causative organism may be slow-growing or non-culturable. Many studies have applied culture-independent broad-range molecular techniques to establish the infecting organism. Goldenberger et al (1997) used broad-range PCR amplification of part of the 16S rRNA gene followed by single-strand sequencing directly to 18 heart valve samples. The heart valve samples were also cultured onto agar media and blood cultures had been collected. Agreement was reached between PCR and either heart valve or blood cultures in 11 samples. PCR results were negative
although blood cultures were positive in 2 samples, PCR positive and culture negative in 2 samples. Amplification was inhibited in 1 sample and sequencing was unsuccessful in 2 samples. The authors concluded that broad-range PCR is a useful tool in IE patients who remain culture-negative. Another study by Breitkopf et al (2004) that tested 52 excised heart valves found 22 positive by broad-range PCR testing – 14 of these were negative by conventional culture methods and 4 were positive by culture. This study also found broad-range molecular methods important in the diagnosis of culture-negative cases and patients receiving antimicrobial treatment. Numerous other studies have also supported these findings (Goldenberger et al 1997, Bosshard et al 2003, Breitkopf et al 2005, Rice 2005, Marin et al 2007).

**Corneal Ulcers**

Corneal ulcers are a serious problem, especially in developing countries, and can be caused by fungal or bacterial organisms. The rapid diagnosis between fungal and bacterial aetiology is important in the treatment of these patients. Conventional diagnosis has been made by culture of corneal scrapings onto agar media, a smear for Gram’s stain and another smear for a potassium hydroxide (KOH) wet mount. Three recent studies by Kim et al (2008), Vengayil et al (2009) and Itahashi M (2010) compared the use of PCR in the diagnosis of fungal and/or bacterial organisms in corneal samples. The usefulness and increased sensitivity of PCR techniques was stated in all studies and that it may be a fast diagnostic tool to be used in conjunction with culture.

**Bone and Joint Infections**

Infections in bone and joints may be difficult to determine due to the nature of the organisms involved. The organisms may be of low virulence, generally associated with skin flora and may be present in low numbers (Esposito & Leone 2008). Long-term antibiotic treatment will also influence recovery of pathogens by culture methods. Many studies have found that the use of a broad-range PCR assay will identify a pathogen in many cases of culture-negative bone and joint infections (Fenollar et al 2006, Moojen et al 2007, Fihman et al 2007). Other studies by Dora et al (2008) and De man et al (2009) found that PCR was able to confirm false culture-positive results and that it should only be used as an additional tool to culture when warranted by clinical symptoms.
Allograft Musculoskeletal Tissue

As discussed above, there have been numerous evaluations on the use of nucleic acid testing to determine the causative organisms of infection in bone and joints. However, as a bioburden assessment tool for allograft musculoskeletal tissue there is very little. The virology testing of blood samples obtained from musculoskeletal allograft donors is being performed by nucleic acid testing as well as by serology tests (Table 1). The bioburden assessment of allograft musculoskeletal tissue from donors for a bacterial presence by nucleic acid testing has not been documented and only one document was found on the detection of fungal contamination in allograft tissue by PCR (Gupta et al 2008). The use of broad-range RT-PCR (bacteria and fungi) as a tool for the bioburden assessment of musculoskeletal tissue requires investigation.

Living and cadaveric musculoskeletal allograft donors are screened via social and medical questionnaires and samples for bioburden assessment are collected from patients who pass this initial assessment. Living donors are generally healthy requiring hip replacements due to injury or age-related factors such as arthritis or osteoporosis. Allografts and samples from living donors are collected in operating room conditions and cadaveric donors are collected using clean room techniques. Subsequently, the bioburden of bacteria and fungi in these types of samples is expected to be low. Studies have indicated that $10^5$ viable organisms per gram of tissue are required for wound infection (Levine 1976), however low numbers of unculturable organisms may be implicated with a range of other factors that prevent the successful transplant of allograft musculoskeletal tissue (Tunney et al 1999, Ince et al 2004).

Bioburden assessment of allograft samples by PCR testing to replace culture-based methods appears to be a logical progression. PCR methods are especially of interest due to the pre-operative antibiotic therapy of living donors and, in some cases, the use of allograft processing washes containing antibiotics, potentially causing a bacteriostatic affect on organisms and recovery by culture methods difficult. Validation studies on bioburden assessment culture methods are generally performed in Australia according to the British Pharmacopoeia with challenge organisms of inoculum sizes of $<100$ CFU. The level of sensitivity of RT-PCR assays in detecting bioburden in allograft musculoskeletal tissue samples is still to be determined.

In allograft musculoskeletal samples, the presence of organisms generally does not indicate infection or disease, although this is not the purpose of testing these samples. These
samples must be considered in terms of risk of infection or infectious disease transmission via transplant of the allograft to recipients.

**Benefits and Challenges Associated with PCR Assays**

The use of molecular testing on allograft musculoskeletal samples presents many benefits and challenges. Samples received may be swabs, tissue biopsies such as bone chips, or other types of samples. Biopsies are irregular shaped with a non-uniform surface. All samples must be assessed for the presence of bacterial and fungal genetic material so a broad-range RT-PCR approach remains the optimal assay.

The detection of nucleic acid does not indicate the presence of viable micro-organisms. This is not a problem with conventional culture methods where organisms must be viable to grow on agar plates and in broth media. However the presence of nucleic acid or growth on media does not indicate clinical infection in a patient. The detection of some organisms indicates a high specificity of infection, such as meningococcus from a CSF, whereas the detection or growth of coagulase-negative staphylococci does not. The results of nucleic acid diagnostic tests need to be considered in the clinical context of the patient. In the threat of bioterrorism, nucleic acid testing of non-living organisms is a benefit as samples may be autoclaved prior to testing eliminating exposure risks to personnel. However, this is not a consideration in the nucleic acid testing of donor allograft musculoskeletal tissue prior to transplant.

RT-PCR techniques are ideal for micro-organisms that are difficult to isolate by culture-based methods. As allograft musculoskeletal tissue samples are retrieved from donors who are considered generally healthy based on exclusion factors, the samples are not expected to have a high bioburden. Nucleic acid techniques such as RT-PCR are ideal for low numbers of organisms due to the amplification process. Prior antibiotic therapy reduces the isolation rate of micro-organisms by culture methods. RT-PCR would be helpful as living donors receive prophylactic antibiotic therapy prior to surgery.

Fungal infections can be difficult to detect using conventional culture methods due to a lack of sensitivity, the need for extended incubation periods and the fastidious nature of some fungi. RT-PCR assays have been developed and are able to detect species-specific fungal targets, genera-specific targets or pan-fungal detection (van Burik et al 1998, Jaeger et al 2000, Klingspor & Jala 2006).
The technology and consumables associated with molecular techniques have generally been more expensive than culture-based methods. However cost-benefits can be generated by replacing a labour-intensive method, rapid diagnosis and subsequent targeted treatment. Further problems may be encountered when comparing sequences obtained in a sample to the existing sequences in a public database such as GenBank where accuracy of existing databases cannot be determined.

Contaminating DNA may be present in a sample or it can come from reagents, water and consumables. This is especially a problem where non-specific genetic material is being looked for, as in broad-range RT-PCR, as it will be amplified and will produce false positive results. False positive results can also be due to dead micro-organisms, in this case the result of testing will be diagnostically correct but clinically will be regarded as a false-positive. To prevent false-positive results due to environmental contamination the laboratory requires the physical separation of the different processes involved in PCR techniques. Reagent preparation, specimen preparation and product detection processes must all be located in different areas of the laboratory, combined with a high level of staff skill and training. Ultraviolet light irradiation of reagents and chemical inactivation of surface contamination with sodium hypochlorite can reduce amplicon laboratory contamination. RT-PCR reduces this risk by the use of a single tube PCR reaction and detection system, with further reduction with the use of automation. False-positive reactions can occur when manual manipulation of the post-amplification PCR product is required. During these manipulations, amplicon can be carried over to other reactions creating false-positive reactions. Using a closed-tube format with automated instruments reduces the likelihood of amplicon carryover.

False-negative reactions can also occur in PCR assays due to the presence of inhibitors in clinical samples. Substances such as polysaccharide, haem and therapeutic drugs may inhibit PCR enzymes (Gilbert et al 1999). Inhibitor checks must be included for each sample to ensure that a negative result is not one that has been inhibited (Speers 2006).
The Role of Microbiology Laboratories in the Determination of the Bioburden Assessment of Allograft Musculoskeletal Tissue

The microbiology laboratory is an integral partner of the tissue bank that it provides testing for. The relationship between staff at both facilities is much more than a professional one as consultations between both regularly occurs to meet challenges faced, for example, by results of testing, review or contracted agreements, regulatory requirements, sample and transport issues or quality improvements.

Many tissue banks, generally the smaller bone banks, do not process retrieved tissue from living donors in any way prior to storage nor is any bioburden reduction method, such as gamma irradiation, performed prior to transplant of the allograft. These bone banks pride themselves on satisfying the requirements of their orthopaedic surgeons in providing a quality tissue with sound biochemical and structural integrity. The role of the microbiology laboratory in the determination of the bioburden assessment of allograft musculoskeletal tissue is therefore a very important one.

The microbiology department has a significant responsibility in ensuring the methods used to determine bioburden assessment are able to meet the expectations of the tissue banks, orthopaedic surgeons, regulatory bodies and, ultimately and most importantly, the recipient of the allograft musculoskeletal tissue. From the literature and information collected from many Australian microbiology laboratories, it is apparent that varying combinations of agar media, broth media, methods and incubation periods are in use (Tables 3 & 4). There is no optimal method, with different media being able to satisfactorily isolate aerobic and anaerobic bacteria and fungi from allograft musculoskeletal tissue.

The advent of nucleic acid based testing, especially RT-PCR, moved clinical microbiology laboratories forward at an exceedingly fast rate from many years of stability. Nucleic acid testing as part of the donor’s viral screen is now routine. The ‘window period’ where false-negative results can be obtained for patients with human immunodeficiency virus (HIV), Hepatitis B (HBV) and Hepatitis C (HCV) has been eliminated by using nucleic acid testing methods such as RT-PCR (Yao et al 2008, Pruss et al 2010). It is only a matter of time before regulatory requirements are changed so that nucleic acid testing replaces the other current serology and virology repertoire of tests, removing the 180-day retesting
requirement. The consequences of this action would allow the release of allograft musculoskeletal tissue much earlier to meet the increasing demand. Allografts are often rejected due to the donor’s inability or reluctance to return for re-testing at 180-days post-donation. This would also result in a greater supply of allografts being available for transplant.

Bioburden assessment methods must also move forward to include RT-PCR techniques and improve the turn-around time of culture results. Currently, fungal cultures are incubated for periods of 4 weeks in some laboratories. However, RT-PCR testing requires careful consideration of workflow practices and validation before any changes are made. The problems associated with RT-PCR such as false positives, false negatives, specialised and separated work areas and trained technical staff requires investigation for the bioburden assessment of allograft musculoskeletal tissue samples. Certainly, any method used must be sensitive and of a high standard. Unlike specific cases where organisms may be unculturable or extremely slow growing, the majority of micro-organisms isolated from allograft musculoskeletal tissue samples appear to be those commonly encountered in aerobic and anaerobic clinical microbiology. The majority of musculoskeletal tissue samples from living donors are culture negative and although the initial processing and data entry of these samples may be time consuming, the interpretation of cultures is generally not. Quite often, more effort is required in finding the space for the many agar plates and broth media involved in their culture and is labour-intensive with broth sub-cultures generally required. Therefore, faster methods may eliminate this problem. Increased sensitivity may be gained using RT-PCR, although organisms cultured so far do not appear to be fastidious or unusual, low numbers of organisms may be missed by culture-based methods. A cost analysis should also be considered as agar and broth culture media is relatively cheap compared to nucleic acid based methods.
Conclusion

Musculoskeletal tissue transplants outnumber all other organ and tissue transplants however the demand for tissue for musculoskeletal tissue is not met in Australia. Musculoskeletal tissue banks have come a long way since their beginnings by orthopaedic surgeons to meet their own requirements for bone during orthopaedic surgery. All tissue bank processes from donor assessment, tissue retrieval, allograft storage and distribution are maintained and regulated through the tissue bank’s quality systems.

The ability of bacteria and fungi to be present as a pathogen within the allograft tissue, to be able to migrate to different parts of the body before tissue retrieval, their ability to contaminate tissues via personnel, reagents and consumables requires microbiology laboratories to perform bioburden assessment on allograft tissue prior to transplant. The bioburden assessment methods used by laboratories in Australia and internationally vary from laboratory to laboratory, all equally validated to detect aerobic and anaerobic bacteria and fungi. The development and widespread use of nucleic acid based techniques, such as RT-PCR, provides an opportunity for laboratories to investigate their use and re-evaluate their current methods in the bioburden assessment of allograft musculoskeletal tissue.

Ensuring that the highest quality and safest possible allograft musculoskeletal tissue is transplanted to recipients is the collaborative pride of tissue banks, regulatory authorities and microbiology laboratories.
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