ASSESSMENT OF MICROBIAL BIOBURDEN METHODOLOGIES FOR TISSUE BANK SPECIMENS

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CERTIFICATE OF AUTHORSHIP / ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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ABSTRACT

Musculoskeletal tissues form part of the skeletal and/or muscular system of the body, vital in providing support and mobility. Musculoskeletal tissue transplants outnumber all other organ and tissue transplants. The bioburden assessment of allograft musculoskeletal tissue must be performed as part of the assessment screening of living and cadaveric donors to minimise the potential risk of transmission of infectious diseases via the allograft to the recipient.

There are no guidelines or standard method for determining the bioburden assessment of allograft musculoskeletal tissue and microbiology laboratories may use different types of samples, culture media and methods. Determining the suitability of the allograft tissue sample and the sensitivity of the bioburden testing methods required investigation especially with the advent of nucleic-acid testing (NAT). Subsequently, this investigation highlighted the lack of information regarding microbiology laboratories and the tissue banking industry in Australia.

A questionnaire was sent to all Australian tissue banks to determine their current status and the types of allograft samples being collected for bioburden assessment. Another questionnaire was designed for Therapeutic Goods Administration (TGA) licensed clinical microbiology laboratories to establish what bioburden assessment methods were being used for allograft samples. The information obtained from these questionnaires guided the evaluations undertaken in this thesis to compare different allograft samples and methods for bioburden assessment.

The current practice of collecting a swab and biopsy sample of allograft musculoskeletal tissue appears optimal for bioburden assessment. Retrospective reviews of isolates recovered from allograft musculoskeletal tissue and from the literature found a wide range of aerobic and anaerobic micro-organisms with fungi infrequently isolated.

An evaluation of the Amies gel swab and the ESwab systems was performed to determine if bioburden recovery could be improved at the pre-analytical stage. Both swab systems were found to be suitable sampling devices for bioburden testing of allograft musculoskeletal tissue.

The most common bioburden assessment methods, agar and broth culture, were compared with a broad-range NAT method. Swab and biopsy samples were inoculated with known quantities of challenge organisms and the percentage recovery of the challenge organisms was compared. In this study, the NAT method was not more sensitive than the culture-based techniques evaluated with broth culture being the most sensitive.

Microbiology laboratories must continue to re-evaluate current methods and investigate new ones to improve sensitivity. Future directions must be cost-effective as the value of maintaining a TGA-licence has become uncertain for some laboratories. Ultimately, tissue banks, clinicians and, most importantly, the allograft recipient must have confidence in the pre-analytical sampling techniques and the testing methods used to determine the bioburden of allograft musculoskeletal tissue prior to transplant.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Musculoskeletal tissue transplantation

The World Health Organisation (WHO) defines transplantation as the 'transfer (engraftment) of human cells, tissues or organs from a donor to a recipient' (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. This chapter provides an insight and an explanation into the processes of musculoskeletal tissue transplantation, with an emphasis on the Australian perspective. It supplies the background information required to understand the importance of musculoskeletal tissue transplantation, the practice of tissue banks and why microbiology is an integral part of the musculoskeletal tissue transplant process.

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

There are some significant differences between human cell, organ and musculoskeletal 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. 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, to remove 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).

1.2 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 (1881) 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. 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.

1.3 Source of allograft musculoskeletal tissue for transplant

Musculoskeletal tissue can be obtained from living or cadaveric donors. Bone is obtained from living donors as a whole femoral head 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.

1.4 Clinical use of allograft musculoskeletal tissue

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).

Orthopaedic surgeons use musculoskeletal tissue 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 (Aro & Aho 1993, Abbas et al. 2007). 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.

Bone 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 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 orthopaedic surgery 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 urogynaecological 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.

1.5 Musculoskeletal tissue banks

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 disease transmission such as hepatitis and HIV, 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.

1.6 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 (NSW), the Australian Capital Territory (ACT), Victoria, South Australia, Western Australia and Queensland. There are no tissue banks 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. b). 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 NSW, 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 2014a). 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.

1.7 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 (Murphy 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. c). 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 Biotherapeutics Association of Australasia (BAA) although these figures are not available on their website.

1.8 Therapeutic Goods Administration

The Therapeutic Goods Administration (TGA) was established in 1990 as part of the Australian Government Department of Health and is responsible for the regulation of therapeutic goods (TGA 2014). Musculoskeletal tissue is considered a 'therapeutic good' as it is manufactured for therapeutic use in humans and includes any stage of the retrieval, processing, storage, testing and distribution of the tissue. Tissue banks and microbiology laboratories are therefore part of the manufacturing process and must be audited on-site for compliance of the Australian Code of Good Manufacturing Practice (GMP) - Human Blood and Blood Components, Human Tissues and Human Cellular Therapies (TGA 2013).

1.9 Donor assessment

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 (bacteriology, virology and serology) and histopathological assessment of donor tissue samples.

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 (CJD), 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. Prions are known to cause CJD and variant CJD and have been implicated in the transplant of dura matter but not with musculoskeletal allograft tissue (Doerr et al. 2003, Delloye et al. 2007). 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 BAA and are available on the BAA website (BAA 2013a 2013b). 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.

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). However, not all donors with high risk behaviour have obvious signs. 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 (van Wijk et al. 2008).

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 in Australia as outlined in the Therapeutic Goods Order No. 88 (TGA 2013). 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.

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 (Cheek et al. 1995).

1.10 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 BAA, however practices may vary between individual tissue banks (BAA 2013c). 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 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℃ or colder while in transport to the tissue bank.

1.11 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. 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 2014b).

1.12 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℃ to -80℃ 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 -80ºC 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.

1.13 Musculoskeletal tissue infection

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 (Woolf & Pfleger 2003, Australian Institute of Health and Welfare 2008). This subsequently places a greater demand on the health system with repeat surgery, access to other services and greater demands on patient carers. 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 these 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).

Micro-organisms that are not commonly associated with infection can become pathogens if the 'opportunity' arises, therefore termed opportunistic infections (Zeller et al. 2007, Bjerkan et al. 2012). 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.

1.14 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.

1.15 Bioburden reduction processes

Micro-organisms function by their metabolic activities, which are dependent on chemical reactions and are 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.

1.15.1 Autoclaving

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.

1.15.2 Antibiotics

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).

1.15.3 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.

1.15.4 Supercritical carbon dioxide

Supercritical carbon dioxide is considered a sterilisation process, also known as dense CO_2. Supercritical CO_2 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 effects 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 terminal sterilisation of allograft bone (Australian Biotechnologies/Products 2014b).

1.15.5 Microwaving

The microwave sterilisation of femoral head allografts was investigated (Dunsmuir 2003) as an alternate to the labour-intensive and expensive bioburden reduction methods. The cores of the femoral heads were inoculated with *S. aureus* and *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.

1.15.6 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 intraarticular reactions (Jackson 1990, Roberts 1991).

1.15.7 Gamma irradiation

The most common process of bioburden reduction in Australia is by gamma irradiation from a Cobalt 60 source. Gamma irradiation has the ability to penetrate through packaging around the tissue which is 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-Goclawska 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.

1.16 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 cadaveric 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. (McAllister 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.

1.17 Thesis overview

It is important that bacterial and fungal bioburden assessment is performed to minimise the risk of post-transplant infections. The work described in this thesis evolved from a lack of information regarding the relationship between microbiology laboratories and musculoskeletal tissue banks in Australia. There was a lack of knowledge regarding how many microbiology laboratories were involved in the bioburden testing of musculoskeletal allografts, the location of the laboratories and of their associated tissue banks. There are currently no guidelines or standard method for determining bioburden assessment and microbiology laboratories may use different types of samples, culture media and methods. Determining the suitability and sensitivity of the allograft tissue sample and bioburden testing methods required investigation.

1.18 Thesis aims

The aims of this research are:

- to determine the current inter-relationship of microbiology laboratories and musculoskeletal tissue banks in Australia;
- to determine the bioburden assessment methods currently in use in microbiology laboratories in Australia;
- to retrospectively review the bacterial and fungal isolation rates from allograft musculoskeletal tissue samples received at the microbiology laboratory of the South Eastern Area Laboratory Services (SEALS);
- to evaluate the different types of samples collected for bioburden testing;
- to evaluate the different testing methods used by Australian laboratories for bioburden assessment.

1.19 Thesis format

This thesis is divided into 13 chapters including a general introduction, a general summary/conclusion, a chapter combining the references from the previous chapters and the final chapter containing the appendices. Each of the chapters describes related but independent studies and progresses from a general overview of musculoskeletal tissue transplant and tissue banking, microbiology laboratories and micro-organisms isolated from allograft samples, to more defined evaluations on the different bioburden testing methods in use in Australian laboratories and the determination of an optimum sample.

A brief summary of each chapter is described below:

Chapter 1: to provide a comprehensive introduction to musculoskeletal tissue transplantation and the current status of tissue banks and tissue banking processes in Australia and internationally. The questionnaire used to survey the tissue banks is attached as Appendix 1;
Chapter 2: to provide current information regarding the Australian microbiology laboratories performing bioburden testing of allograft musculoskeletal samples. The questionnaire used to survey the bacteriology laboratories is attached as Appendix 2;
Chapter 3: to perform a retrospective review of the micro-organisms isolated from samples of allograft musculoskeletal tissue retrieved from living donors;
Chapter 4: to perform a retrospective review of the type of the micro-organisms isolated from samples of allograft musculoskeletal tissue retrieved from cadaveric donors;
Chapter 5: to perform a retrospective review of fungal isolates and determine the relevance of fungal culture on allograft musculoskeletal tissue samples;
Chapter 6: to establish the current culture media and methods being used by Australian laboratories for bioburden testing of allograft musculoskeletal tissue samples;
Chapter 7: to evaluate the use of solid agar and broth media for the culture allograft samples;
Chapter 8: to determine if there is an optimum musculoskeletal tissue sample for bioburden testing;
Chapter 9: to compare two types of swab sampling systems and determine if micro-organism recovery from musculoskeletal tissue can be improved;
Chapter 10: to compare the traditional culture-based methods currently in use with nucleic-acid based testing for bioburden assessment;
Chapter 11: a general summary and conclusion of the thesis;
Chapter 12: thesis references.
Chapter 13: Appendices

Chapters 2–10 have been individually published and are included unchanged from the published version, except for the formatting style. Subsequently, some repetition will result between each chapter, especially in the introduction and methods sections. The identification of bacteria in Chapter 2 and Chapter 3 was performed using traditional biochemical testing or purchased API biochemical kits (bioMerieux Inc., Durham, N.C.). Chapter 11 provides a general summary and conclusion of the thesis and Chapter 12 provides the references used throughout. The Tissue Bank and Bacteriology laboratory questionnaires used in this thesis have been attached as Appendices in Chapter 13.

CHAPTER 2: BACTERIOLOGY LABORATORIES AND MUSCULOSKELETAL TISSUE BANKS IN AUSTRALIA

This chapter has been published in The Australian & New Zealand Journal of Surgery:

Varettas K (2012)
Bacteriology laboratories and musculoskeletal tissue banks in Australia.
Australian New Zealand Journal of Surgery 82: 775-779

Except for the formatting style, this chapter is unchanged from the published version.

2.1 Abstract

In Australia, there are six Therapeutic Goods Administration–licensed clinical bacteriology laboratories providing bacterial and fungal bioburden testing of allograft musculoskeletal samples sent from 10 tissue banks. Musculoskeletal swab and/or tissue biopsy samples are collected at the time of allograft retrieval and sent to bacteriology laboratories for bioburden testing, in some cases requiring interstate transport. Bacteria and fungi may be present within the allograft at the time of retrieval or contaminated from an external source. The type of organism recovered will determine if the allograft is rejected for transplant, which may include all allografts from the same donor. Bacteriology staff also provides unpaid support of tissue banks through meeting involvement, consultations, licence-related activities, validations and research funded by their organisation and not part of any contractual agreement. Bacteriology laboratories and tissue banks must be compliant to the Code of Good Manufacturing Practice – Human Blood and Tissues and regulated by the Therapeutic Goods Administration. Clinical bacteriology laboratories also require mandatory accreditation to Standards Australia International Organisation for Standardisation (ISO) 15189:2009 medical laboratories – particular requirements for quality and competence, and may also attain Standards Australia/New Zealand Standard ISO 9001:2000 quality management systems certification. Bacteriology laboratories and musculoskeletal tissue banks are integral partners in providing safe allograft musculoskeletal tissue for transplant.

Varettas K. (2012) Bacteriology laboratories and musculoskeletal tissue banks in Australia. *ANZ J Surg*. 82: 775-779. DOI: 10.1111/j.1445-2197.2012.06145.x

CHAPTER 3: BIOBURDEN ASSESSMENT OF BANKED BONE USED FOR ALLOGRAFTS

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K, Taylor PC (2011)
Bioburden assessment of banked bone used for allografts.
Cell and Tissue Banking 12: 37-43

Except for the formatting style, this chapter is unchanged from the published version.

3.1 Abstract

Allograft bone is commonly used in reconstructive orthopaedic surgery and needs to be assessed for bioburden before transplant. The Microbiology Department of the South Eastern Area Laboratory Services (SEALS), located at the St. George Hospital, Sydney, has provided this service to the New South Wales (NSW) Bone Bank. This study reviewed the organisms isolated from femoral head allografts of living donors from the NSW Bone Bank over a 7-year period. It was found that growth was reported from 4.9% of samples with the predominant organism being coagulase-negative staphylococci. This review will focus on the micro-organisms isolated, the interaction of the laboratory with the bone bank, the relevance of the bioburden assessment in the overall quality process and patient safety.

Varettas K, Taylor P. (2011) Bioburden assessment of banked bone used for allografts. *Cell Tissue Banking*. 12:37–43. DOI:10.1007/s10561-009-9154-z

CHAPTER 4: MICRO-ORGANISMS ISOLATED FROM CADAVERIC SAMPLES OF ALLOGRAFT MUSCULOSKELETAL TISSUE

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K (2013)
Micro-organisms Isolated from Cadaveric Samples of Allograft Musculoskeletal Tissue.
Cell and Tissue Banking 14: 621-625

Except for the formatting style, this chapter is unchanged from the published version.

4.1 Abstract

Allograft musculoskeletal tissue is commonly used in orthopaedic surgical procedures. Cadaveric donors of musculoskeletal tissue supply multiple allografts such as tendons, ligaments and bone. The microbiology laboratory of the South Eastern Area Laboratory Services (SEALS, Australia) has cultured cadaveric allograft musculoskeletal tissue samples for bacterial and fungal isolates since 2006. This study will retrospectively review the micro-organisms isolated over a 6-year period, 2006-2011.

Swab and tissue samples were received for bioburden testing and were inoculated onto agar and/or broth culture media. Growth was obtained from 25.1% of cadaveric allograft musculoskeletal tissue samples received. The predominant organisms isolated were coagulase-negative staphylococci and coliforms, with the heaviest bioburden recovered from the hemipelvis.

The rate of bacterial and fungal isolates from cadaveric allograft musculoskeletal tissue samples is higher than that from living donors. The type of organism isolated may influence the suitability of the allograft for transplant.

Keywords: cadaveric; musculoskeletal; allograft; bioburden; contamination

Varettas K. (2013) Micro-organisms isolated from cadaveric samples of allograft musculoskeletal tissue. *Cell Tissue Bank*. 14: 621-625. DOI: 10.1007/s10561-013-9363-3

CHAPTER 5: FUNGAL CULTURE OF MUSCULOSKELETAL TISSUE: WHAT'S THE POINT?

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K, Taylor P (2012)
Fungal Culture of Musculoskeletal Tissue: What's the Point?
Cell and Tissue Banking 13: 415–420

Except for the formatting style, this chapter is unchanged from the published version.

5.1 Abstract

There have not been any studies that review the prevalence of fungal isolates using selective media from samples of banked musculoskeletal tissue retrieved from living and cadaveric donors. A total of 2036 swab and 2621 biopsy samples of musculoskeletal tissue from tissue banks were received from the 1st August 2008 till 31st December 2010. Routine culture for fungi using selective media with a prolonged incubation period failed to demonstrate a greater prevalence of fungal isolates than by using non-selective culture media alone. Using selective culture fungi were recovered from only two Sabouraud agar (SAB) plates (0.1%) but not from non-selective media. During the same period fungi were isolated from three graft samples cultured in non-selective broth media only (0.1%). There was no correlation of fungal isolates from selective or non-selective media inoculated at the same time nor from multiple graft samples collected from the same donor supporting the possibility of an exogenous source for fungal isolates rather than an endogenous source.

Keywords: musculoskeletal; graft; fungal; bioburden; contamination

Varettas K, Taylor P. (2012) Fungal culture of musculoskeletal tissue: What's the point? *Cell Tissue Banking*. 13:415-420. DOI: 10.1007/s10561-011-9287-8

CHAPTER 6: CULTURE METHODS OF ALLOGRAFT MUSCULOSKELETAL TISSUE SAMPLES IN AUSTRALIAN BACTERIOLOGY LABORATORIES

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K (2013)
Culture Methods of Allograft Musculoskeletal Tissue Samples in Australian Bacteriology Laboratories
Cell and Tissue Banking 14: 609-614

Except for the formatting style, this chapter is unchanged from the published version.

6.1 Abstract

Samples of allograft musculoskeletal tissue are cultured by bacteriology laboratories to determine the presence of bacteria and fungi. In Australia, this testing is performed by 6 TGA-licensed clinical bacteriology laboratories with samples received from 10 tissue banks.

Culture methods of swab and tissue samples employ a combination of solid agar and/or broth media to enhance micro-organism growth and maximise recovery. All six Australian laboratories receive Amies transport swabs and, except for one laboratory, a corresponding biopsy sample for testing. Three of the 6 laboratories culture at least one allograft sample directly onto solid agar. Only one laboratory did not use a broth culture for any sample received. An international literature review found that a similar combination of musculoskeletal tissue samples were cultured onto solid agar and/or broth media.

Although variations of allograft musculoskeletal tissue samples, culture media and methods are used in Australian and international bacteriology laboratories, validation studies and method evaluations have challenged and supported their use in recovering fungi and aerobic and anaerobic bacteria.

Keywords: allograft; bioburden; contamination; culture; musculoskeletal

Varettas K. (2013) Culture methods of allograft musculoskeletal tissue samples in Australian bacteriology laboratories. *Cell Tissue Banking*. 14: 609-614.
DOI: 10.1007/s10561-012-9361-x

CHAPTER 7: BROTH VERSUS SOLID AGAR CULTURE OF SWAB SAMPLES OF CADAVERIC ALLOGRAFT MUSCULOSKELETAL TISSUE

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K (2013)
Broth versus Solid Agar Culture of Swab Samples of Cadaveric Allograft Musculoskeletal Tissue
Cell Tissue Banking 14: 627-631

Except for the formatting style, this chapter is unchanged from the published version.

7.1 Abstract

As part of the donor assessment protocol, bioburden assessment must be performed on allograft musculoskeletal tissue samples collected at the time of tissue retrieval. Swab samples of musculoskeletal tissue allografts from cadaveric donors are received at the microbiology department of the South Eastern Area Laboratory Services (SEALS, Australia) to determine the presence of bacteria and fungi. This study will review the isolation rate of organisms from solid agar and broth culture of swab samples of cadaveric allograft musculoskeletal tissue over a 6-year period, 2006-2011.

Swabs were inoculated onto horse blood agar (anaerobic, 35℃) and chocolate agar (CO_2, 35℃) and then placed into a cooked meat broth (aerobic, 35℃). A total of 1912 swabs from 389 donors were received during the study period. 557 (29.1%) swabs were culture positive with the isolation of 713 organisms, 249 (34.9%) from solid agar culture and an additional 464 (65.1%) from broth culture only.

This study has shown that the broth culture of cadaveric allograft musculoskeletal swab samples recovered a greater amount of organisms than solid agar culture. Isolates such as *Clostridium* species and *Staphylococcus aureus* would not have been isolated from solid agar culture alone. Broth culture is an essential part of the bioburden assessment protocol of swab samples of cadaveric allograft musculoskeletal tissue in this laboratory.

Keywords: allograft; musculoskeletal, bioburden; swab; broth; culture

Varettas K. (2013) Broth vs solid agar culture of swab samples of cadaveric allograft musculoskeletal tissue. *Cell Tiss Banking*. 14: 627-631. DOI: 10.1007/s10561-013-9365-1

CHAPTER 8: SWAB OR BIOPSY SAMPLES FOR BIOBURDEN TESTING OF ALLOGRAFT MUSCULOSKELETAL TISSUE?

This chapter has been published in The Cell and Tissue Banking Journal:

Varettas K (2014)
Swab or biopsy samples for bioburden testing of allograft musculoskeletal tissue?
Cell Tissue Banking 15:613-618

Except for the formatting style, this chapter is unchanged from the published version.

8.1 Abstract

Swab and biopsy samples of allograft musculoskeletal tissue are most commonly collected by tissue banks for bacterial and fungal bioburden testing. An in-vitro study was performed using the NCCLS standard 'Quality control of microbiological transport systems' (2003) to validate and evaluate the recovery of six challenge organisms from swab and biopsy samples of allograft musculoskeletal tissue. On average, 8.4 - >100% and 7.2 - >100% of the inoculum was recovered from swab and biopsy samples respectively.

A retrospective review of donor episodes was also performed, consisting of paired swab and biopsy samples received in this laboratory during the period 2001-2012. Samples of allograft femoral heads were collected from living donors during hip operations. From the 3859 donor episodes received, 21 paired swab and biopsy samples each recovered an isolate, 247 swab samples only and 79 biopsy samples only were culture positive.

Low numbers of challenge organisms were recovered from inoculated swab and biopsy samples in the in-vitro study and validated their use for bioburden testing of allograft musculoskeletal tissue. Skin commensals were the most common group of organisms isolated during a 12-year retrospective review of paired swab and biopsy samples from living donor allograft femoral heads. Paired swab and biopsy samples are a suitable representative sample of allograft musculoskeletal tissue for bioburden testing.

Keywords: allograft; musculoskeletal; bioburden; swab; biopsy

Varettas K. (2014) Swab or biopsy samples for bioburden testing of allograft musculoskeletal tissue? Cell Tissue Banking. 15:613-618. DOI:10.1007/s10561-014-9435-z

CHAPTER 9: EVALUATION OF TWO TYPES OF SWABS FOR SAMPLING ALLOGRAFT MUSCULOSKELETAL TISSUE

This chapter has been published in The Australian & New Zealand Journal of Surgery:

Varettas K (2014)
Evaluation of two types of swabs for sampling allograft musculoskeletal tissue
Australian New Zealand Journal of Surgery DOI: 10.1111/ans.12661

Except for the formatting style, this chapter is unchanged from the published version.

9.1 Abstract

Allograft musculoskeletal tissue is commonly sampled by a swab for bioburden screening. To determine if bioburden recovery could be improved at the pre-analytical stage, two swab systems were evaluated, the Amies gel swab and the Eswab. In-vitro studies were performed to determine the recovery of each swab system with <100 CFU of challenge organisms using inoculated swabs and by sampling inoculated femoral heads. The standard culture protocol used in this laboratory was also evaluated after the sampling of inoculated femoral heads. A prospective study was performed with both swab systems used in parallel to sample cadaveric allograft musculoskeletal tissue. The challenge organisms could be recovered from the in-vitro inoculated studies. The standard culture protocol in this laboratory recovered all challenge organisms from both swab systems. 106 paired Amies and ESwabs were collected from 8 cadaveric donors with skin commensals the predominant isolates. The sampling of an inoculated femoral head was included to reflect routine swab sampling practice as was the inclusion of the standard method used in this laboratory. This appears to be the first study to compare Amies gel swabs with ESwabs to sample allograft femoral heads and in a prospective study with cadaveric allograft musculoskeletal tissue. Other comparative studies of swab systems have used a much higher inoculum to mimic an infection however sepsis is an exclusion criterion for allograft donors. It was found that the Amies gel swab and Eswab are both suitable sampling devices for bioburden testing of allograft musculoskeletal tissue.

Keywords: allograft; musculoskeletal, bioburden; Amies; ESwab

Varettas K. (2014) Evaluation of two types of swabs for sampling allograft musculoskeletal tissue. *ANZ Journal of Surgery*. DOI:10.1111/ans.12661

CHAPTER 10: RT-PCR TESTING OF ALLOGRAFT MUSCULOSKELETAL TISSUE – IS IT TIME FOR CULTURE-BASED METHODS TO MOVE OVER?

This chapter has been published in Pathology, the Journal of the Royal College of Pathologists of Australasia:

Varettas K (2014)
RT-PCR testing of allograft musculoskeletal tissue – is it time for culture-based methods to move over?
Pathology *In Press*

Except for the formatting style, this chapter is unchanged from the published version.

10.1 Abstract

Allograft musculoskeletal tissue samples are assessed for microbial bioburden to reduce the risk of post-transplant infection. Traditionally, solid agar and broth culture media have been used however nucleic acid testing, such as real-time PCR, has been described as more sensitive. This study evaluated the recovery of low numbers of challenge organisms from inoculated swab and musculoskeletal biopsy samples using solid agar culture, cooked meat medium, blood culture bottles and a RT-PCR assay. It was found that broth culture methods were the most sensitive with RT-PCR unable to detect low numbers of bacteria from these samples. Investigation of other non-culture methods may be worthwhile.

Varettas K. (2014) RT-PCR testing of allograft musculoskeletal tissue - is it time for culture-based methods to move over? *Pathology*. 46:640-643.

DOI: 10.1097/PAT.0000000000000163

CHAPTER 11: GENERAL SUMMARY & CONCLUSIONS

This thesis set out to provide an evaluation of the bioburden testing methods of allograft musculoskeletal tissue in Australian microbiology laboratories. The primary aims were to determine the optimal type of sample required for culture and evaluate culture media and testing methods, with the inclusion of nucleic-acid based testing (NAT), which had not previously been evaluated for the bioburden testing of allograft samples. Preliminary investigations highlighted the lack of information available, not only on the Australian tissue banking industry, but also the microbiology laboratories providing testing. As a result, this thesis begins with comprehensive background information on the Australian tissue banking industry and progresses onto specific information on microbiology laboratories and the micro-organisms encountered in allograft samples before evaluating allograft sample collection, types of allograft samples and bioburden testing protocols.

Musculoskeletal tissue transplantation is a common procedure in Australia and worldwide. Chapter 1 provided an introduction on the variety of allograft musculoskeletal tissue that can be transplanted and used for a range of debilitating conditions ranging from constructive orthopaedic surgery to urogynaecological conditions. Musculoskeletal tissue can be retrieved from living or cadaveric donors and can be transplanted from a donor to a recipient without the need for compatibility testing. Tissue banks are involved in the donor assessment, tissue retrieval, processing, storage and distribution of allografts. Bioburden testing of allograft musculoskeletal tissue is part of the donor assessment and is performed by microbiology laboratories licenced by the Therapeutic Goods Administration (TGA) to the code of Good Manufacturing Practice (GMP) - human blood and blood components, human tissues and human cellular therapies (2013).

Chapter 2 focused on the inter-relationship between Australian microbiology laboratories and tissue banks previously mentioned in chapter 1. The differences in the allograft samples collected for bioburden testing and the culture media and testing methods used between the laboratories was highlighted. In such a heavily regulated area such as tissue banking, there is no harmonisation of sample types or methods used for bioburden testing in microbiology laboratories. The TGA acts purely as a regulatory body, ensuring compliance to the Australian Code of GMP - Human Blood and Blood Components, Human Tissues and Human Cellular Therapies (TGA 2013). The TGA does not provide any guidelines or recommendations on bioburden testing. However, laboratories and tissue banks are required to validate and justify allograft sample types and bioburden testing methods in use. Tissue banks are not able to fulfil

this requirement without the involvement of the microbiology laboratory. The Biotherapeutics Association of Australasia (BAA) provides guidelines and information regarding tissue retrieval and donor assessment questionnaires to tissue bank members however there is no guidance document for microbiology laboratories. This is not surprising as this is not the role of the BAA who must rely on the microbiology laboratories for their expertise. This thesis provides current information and evaluations to tissue banks and microbiology laboratories, thereby providing a comparison for other facilities and encouraging further evaluations in the area of bioburden testing.

It is essential to understand the importance of bioburden testing for allograft musculoskeletal tissue. Allograft samples must be considered important clinical samples in the microbiology laboratory and not just 'environmental' samples without any clinical relevance. Often bioburden testing of allografts is referred to as contamination testing, implying that organisms isolated from allografts are from external sources, but this is not always the case. Determining the presence of an organism in an allograft is more important than knowing its source. False-negative results produced by insensitive sampling techniques, culture media and testing methods can be potentially debilitating, if not fatal, to transplant recipients. The range of organisms isolated from allografts is very broad and were described in Chapters 3, 4 and 5 from living and cadaveric donors

Microbiology laboratories culture allograft musculoskeletal samples using a range of culture methods. The use of broth and/or solid agar culture media is up to the discretion of the laboratory. The methods used by each laboratory in Australia have not been previously described and were provided in Chapter 6. By using a questionnaire (Appendix 2) and keeping the laboratory identities confidential this chapter was able to promote discussion at the laboratory workshop held at the annual BAA conference in 2012. The importance of including a broth culture was highlighted in the 6-year retrospective analysis of swab samples in Chapter 7. Chapters 6 and 7 provide essential references for a laboratory's method review.

The microbiology laboratory has a duty of care to tissue banks, clinicians and allograft recipients who must be confident that the bioburden results reported by the laboratory are accurate. Microbiology testing requires interpretive judgement and the interpretation of results will be affected by the quality of the samples received for testing, the culture media and methods used. However, the quality of the sample collected is not under the control of the laboratory. The attention to pre-testing

conditions is just as important as conditions during testing. Inferior or inadequate sample collection can compromise organism growth and culture interpretation. Micro-organisms will grow and multiply in an allograft sample but may die before testing commences. Alternatively, organisms may multiply to the point of overgrowth, masking other less numerous organisms which are no less significant. The collection of a biopsy or swab allograft sample for bioburden testing was assessed in Chapter 8. A greater number of organisms were isolated from swab samples than biopsy samples and collecting both samples at the time of retrieval appears optimal.

Chapter 9 continued to focus on pre-testing factors and enhancing the quality of the sample collected. A new type of swab was evaluated, the ESwab, which had not previously been reported to sample allograft musculoskeletal tissue. The ESwab was claimed to increase the capture of organisms during sampling and the release of organisms during culture. It was found that the Amies gel swab and Eswab are both suitable sampling devices for bioburden testing of allograft musculoskeletal tissue. The inoculum from the Amies gel swab was not uniform across different culture media. The advantage of the ESwab is that it is able to inoculate culture media with an equal volume of inoculum and is optimal for automated inoculation and plate streaking instruments. However, the price of the ESwab may discourage its use in tissue banks with limited funding.

The comparison of culture-based methods with nucleic-acid based testing in Chapter 10 was an exciting part of this thesis. As with any laboratory method, the ultimate utility and application of direct molecular diagnostic methods based on amplification will depend on their accuracy, potential impact on patient care, advantages over currently available methods, and resources required to establish and maintain their use in the diagnostic setting. The lack of sensitivity of the real time polymerase chain reaction (RT-PCR) method compared to the culture-based methods was unexpected. The purpose of performing RT-PCR testing directly on samples was that it would act as a screening test and allow negative results to be reported without the need for further handling and potential contamination. Culture-based methods allow organisms to multiply after incubation periods of 18-24 hours, therefore low numbers of organisms can be isolated. Performing RT-PCR testing on a post-incubation medium, such as a broth culture, would not be of value as allograft samples would have had to undergo the labour intensive set-up process, with positive samples appearing turbid without the need for RT-PCR testing. Unlike other clinical samples from patients awaiting

treatment, turn-around-times are not the most important factor in reporting bioburden results of allograft samples.

Overall, a limitation encountered in this study was that the majority of tissue used during evaluations was bone from femoral heads, either as a biopsy or whole. Other musculoskeletal tissue was not available in the quantity required. Only in the prospective study comparing the two swab systems in Chapter 9 were other types of allograft musculoskeletal tissue included. Another limitation was the small range of challenge organisms used however a good cross-section of bacteria and fungi were used as recommended by the British Pharmacopoeia (2013).

Transplants of allograft musculoskeletal tissue will increase in the future as awareness is raised via the DonateLife network in Australia and other groups worldwide. The resulting increase in sampling and bioburden testing will impact on the few licensed clinical microbiology laboratories in Australia, placing additional strains on stretched resources. Many clinical laboratories have questioned the continuation of their expensive TGA licence, which is certainly not essential for their core business in a clinical setting.

This thesis investigated several factors that can affect the bioburden assessment of allograft musculoskeletal tissue. This study was successful in achieving its aims and the findings of this study have led to a greater understanding of the tissue banking industry in Australia and challenged sample types and methods used in the bioburden testing of allografts. Allograft musculoskeletal tissue must be considered ‘safe’ before being transplanted, that it is free of infectious agents such as bacteria and fungi. It is important that all parts of the tissue banking process continue to be improved and raise the benchmark on current accepted practices. Patients and surgeons must have confidence in the use of allograft musculoskeletal tissues and the best outcomes for allograft recipients must be foremost in the processes of the tissue banks and microbiology laboratories. A good relationship between the laboratory and the tissue bank is therefore important and collaborative investigations should be encouraged. The challenge is to continually revise and improve practices to maximise sensitivity of sampling and testing to determine the bioburden of allograft musculoskeletal tissues.

In summary, this study found that bioburden testing of allograft musculoskeletal tissue by microbiology laboratories is an important part of the tissue transplant process. The organisms isolated can range from commensal organisms such as diphtheroids to

clinically significant organisms such as *S. aureus* and *Clostridium* sp. Culture methods of musculoskeletal tissue samples vary between microbiology laboratories however this study highlighted that broth culture was essential. The combination of swab and biopsy samples for bioburden testing, commonly collected by tissue banks, increases organism isolation. Both the Amies gel swab and the Eswab system were found to be satisfactory sampling tools of musculoskeletal tissue. The detection of bioburden in samples of allograft musculoskeletal tissue was found to be optimal with culture-based methodology. The future direction of bioburden testing must be able to test and report on large numbers of samples with minimal handling and increased sensitivity. Further studies on non-culture methods may be beneficial.

CHAPTER 12: BIBLIOGRAPHY

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CHAPTER 13: APPENDICES

13.1 Appendix 1: Tissue Bank Questionnaire

1. What is the name of the person completing the questionnaire?
2. What are your contact details?
3. What is your position in the organisation?
4. Date questionnaire completed.
5. When was your tissue bank established?
6. Is your tissue bank involved in all aspects of bone banking? If not, what parts of the process are you involved in?
7. Are tissue (bone) samples retrieved from living donors?
8. For living donors, where is the tissue retrieval performed and who processes, samples and packages the tissue?
9. Are tissue samples retrieved from cadaveric donors?
10. Where are cadaveric retrievals performed?
11. Who is on the cadaveric retrieval team?
12. What are the cadaveric rules for retrieval - how long after death can tissue be collected, what is the order of collection?
13. Describe briefly the method used for processing of these samples after retrieval (e.g. what is involved, saline wash only, is an antibiotic wash solution used, which antibiotics?).
14. If relevant, are all tissue samples processed in the same way, for example, femoral heads and tendons?
15. What samples are retrieved for microbiology contamination testing – swab only, bone chip only, both swab and bone chip; other?
16. What is the name of the Microbiology laboratory that performs contamination testing for your tissue bank?
17. If a swab is used, what brand and type of swab is used for contamination testing – e.g. Copan Amies transport media without charcoal?
18. If a swab is used, when is the tissue sampled – before or after processing of the tissue?
19. At what temperature are microbiology samples stored (swab and/or tissue) until sent to the microbiology laboratory - fridge, freezer, room temp?
20. What type of sterilisaiton is performed, if any (irradiation, supercritical CO2)? If irradiated, what kGy is used? Is there a difference in the sterilisation level between living and cadaveric tissues?
21. Are all types of tissue sterilised after laboratory testing has been performed, regardless of culture results?

22. How is the tissue stored until required for transplant and for how long? (frozen at -80℃, freeze dried, 5 years)
23. Is any cryoprotectant or any other product added to the tissue before storing / freezing?
24. Are non-mandatory blood tests performed on musculoskeletal donors? (such as cytomegalovirus - CMV, Epstein Barr virus – EBV, toxoplasmosis).
25. Who distributes the tissue to surgeons requiring it for transplant?
26. Is it OK if your tissue bank is identified in regards to any of the information above or do you wish to remain anonymous?

13.2 Appendix 2: Bacteriology Laboratory Questionnaire

1. What is the name of the person completing the questionnaire?
2. What are your contact details?
3. What is your position in the organisation?
4. Date questionnaire completed.
5. What was the date when your laboratory was first licenced by TGA for contamination testing?
6. Are tissue samples received from living or cadaveric donors or both?
7. What types of samples are received from tissue (bone) banks?
   e.g. swab, bone piece, other?
8. Describe briefly the method used for testing of all samples received. e.g. what agar plates are used?
9. Is a broth medium used – which one?
10. Are swabs inoculated onto agar and broth media or agar plates only?
11. Are tissue pieces inoculated into broth media only?
12. What is the incubation period of each media?
13. What media is used to isolate fungi?
14. Does the laboratory process other non-tissue bank clinical or non-clinical samples (e.g. clinical samples from hospital in-patient)?
15. If applicable, is the set-up of samples from tissue banks integrated with the set-up of other patient samples or are tissue banking samples in a separate section?
16. Is it OK if your laboratory is identified in regards to any of the information above or do you wish to remain anonymous?