CONVENTIONAL AND MOLECULAR APPROACHES FOR BACTERIAL IDENTIFICATION AND QUANTIFICATION IN CHRONIC WOUNDS

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For My Grandmother
Lucyna Kamienska
(1939-2012)

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Khan K, Stuczen M, Jones H, Shedden D - Evaluation of a New Combined Chromogenic Medium and Collection Device for Methicillin –resistant *Staphylococcus aureus* (MRSA) with Confirmation by GeneXpert Polymerase Chain Reaction (PCR). ECCMID, Berlin, Germany, April 2013 – poster presentation.

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Stuczen M, Bowling FL, Edwards-Jones V - Evaluation of the New Sigma Transwab for maintaining viability of aerobic and anaerobic bacteria. American Society for Microbiology, New Orleans, USA (2011) – poster presentation.

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Stuczen M, Edwards-Jones V - Maintaining Viability of Aerobic and Anaerobic Bacteria from Wounds Using the New Sigma-Swab Transport System. American Society for Microbiology, Philadelphia, 2009 – poster presentation.

Edwards- Jones V, Stuczen M - Efficacy of a New Sigma-Swab Transport System in Maintaining Viability of Wound Pathogens. European Wound Management Association, 19th Conference, Helsinki, May 2009 – poster presentation.

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Stuczen M, Edwards-Jones V - Comparison of the New Sigma Swab with Amies Transport System for Maintenance of Bacteria Viability. IBMS Congress, September 2009 – oral presentation.

Pisanelli A, Persaud K, Bailey A, Stuczen M, Duncan R, Dunn K - Development of a Diagnostic Aid for Bacterial Infection in Wounds. AIP, Volume 1137: 133-135 (2009).

Stuczen M, Bowling FL, Boulton AJM, Edwards-Jones V - Use of Maggots Therapy to Reduce MRSA in Diabetic Foot Ulcers. Presented at Annual Research Student Conference, MMU, September 2008 – poster presentation.

Abstract

Wounds present an ideal environment for the growth of bacteria as they are usually moist and warm. The impact of bacteria on wound healing and developing infection is debatable and only partially understood. Some clinicians believe that the number of bacteria is a crucial factor in determining whether the wound is likely to heal. Others argue that the presence of specific pathogens and their interactions are the main cause of non-healing wounds. Also, the methodology of wound culture has been prone to controversy for many years. Most diagnostic laboratories use conventional microbiological techniques to indicate if there are pathogens in a wound. Some specialists still argue that greater than 10^5 organisms per gram of tissue is diagnostic for infection. Introduction of new molecular techniques have shown that only a small percentage of bacteria are identified and they grown in biofilms, which makes sampling difficult. In this project, the aspect of sample collection and transport was investigated as well as the conventional and molecular approaches for bacteria identification and quantification. Four different swab transport systems were tested for their ability to maintain viability of the most common wound bacteria during transport and their performance with molecular methods in order to establish the best swab transport devices for further testing on patients. The most satisfactory results were achieved with Sigma dry swabs and this swab remained the best choice for further *in vivo* studies involving both conventional and molecular techniques of bacteria identification and quantification. The semi-quantitative swab and biopsy culture was compared with quantitative culture to establish the best method for bacteria culture. Our findings demonstrated a statistically strong significant correlation between semi-quantitative and quantitative swab and biopsy methods and use of semi-quantitative count as a cost effective method compared to quantitative serial dilutions. However, when time is important rapid methods should be employed thus Real-Time PCR (RT-PCR) assay was developed for the direct and rapid detection of MRSA and compared with conventional methods. The diagnostic values of the RT-PCR assay for the detection of *mec*A and *fem*B genes were as follows: sensitivity 83.3%, specificity 88.5%, PPV 62.5% and NPV 95.8%. Quantitative analysis revealed that the average difference between the MRSA counts obtained using the RT-PCR and conventional culture results was 0.61 log. These findings show the potential of the RT-PCR assay in rapid detection and quantification of MRSA. Development of a RT-PCR assay for MRSA detection was the first step in developing a multiplex RT-PCR assay for chronic wound samples. In further studies, a DGGE-sequencing method was developed for the analysis of the diversity of microflora in chronic wounds and healthy feet and compared with conventional methods. DGGE-sequencing allowed identification of a number of strains not detected by culture techniques with 43% of the DGGE fragments representing organisms not cultured from the wound from which they had been amplified. This highlights the fact that a significant proportion of the resident microflora was not able to be analysed by culture. Development of PCR-DGGE sequencing and investigation of the diversity of microflora in chronic wounds allowed us to select the panel of microorganisms for the further development of multiplex RT-PCR assay for the rapid detection of bacteria in chronic wounds.

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Abbreviations

AHL – acyl-homoserine-lactone

Al-2 – autoinducer -2 signalling molecule

Api 20 NE – standardised system for the identification of non-fastidious, non-enteric Gram-negative rods

Api 20E – standardised system for the identification of of enteric bacteria

Api Staph – standardised system for the identification of the genera *Staphylococcus*, *Micrococcus* and *Kocuria*, which uses miniaturized biochemical tests and a specially adapted database

APS – Ammonium persulfate

ATCC – American Type Culture Collection

bFGF – basic fibroblast growth factor

bla – antibiotic resistance gene encoding a beta-lactamase

BLAST – Basic Local Alignment Search Tool

BRCA 1 – Breast Cancer 1 gene belonging to a class of genes known as tumor suppressor genes

BRCA 2 – Breast Cancer 2 gene belonging to a class of genes known as tumor suppressor genes

bTEFAP – Bacterial tag-encoded FLX amplicon pyrosequencing

C5A – protein fragment released from complement component C5

CA-MRSA – Community Acquired Methicillin Resistant *Staphylococcus aureus*

CBA – Columbia Blood Agar

CFU – Colony Forming Units

CNS – Coagulase Negative Staphylococci

Ct – threshold cycle

ddNTP – dideoxynucleotide triphosphate

DETC – Dendric Epidermal T-cells

DFU – Diabetic Foot Ulcer

DGGE – Denaturing Gradient Gel Electrophoresis

DNA – Deoxyribonucleic acid

dNTP – dinucleotide triphosphate

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EMRSA15 – Epidemic Methicillin Resistant *Staphylococcus aureus* strain 15

EMRSA16 – Epidemic Methicillin Resistant *Staphylococcus aureus* strain 16

EMRSA17 - Epidemic Methicillin Resistant *Staphylococcus aureus* strain 17

FAA – Fastidious Anaerobic Agar

FAM – BHQ – Black Hole Quencher

***fem* A – E -** genes encode proteins which influence the level of methicillin resistance of *S. aureus*

FEP – pathogenic group

FLX – GS FLX the latest pyrosequencing platform by 454 Life Sciences

FRET – Fluorescence Resonance Energy Transfer

GDM – Gestational Diabetes Mellitus

GF – Growth factor

GI – Gastrointestinal Tract

GISA – Intermediate Glicopeptide Resistant *Staphylococcus aureus*

GRE – Glycopeptide Resistant Enterococci

H5N1 – Avian influenza

HBOT – Hyperbaric Oxygen Therapy

HEX – Hexachlorofluorescein, popular amino-reactive fluorescent probe that is widely used in nucleic acid sequencing and related research

HLT – Healthlink Transporter Swab (Copan)

ICU – Intensive Care Unit

IGF-1 – Insulin-like growth factor

IgG – Immunoglobulin G

KGF – Keratinocyte Growth Factor

M40-A – Laboratory standard for Swab Transport Systems Evaluation

***mec*A –** antibiotic resistance gene

***mec*R1 –** methicillin resistance gene

MGB – Minor Groove Binding

MMPs – Matrix metalloproteinases

MR-CNS – Methicillin Resistant Coagulase Negative Staphylococci

mRNA – messenger RNA

MRSA – Methicillin Resistant *Staphylococcus aureus*

MSA – Mannitol Salt Agar

MSSA – Methicillin Sensitive *Staphylococcus aureus*

NCCLS – National Committee for Clinical Laboratory Standards

NCTC – National Collection of Type Culture

NPV – Negative Predictive Value

NPWT – Negative pressure wound therapy

NTC – no-template control

***Nuc* –** gene encoding thermonuclease of Staphylococci

PBP – Penicillin Binding Protein

PCR – Polymerase Chain reaction

PDGF – Platelet Deriver Growth Factor

PMN – Polymorphonuclear neutrophils

PPV – Positive Predictive value

PRAPS – Partial Ribosomal Amplification and Pyrosequencing

PSC – Pluripotential Stem Cell

PvP-iodine – Povidone iodine

QPCR – Quantitative Polymerase Chain Reaction

rDNA – ribosomal deoxyribonucleic acid

ROS – Reactive oxygen species

rRNA – ribosomal ribonucleic acid

Rsq – amplification efficiency value

RT-PCR – Real Time Polymerase Chain Reaction

Sa442 – small DNA fragment, popular DNA target for identification of *Staphylococcus aureus* by PCR

SAB – Sabouraud Agar

SARS – Severe Respiratory Acute Syndrome

SCC – Staphylococcal cassette chromosome

SCCmec – Staphylococcal cassette chromosome mec elements encoding methicillin-resistance

SCPI – Secretory Leucocyte Protease Inhibitor

STSs – Swab Transport Systems

SYBR green - asymmetrical cyanine dye used as a nucleic acid stain in molecular biology

TAE – is a buffer solution containing a mixture of Tris base, acetic acid and EDTA

TAMRA – Tetramethylrhodamine fluorophore

TBC – Total Bacterial Count

TEMED – Tetramethylethylenediamine

TGF-β – Transforming Growth Factor beta

tRNA – transfer Ribonucleic acid

TthDNA polymerase – is a thermostable enzyme that replicates DNA at 74°C and exhibits a half-life of 20 minutes at 95°C

VEGF – Vascular Endothelial Growth Factors

VISA – Intermediate Vancomycin Resistant *Staphylococcus aureus*

VRSA - Vancomycin Resistant *Staphylococcus aureus*

WESDAR – Wisconsin Epidemiological Study of Diabetic Retinopathy

WHO – World Health Organisation

Declaration

With the exception of any statements to the contrary, all data presented in this report are the results of my own efforts. No part of this report has been copied from other sources. In addition, no portion of the work included in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institution of learning.

Monika Stuczen

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CHAPTER 1

Literature overview

1.1 Background

One function of intact skin is to protect underlying tissue against infection by potential pathogens. Injured skin can provide a moist, warm and an environment rich in nutrients for bacterial colonisation and proliferation. The types and abundance of bacteria in any wound are influenced by factors such as wound type, depth, location and the level of tissue perfusion. Burn wounds and clean surgical wounds usually contain minimal numbers of bacteria, but the presence of devitalised tissue or foreign material can increase the likelihood of microbial proliferation (Bowler *et al*., 2001). Although most traumatic wounds heal without difficulty, some will become infected and cause increased morbidity such as pain and loss of function of the affected limb. Infected wounds often fail to heal causing increased trauma to the wound area. In some cases, wound infection leads to mortality and in all cases, increased intensity of health-care intervention and treatment cost (White, 2002). The more wound healing is delayed the greater the impact on the patient. For patients, this requires learning to live with the pain, emotional problems and social isolation associated with delayed healing (Franks and Moffatt, 1998; Hopkins, 2004). Costs associated with hard to heal wounds are higher as the frequency of therapy, staff time and product use increases (Tennvall and Hjelmgren, 2005; Tennvall *et al*., 2006).

A common factor associated with wound infection and a dysfunctional healing process is the presence of a heavy bioburden in the wound. There is currently widespread debate regarding the exact mechanism by which bacteria cause overt wound infection and also their significance in non-healing wounds that do not exhibit clinical signs of infection. One argument is that the numbers of bacteria is the critical factor in determining whether a wound is likely to heal (Heggers, 1998; Mangram *et al*., 1999). Alternatively, the presence of specific pathogens is argued to be of primary importance in delayed healing (Pallua *et al*., 1999; Schraibman, 1990). Some studies have

suggested the presence of bacteria to be of minimal importance in delayed healing (Trengove *et al*., 1996). A recent study suggests that bacteria produce specific types of communication molecules – autoinducer-2 (AI-2) and acyl-homoserine-lactones (AHLs), which are responsible for delayed healing. Manipulation of the cell-cell signalling pathways, especially in antibiotic - resistant pathogenic microorganisms, has the potential to be an effective strategy for wound healing (Rickard *et al.*, 2010). There is also a continued debate as to whether the method of wound sampling should be with a superficial swab or tissue biopsy to determine the presence of the potential pathogens in the wound and their role in subsequent treatment (Bowler *et al*., 2001).

1.2 Wound healing

Wound healing involves a variety of different processes (cellular, physiological, biochemical and molecular) which result ultimately in connective tissue repair and fibrous scar formation (Cockbill, 2002). This is a dynamic process coordinated by an array of cytokines and growth factors. It involves a complex interaction between epidermal and dermal cells, the extracellular matrix (ECM), controlled angiogenesis, and plasma-derived-proteins. Wound healing is divided into three overlapping phases – inflammation, proliferation and remodelling (Harding *et al*., 2002). The inflammatory phase begins immediately after injury and its purpose is to destroy, dilute or isolate the injurious agents and the injured tissue. It is characterised by pain, heat, redness, swelling and loss of function at the site of the wound and these classic signs of inflammation are also characteristic and can be confused with an impending wound infection. These can be seen almost immediately after injury (Mustoe, 2005). The initial response to the disruption of blood vessels is bleeding and the homeostatic response to this is clot formation to stop haemorrhage. Collagen and basement membrane proteins exposed by the injury activate Hageman factor XII, which is responsible for activation

of the healing cascade (Barker, 1986). There are several effector systems within the healing cascade such as the plasminogen cascade, the complement cascade, the kinin cascade and the clotting cascade. They release complement C5A, fibrin degradation factors, platelet activity factors and chemical mediators- serotonin and histamine, which interlink to control infection and regenerate tissue (Leaper, 1986).

Immediately after tissue injury, blood comes in contact with collagen triggering platelets to begin secreting inflammatory factors. Also, expression of glycoproteins on platelet cell membranes allows them to stick to one another and to aggregate forming a mass. Fibrin and fibronectin form a plug by binding together. This plug prevents further loss of blood, traps proteins and particles and also provides the main structural support for the wound until collagen is deposited and is used by the migratory cells as a matrix to crawl across (Midwood *et al*., 2004). Platelets adhere to it and secrete ECM proteins and cytokines including growth factors, which stimulate cells to speed up their rate of division. Platelets also release serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane and histamine. These factors increase cell proliferation and migration to the area and cause blood vessels to become dilated and porous. These factors cause vasoconstriction to prevent blood loss and also to collect inflammatory cells and factors in the area of injury. This process lasts about 20 minutes post-wounding. Histamine is the main factor causing vasodilation and allows the tissue to become oedematous as proteins from the bloodstream leak into extravascular space increasing its osmotic load and drawing water into the area. Increased porosity of blood vessels allows the entry of inflammatory cells from the bloodstream into the wound site. Within an hour of wounding, polymorphonuclear neutrophils (PMNs) arrive at the wound site, attaining large numbers within 24 hours (Mustoe, 2004). Neutrophils have a large impact on wounds (Davies, 2008). They phagocytise debris and kill microorganisms by releasing free radicals in the process called a respiratory burst (Greenhalgh, 1998). They also

clean the wound by secreting proteases breaking down damaged tissue. Patients with neutrophil immunodeficiency such as chronic granulomatous disease (CGD) are susceptible to bacterial infections of the skin and mucosal membranes (Davies, 2008). The polymorphonuclear neutrophils undergo apoptosis after completing their phagocytic tasks. They are followed temporarily by macrophages that appear in significant numbers within 2 or 3 days. Domination of macrophages in the wound indicates healing progress and recovery, whereas a large percentage of PMNs is an indication of existing problems (Diegelmann and Evans, 2004). Macrophages phagocytose bacteria and debride damaged tissue by releasing proteases such as metalloproteinases (MMPs), which degrade collagen, elastin, fibronectin, and other ECM components (Chizzolini *et al*., 2000). They are also the source of more than 30 different growth factors and cytokines, which attract cells involved in the proliferation stage of wound healing. The next stage of healing process is the formation of a provisional matrix of fibrin and fibronectin and is called the proliferation phase. By day 3, fibroblasts appear in the fibrin-fibronectin framework and initiate collagen synthesis and disposition, which is important because it increases the strength of the wound. The fibrin-fibronectin clot does not provide much resistance to traumatic injury. Inflammatory cells, cells involved in angiogenessis and connective tissue construction attach, grow and differentiate on the collagen matrix laid down by fibroblasts (Ruszczak, 2003). Fibroblasts proliferate in response to growth factors and become the dominant cell type during this phase. They secrete a range of growth factors - insulin-like growth factor one (IGF-1), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β) and keratinocyte growth factor (KGF). Keratinocyte growth factor (KGF) and transforming growth factor beta induce angiogenesis, which induces ingrowth and proliferation of endothelial cells, forming new capillaries. The stem cells of endothelial cells, originating from parts of uninjured blood vessels, develop

pseudopodia and push through the ECM. They stimulate connective tissue formation and directly enhance epithelialisation while KGF stimulates keratinocyte proliferation into the wound site to establish new blood vessels. This neovascularity is visible through the epithelium and gives the wound a pink or purple-red appearance. Capillary ingrowth provides the fibroblasts with oxygen and nutrients to sustain cell proliferation and support the production of the permament wound matrix. This is composed of collagen and proteoglycans or ground substance and replaces the provisional fibronectin-fibin matrix. Granulation tissue begins to appear during the inflammatory phase and continues growing until the wound is covered. It consists of new blood vessels, inflammatory cells, fibroblasts, endothelial cells, myofibroblasts and the component of a new provisional matrix (ECM), which is different in composition from ECM in normal tissue. The provisional matrix is composed predominantly of covalently cross-linked fibrin and plasma fibronectin (FN). It is remodeled over time to recapitulate normal tissue and this remodeling involves cell mediated contraction and deposition of new rich in fibronectin matrix (Clark, 1996; Midwood *et al.,* 2004). Over time the provisional matrix is replaced with extracellular matrix similar to ECM found in non-injured tissue (Lorenz and Longaker, 2003).

The remodelling phase begins when the collagen accumulation within the wound reaches a maximum. It usually happens within 2 to 3 weeks after wounding. Scars do not achieve the strength achieved by collagen in normal unbroken skin. They increase in strength over 6 months or longer, however they can only reach 70% of the strength of unwounded skin (Mustoe, 2005). This means that the post-wounded area is more sensitive and can more easily break down again.

There are several types of T-cells that have an impact on wounds. These are most prominent after wound closure, when microbial invasion is no longer a significant factor, suggesting that they play a role in the post-closure tissue remodelling phase

(Eming *et al*, 2007). The role of T helper 1- and T helper 2-cell subsets in wound repair is not well documented, though it is likely that they regulate the wound micro-environment at particular times and in particular ways by secreting their own distinctive cytokine profiles (Hart, 2002).

Another type of T-cell associated with the skin is γδ T-cells or dendric epidermal T cells (DETC). They are found in the epidermis in association with damaged, stressed or transformed keratinocytes. Studies in mouse wound models have shown that healing is delayed if DETC are deficient, and it is evident that they provide crucial signalling molecules controlling keratinocyte actions and macrophage infiltration (Jameson and Havran, 2007).

Reconstruction of the epithelial barrier (epithelialisation) begins within hours of the initial injury, when epithelial cells from the basal layer at the wound edge flatten and migrate across the wound (Mustoe, 2005). The epidermal cells in open wounds must migrate across a surface of granulation tissue. If there is a crust or eschar, then collagenases and other metalloproteinases must be released to allow the epidermal cells to burrow underneath and lift the eschar. The accurate level of moisture is important in dermal ulcers healing. It prevents formation of crusts and eschars and speeds epithelialisation (Mustoe, 2005). As soon as the first layer of cells restores the epithelial barrier, additional layers develop, restoring the basilar-to-apical order. As the cells mature, they restart keratin formation what regenerates the *stratum corneum* of the epidermis. This completes the restorative process of epithelialisation and also provides stable coverage. The process of epidermis reconstruction induces apoptosis of cells responsible for inflammation, and mesenchymal cells in the underlying dermis with initiation of the maturation process.

In a younger patient with prolonged inflammation, delayed epithelialisation often results in hypertrophic excessive scarring. In regulating the scarring process the epithelial

mesenchymal cell factors released by the epidermis are important. Homeostasis is not achieved until a mature *stratum corneum* fully restores the normal barrier to evaporation. The epidermis continues to release growth factors, which stimulate collagen production and scar formation (Mustoe, 2005).

After collagen deposition begins, the wound starts to contract and this process is an important event of the healing in open wounds. The surrounding skin is pulled over the wound to reduce its size when the open wound contracts. Wound contraction occurs much faster than epithelialisation and depends on size, location of the wound and on how loose the tissue in the wounded area is. Contraction happens at a speed of up to 0.75 mm per day (Romo and Pearson, 2006). The open wound is resurfaced by normal unbroken skin surrounding the wound. This is also an advantage in addition to increasing the speed of wound closure. Human skin adheres tightly to subdermal structures and is less elastic especially in lower leg. While contraction generally accounts for 90% of the reduction in wound size on the perineum, it only accounts for at most 30% to 40% of the healing of a lower leg ulcer. This is one of the most important reasons of delay in the healing of leg ulcers (O'Leary *et al*., 2002).

1.3 Wound types

1.3.1 Acute wounds

Acute wounds are caused by injury to intact skin and include bites, minor cuts, burns, surgical wounds, abrasions and also more traumatic wounds such as lacerations and gun-shot injures (Davis and Dunkley, 1992). Wounds are classified according to the number of layers affected. Injury limited only to the epithelial tissue is classified as a superficial wound and will heal rapidly by regeneration of epithelial cells. A partial thickness wound involves the deeper dermal layer and includes damage to the blood

vessels. A full thickness wound affects the subcutaneous fat layer and beyond and its healing will take longer as it requires the synthesis of new connective tissue (Cockbill, 2002). Acute wounds heal within a reasonable time frame after injury. Clean surgical wounds usually require minimal intervention to enable quick healing. However in more traumatic wounds, the presence of dead tissue, viable bacteria and non-viable foreign material is likely to require surgical intervention and in some cases antimicrobial treatment to progress natural healing (Leaper and Harding, 1998).

1.3.2 Chronic wounds

Acute wounds can transform into chronic wounds if the wound healing process is disrupted and they do not heal over the expected period of time irrespective of the cause. Additionally, they may form as the result of systemic infection, immune, vascular or nerve insufficiency or metabolic disorders such as diabetes (Crovetti *et al*., 2004). They are difficult to heal, may never heal or take years to do so. In general, a wound that does not heal within 3 months is termed a chronic wound (Mustoe, 2005). In chronic wounds the balance between production and degradation of collagen is lost and this is frequently caused by endogenous mechanisms, which disturb the integrity of dermal and epidermal tissue (Davis and Dunkley, 1992). There are several factors that may have an impact on wound healing. Such factors include age due to a decrease in inflammatory response and physiological processes such as blood circulation, reduction in collagen formation and basement membrane degradation (Doughty *et al*., 2007). Malnutrition can also prevent wound healing by decreasing collagen production and other proteins needed for wound repair. Bacteria in high numbers at the wound bed produce toxic end products and complete with cells in the granulation tissue for available nutrients (Evans, 2005). Stress has also been implicated in the impaired healing process with decreased wound healing associated with pain and noise (Kane,

2007). Chronic wounds may affect only the epidermis and dermis or they may affect tissue to the fascia. The majority of chronic wounds can be classified into three broad categories: venous leg ulcers (VLUs), pressure ulcers and diabetic foot ulcers (DFUs).

Approximately 20% of diabetic patients develop DFUs due to peripheral neuropathy, muscle atrophy, foot deformity and neuropathic fractures (Raja, 2007). These ulcers eventually became colonised or infected with different bacteria. Diabetic foot ulcers (DFUs) precede 85% of all diabetes-related lower-leg amputations (Reiber *et al*., 1995). The moment a person with diabetes suffers a breach in the skin of their foot, they are at danger of amputation. Currently, there are approximately 100,000 limb amputations performed in the United States every year. It is estimated that more than a million people with diabetes require limb amputation each year globally, an indication that an amputation is performed worldwide every 30 seconds (Jeffcoate and Bakker, 2005).

Venous leg ulcers (VLU) are localised in the lower limb or an area of damaged skin below the knee. They are thought to be due to venous hypertension caused by the improper function of the valves that exist in the veins to prevent blood from flowing backward. The body needs the pressure gradient between arteries and veins in order for the heart to pump blood forward through the arteries and into veins. When venous hypertension exists, arteries no longer have significantly higher pressure than veins and blood is not pumped as effectively into or out the area and it pools (Mustoe, 2005; Moreo, 2005). Venous hypertension may also stretch veins and allow blood proteins to leak into the extravascular space, isolating extracellular matrix molecules and growth factors, preventing them from healing the wound (Brem *et al*., 2004). Leakage of fibrinogen from veins as well as deficiencies in fibrinolysis may also cause fibrin to build up around the vessels, preventing oxygen and nutrients from reaching calls (Brem *et al*, 2004). There are also other factors that may contribute to venous leg ulcers such as

arterial disease, trauma, obesity, immobility, vasculitis and diabetes (Simon *et al.*, 2004). VLUs are associated with age and are less common among individuals who are less than 45 years old but the risk increases with age. Studies have shown that one in every 50 persons over the age of 80 years is affected by venous leg ulcers (Kane, 2007).

Pressure ulcers usually occur in patients with paralysis (temporary or permanent) that inhibit movement of body parts that are commonly subjected to pressure such as the heels, shoulder blades, and sacrum (Thomas *et al.*, 2005; Wilhelmi and Neumeister, 2008). Pressure ulcers are caused by ischaemia that occurs when pressure on the tissue is greater than the pressure in capillaries causing obstruction of blood flow into the area. Muscle tissue, which needs more oxygen and nutrients than skin shows the worst effects from prolonged pressure (Wilhelmi and Neumeister, 2008). In the UK almost 4-10% of patients admitted to hospital develop one or more ulcers of which the elderly are the most at risk with a high incidence rate of up to 79% (Lyder, 2003).

1.3.2.1 Pathophysiology of chronic wounds

The reasons why wounds do not heal are related to local factors associated with the wound itself and also with co-morbidities (e.g. diabetes). Based on evidence that chronic wounds are associated with high levels of pro-inflammatory cytokines (Warner and Grose, 2003) along with low levels of growth factors (Trengove *et al.*, 1999; Lauer *et al.*, 2000; Ludwig *et al.*, 2002) and high levels of matrix metalloproteinases (MMPs) (Trengove *et al.*, 1999) chronic wounds may be stuck in the inflammatory phase. There is also evidence to suggest that a subset of chronic wounds do not heal because they fail to complete epithelialisation especially in elderly patients. For example, keratinocytes have been shown to exhibit an age-related reduction in mitogenic response and *in vivo* studies have shown that the rate of re-epithelialisation is reduced in both aged rat and mouse models (Ashcroft *et al.*, 2003) and in humans (Holt *et al.*, 1992). Ashcroft *et al.*,

(2002) have suggested that this may be related to low level of growth factors including epidermal growth factor (EGF). There are other major factors that can lead to chronic wounds such as ischaemia, reperfusion injury, and bacterial colonisation (Mustoe, 2004).

1.3.2.1.1 Ischaemia

The interruption of blood supply results in ischaemia, which rapidly damages metabolically active tissues (Mallick *et al*., 2004). Ischaemia causes tissue inflammation and affected cells to release factors attracting neutrophils such as interleukins (Clark, 2005), chemokines, leukotrienes, and complement factors which lead to tissue inflammation. Ischaemia is an important factor in the formation of wounds and their persistance, especially when it occurs repetitively or when combined with a patient’s old age (Mustoe, 2004).

Neutrophils, while fighting pathogens, release damaging enzymes and inflammatory cytokines (Snyder, 2005) and also produce reactive oxygen species (ROS) to kill bacteria, for which they use an enzyme called myeloperoxidase (Mustoe, 2004). The enzymes and ROS produced by neutrophils and other leukocytes destroy cells, prevent cell proliferation and wound closure by damaging DNA, lipids, proteins (Alleva *et al*., 2005), the extracellular matrix, and cytokines that speed healing (Mustoe, 2004). Neutrophils remain in chronic wounds for longer than they do in acute wounds, and contribute to the fact that chronic wounds have higher levels of inflammatory cytokines and ROS (Schönfelder *et al*., 2005; Taylor *et al.,* 2005). Since wound fluid from chronic wounds has an excess of proteases and ROS, the fluid itself can inhibit healing by inhibiting cell growth and breaking down growth factors and proteins in the extracellular matrix (Snyder, 2005).

1.3.2.1.2 Bacterial colonisation

Bacterial colonisation is another major factor that influences wound healing. It is known that healing and bacterial load is a complex equation involving the type(s) and number of bacteria, and the patient's own immune system (Sibbald *et al.*, 2003; Bowler, 2003). In this context wounds may not necessarily show the clinical signs of infection but bacterial numbers (bioburden) may be sufficiently high to prevent normal wound healing.

Patients with decreased tissue oxygenation such as those who suffered hypothermia during surgery or diabetic patients have a higher risk for infection. Low levels of oxygen in the wound environment prevents white blood cells from producing reactive oxygen species essential for killing bacteria (Mustoe, 2004). The host's immune response to the presence of bacteria delays healing by prolonging inflammation and causing damage to the tissue. Infection can cause not only the wound to become chronic but can cause also gangrene, loss of the infected limb, and death of the patient (Dow, 2001).

Microbial colonisation and infection can further damage tissue by attracting a greater number of neutrophils to enter the wound site (Snyder, 2005). In chronic wounds, bacteria resistant to antibiotics such as Methicillin Resistant *Staphylococcus aureus* (MRSA) may have time to develop, colonise and infect the wound (Halcón and Milkus, 2004). Bjarnsholt *et al*., (2008) proposed a hypothesis to explain the involvement of bacteria in wound chronicity. They proposed that the presence of *Pseudomonas aeruginosa* in a biofilm and the lack of existing concurrent elimination by attended PMNs were the main causes of inefficient eradication by antibiotic treatment and antimicrobial activity of the immune system respectively. They used fluorescence *in situ* hybridization (FISH) to analyze sections from chronic wounds and found distinct microcolonies—the basal structures of bacterial biofilms. Studies have

previously reported increased tolerance to various antimicrobial measures and treatments as an indicator of biofilm formation. It has been shown that *in vitro* biofilms of *P. aeruginosa* produce a shielding mechanism that offers protection from the phagocytic activity of PMNs (Jensen *et al*., 2006; Bjarnsholt *et al.,* 2005).

1.3.2.1.3 Growth factors and proteolytic enzymes

The levels of proteolytic enzymes such as matrix metalloproteinases (MMPs) and elastase (Edwards *et al*., 2004; Schönfelder *et al*., 2005; Snyder, 2005) in chronic wounds are higher than in acute wounds, while the concentration of growth factors such as KGF and PDGF are lower (Crovetti *et al*., 2004; Schönfelder *et al*., 2005; Snyder, 2005). Therefore**,** an important factor in chronic wound formation may be inadequate growth factors levels (Crovetti *et al*., 2004). The formation and release of growth factors in chronic wounds may be prevented or the factors may be sequestered and unable to perform their metabolic roles. Also they may be degraded in excess by cellular or bacterial proteases (Crovetti *et al*., 2004).

Chronic wounds such as venous ulcers or diabetic foot ulcers may also be caused by a failure of fibroblasts to produce adequate ECM proteins due to a different gene expression in chronic wounds than in acute wounds (Foy *et al*., 2004). Epidermal growth factor has been shown to be degraded in chronic wound fluid compared to acute fluid, which suggests again a direct link between high protease activity and poor tissue regeneration (Trengove *et al*., 1999).

For full healing, wounds require a certain level of elastase and proteases. However, too high a concentration of these enzymes is damaging (Edwards *et al*., 2004). Elastase is released by leukocytes in the wound area (Edwards *et al*., 2004; Schönfelder *et al*., 2005). This enzyme increases inflammation, destroys tissue, proteoglycans and collagen (Kanda and Watanabe, 2005), and damages growth factors,

fibronectin, and factors that inhibit proteases (Edwards *et al*., 2004). The activity of elastase is increased by albumin, which is the most abundant protein found in chronic wounds. However, chronic wounds with inadequate albumin are especially unlikely to heal, so regulating the wound's levels of that protein may in the future prove helpful in healing chronic wounds (Edwards *et al*., 2004). High levels of matrix metalloproteinases (MMPs) released by leukocytes, may also cause wounds to become chronic (Stanley *et al*., 2005). Matrix metalloproteinases destroy growth factors, ECM (Stanley *et al*., 2005) and protease inhibitors. They increase degradation and reduce construction processes, which leads to balance disturbance (Lai *et al*., 2004; Schönfelder *et al*., 2005). There is strong evidence that activity of MMP decreases as the wound heals (Trengove *et al*., 1999). TiMP1 (Tissue inhibitor of metallopeptidase 1) is a glycoprotein that is expressed in tissue and involved in the degradation of the extracellular matrix. It is able to promote cell proliferation in a wide veriety of cells. Ladwig *et al* (2002), have shown that the ratio of MMP to TiMP1 may be an important factor, which allows a prediction of a wound's ability to heal. Many common wound bacterial species produce a wide array of MMPs that additionally have a negative impact on wound healing (Ladwig *et al*., 2002).

1.4 The Diabetic foot

1.4.1 Diabetes and its epidemiology

Diabetes mellitus is a group of metabolic diseases characterised by hyperglycaemia caused either by an impaired response of body cells to insulin or because the body does not produce enough insulin. In 2000, according to the World Health Organization, at least 171 million people worldwide (2.8% of the population) suffered from diabetes. The number of diabetic patients is increasing rapidly, and it is estimated that by 2030, it will double (Wild *et al*., 2004). There are three main types of

diabetes: Type 1, Type 2 and gestational. Type 1 is caused by the loss of beta cells of the Islets of Langerhans which produce insulin in the pancreas. The body then does not produce insulin, which leads to insulin deficiency. This type of diabetes is further classified as idiopathic or immune-mediated. The majority of type 1 diabetes is of the immune-mediated nature, where beta cell loss is a T-cell mediated autoimmune attack (Rother, 2007). Type 2 diabetes is characterized by insulin resistance. It may be combined with relatively reduced insulin secretion. The specific defects are still unknown, however the defective response of body tissues to insulin is believed to involve the insulin receptor. *Diabetes mellitus* due to a known defect is classified separately. Type 2 diabetes is the most common type (Tripathi and Srivastava, 2006). Gestational *Diabetes mellitus* (GDM) is diagnosed when pregnant women have high blood glucose and have never had diabetes before. It occurs in about 2% - 5% of all pregnancies and usually improves or disappears after delivery. In several respects it resembles type 2 diabetes as it involves a combination of insufficient insulin secretion and responsiveness (Lawrence *et al*., 2008).

1.4.2. Aetiology of diabetic foot ulceration

1.4.2.1 Neuropathy

Poorly controlled and mismanaged diabetes may cause damage to the body. Healthy diet or physical activity alone, or in combination with injections and/or tablets, is important in the prevention of diabetes complications. Persistent hyperglycemia can damage the small and large blood vessels and nerves and the most common diabetes complication is neuropathy. Up to 50% people with diabetes are affected by neuropathy (Adler *et al*., 1997). Diabetic neuropathies result from injury to small blood vessels that supply nerves in addition to macrovascular conditions that can culminate. Metabolic and neurovascular factors are the main reasons for causing neuropathy in diabetic patients.

Peripheral neuropathy is a type of neuropathy causing loss of pain or feeling in the legs and arms, feet and toes due to low blood perfusion and distal nerve damage (Wu *et al*., 2007). As the neuropathy progresses, patients lose feeling and in many cases are not aware of blisters and sores appearing on numb areas of the feet and legs. Any unnoticed pressure or injury to such areas may lead to bacteria colonisation and consequently infection. Diabetic neuropathy and ischaemia are the main risk factors associated with the development of diabetic foot ulcers (Wu *et al*., 2007). More than 80% of amputations occur after foot ulceration or injury resulting from diabetic neuropathy (Boulton *et al*., 2005). Loss of neural supply to the intrinsic muscles of the foot produces an imbalance of the long flexor and extensor tendons. Contraction of the more powerful flexors of the lower limb induces the classic high-arched foot and claw-toe deformity seen in as many as 50% of patients with diabetes (Borssen *et al*., 1990). Hyperextension of the toes with resultant overriding of the metatarsal-phalangeal joints forces the metatarsal heads downward, thereby increasing their prominence. Hyperextension of the toes displaces the metatarsal fat pads distally, further reducing the natural cushioning of the metatarsal heads. These mechanical changes increase plantar pressures inducing callus formation and underlying skin breakdown (Bowering, 2001).

1.4.2.2 Peripheral Vascular Disease

Peripheral vascular disease is the second major factor that contributes to the development of infection in the diabetic foot (Larkin *et al*., 1985; LoGerfo and Coffman, 1984). Chronic occlusive arterial disease and microangiopathy may play a role in this process (Lippmann, 1979). However, although microvascular disease is often cited as a cause of poor outcome in cases of diabetic foot wounds, there is little evidence to support its existence (LoGerfo and Coffman, 1984) and its importance has

been challenged. Macrovascular disease or atheroma occurs commonly in diabetics and is a major factor in the development of foot lesions. Atherosclerosis in the diabetic population tends to occur at an early age and with greater severity than in the non-diabetic population (Warren *et al*., 1966).

1.4.2.3 Immunological Aspects

Immunological Impairment Accompanying Hyperglycaemia (IIAH) is another pathophysiological factor important in the aetiology of foot ulceration. For many years there has been a general clinical impression that diabetic patients are more susceptible to bacterial infections than non-diabetic individuals (Savin, 1974). It is well known that acute infections lead to difficulty in controlling blood-sugar levels (Colwell, 1970) and that infection is the most frequently documented cause of ketoacidosis (Nabarro, 1965). However, controversy persists over whether well controlled patients with diabetes have an increased incidence of infection. It has been suggested that infection in these patients is not due to increased susceptibility but, once the dermal barrier of the foot has been broken down and a portal for infection of the deep tissue has been opened, alterations in their immune system renders the individual unable to fight infection (Brodsky and Schneidler, 1991). Various aspects of the immune system that are deficient in diabetic patients include neutrophil chemotaxis (Mowat and Baum, 1971), adherence to vascular endothelium, phagocytosis (Bagdade *et al*., 1974), intracellular killing (Nolan *et al*., 1978), serum opsonins (Rayfield *et al*., 1978) and cell-mediated immunity (MacCuish *et al*., 1974).

1.4.2.4 Other factors

Other contributory factors have been identified as possible causes of diabetic foot ulceration. These have included, duration of diabetes and glycaemic control (Moss

et al., 1992), abnormally high foot pressures (Veves *et al*., 1992), humoral immunity and social factors (Young, 1987; Reiber *et al*., 1992).

Diabetic patients have normal levels of immunoglobulins (Johnson, 1970) however, Ludwig *et al*., (1976) reported an increased number of diabetics without antibodies to *Bordetella pertussis* and diphtheria toxoid compared to controls. Also, a significantly lower number of juvenile-onset diabetics had agglutinins to *E. coli* and staphylococcal antigens.

Racial and ethnic differences in ulceration and amputation rates in diabetic individuals have been reported. Nelson *et al*., (1988) found that the rate of amputation in diabetic Pima Indians was higher than that reported in other diabetic populations and was significantly related to the duration of diabetes. Gujral (1994) reported that diabetic patients of an Asian origin had a lower incidence of amputation. The risk of amputation was found to be 2.3 times greater for the black population compared to the white population (Most and Sinnock, 1983).

The Wisconsin WESDR (Wisconsin Epidemiological Study of Diabetic Neuropathy) study reported that high glycosylated haemoglobin levels were associated with increased risk of foot ulceration (Moss *et al*., 1992). Moreover, Janka *et al*., (1980) found that there was an increased rate of peripheral vascular disease in diabetic individuals with poor glycaemic control.

Veves *et al.,* (1992) found that abnormally high foot pressure was positively associated with diabetic ulcer occurrence. Boulton (1996) reported that several factors, including orthopaedic problems, decreased pain and proprioception, limited joint mobility and small muscle wasting lead to increased pressure and loads under the diabetic foot. Extensive callus formation occurs at sites of increased pressure and intensifies the forces on the subcutaneous tissue and ultimately leads to foot ulceration.

Reiber *et al*., (1992) reported that single diabetics had an increased risk of amputation when compared to married patients. Lack of social support was also associated with lower extremity amputation. Patients who recognised the importance of diabetes self-care were less likely to have ulcers leading to amputation.

Thus it can be seen that critical factors which proceed ulceration and amputation may include not only pathophysiological conditions such as neuropathy, ischaemia and faulty wound healing but also include social, healthcare and environmental factors (Reiber *et al*., 1992). Each risk factor alone does not cause ulceration but when several factors act together it culminates in foot ulceration (Pecoraro *et al*., 1990). In most diabetic patients with foot ulceration there is a combination of circumstances which leads to the ultimate clinical lesion.

1.5 Microbiology of healthy skin

Skin is an integral part of the immune system and forms the first line of defence against pathogens by reducing microbial adherence and invasion (Nizet *et al*., 2001; Schroder and Harder, 2006). The outer surface of adult skin is colonised by a small number of culturable bacteria, which can be regulary detected and they represent a population refered to as the resident or normal microflora (Noble, 1980; Mackowiak, 1982). Skin also provides a supportive environment for other microorganisms, which are called transient bacteria and are not permanent residents of skin (Price, 1938). The role of these microorganisms in infection is still unknown, although it is highly likely that they influence the infection life cycle (Percival *et al*., 2012). The composition and the density of skin microflora varies with anatomical site. The highest density has been reported in moist regions such as the axillae, groin and between toes (Percival *et al*., 2012).

The Human Microbiome Project (HMP) was initiated in 2007 to analyse the microflora of human skin using molecular approches. The aim of the project was to better define molecular tools, indicate the limitations of standard culture techniques and redefine the microflora of various body sites including sebaceous, moist and dry location. The study found using standard cultural techniques, that the majority of microorganisms inhabiting the skin are viable, but nonculturable (VBNC).

There are many factors affecting microbiology of human skin such as age (Noble and Somerville, 1974), sex (Wilburg *et al*., 1984), skin site, level of hygiene and type of cleansers used, climate, race, occupation and whether an individual is hospitalized (Larson *et al*., 2000). The most frequently identified bacteria from human skin are coagulase negative staphylococci (CNS). Fifty percent of CNS are identified as *S. epidermidis*, which are particulary abundant in the upper regions of hair follicles (Harmory and Parisis, 1987; Vuong and Otto, 2002). Other CNS isolated include *S. saprophyticus*, *S. hominis*, *S. warneri, S. haemolyticus* and *S. capitis*. *S. aureus* is a commonly isolated bacteria and pathogen particularly prevalent in the anterior nares of humans (Nagase *et al*., 2002). The most predominant species isolated from the head, legs and arms are Coryneforms, micrococci and *Bacillus* spp (Kloos, 1981). Some studies report Gram negative bacteria such as *Acinetobacter* and *Pseudomonas* spp isolated from human skin particulary during the warmer months of the year (Seifert *et al.,* 1997; Berlau *et al.,* 1999). The most common microorganisms identified from human skin include *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Propionobacterium*, *Malassezi, Brevibacterium*, *Acinetobacter* and *Dermabacter* (Percival *et al*., 2012).

Skin has many defensive mechanisms that protect the body from invasion by strict and opportunistic pathogens (Barak *et al*., 2005). Dead keratinised cells in the upper layer of epidermis inhibit microbial adherence. The low level of nutrients and

high levels of keratin in the *stratum corneum* limits bacterial density. The continuous shedding of squamous epithelial cells from the skin serves to remove attached microorganisms from the skin surface and within the skin layers. Skin's lymphoid tissue is composed of Langerhans cells and dendritic cells. These cells are antigen presenting cells and they possess surface molecules that recognise specific markers associated with pathogens. They are involved in mediating both the humoral and cell mediated responses of the immune system. Over 20 antimicrobial peptides (AMPs) have been reported on the surface of human skin (Schauber and Gallo, 2009) and these generally exhibit a broad spectrum antimicrobial activity. AMPs are produced by many types of skin cells including mast cells and keratinocytes (Braff *et al*., 2005) and protect skin from microbial invasion. Finally, the high salt concentration on skin is also antimicrobial and this occurs partly due to sweat evaporation.

1.6 Microbiology of chronic and acute wounds

The human host and microorganisms normally exist in a balanced relationship (Cooper, 2005). Infection occurs when microorganisms overcome the host's natural immune system and subsequent invasion of bacteria in viable tissue provoke a series of local and systemic host responses (Thomas, 2008). Microorganisms are likely to enter the wound from three main sources: the environment (exogenous microorganisms in the air or those introduced by traumatic injury), the surrounding skin (involving normal skin flora such as *Staphylococcus epidermidis,* micrococci, propionibacteria), and endogenous sources involving mucous membranes (gastrointestinal, oropharyngeal, genitourinary mucosae). The normal microflora of the gut, the oral cavity, and the vagina are both diverse and abundant and these sources supply the vast majority of bacteria that colonise wounds. According to many studies that have investigated the role of microorganisms in wound healing, the most common pathogens in chronic and

acute wounds are aerobic and facultative bacteria such as *Staphylococcus aureus, Pseudomonas aeruginosa* and β- haemolytic streptococci (Danielsen *et al*., 1998; Halbert *et al*., 1992; Pal'tsyn *et al*., 1996; Sehgal and Arunkumar, 1992; Bowler *et al*., 2001). The presence of anaerobic bacteria in wounds may be significant, but they are often overlooked as many standard laboratories do not routinely screen for them (Thomas, 2005), although many are potentially highly virulent.

Bacterial species in chronic wounds usually exist in synergistic relationships with other bacteria. They rarely exist in pure culture. In many chronic wounds, when using conventional cultural techniques, the number of species of aerobic bacteria recovered range from 1 to 8 with an average of 2.7 different bacterial species per wound (Hutchinson, 1994).

Different wounds support different communities of microorganisms (Bowler *et al.,* 2001). The presence of bacteria in a wound may result in three clearly defined outcomes such as contamination, colonisation and infection. Contamination exists when the bacteria do not replicate in the wound and wound healing is not delayed. There are no suitable nutritive and physical conditions for bacteria and they are not able to successfully evade host defences. The wound is colonised when bacteria grow and multiply, but do not cause damage to the host or initiate wound infection. When bacteria multiply they can release extracellular products such as toxins and enzymes into the local environment and the wound healing becomes disrupted, wound tissue is damaged and there are signs of local infection.

Over 10 years ago the term "critical colonisation" was introduced as a stage between colonisation and infection. Davis (1996) defined critical colonisation as "multiplication of organisms without invasion but interfering with wound healing". White, Cutting and Kingsley (2006), explained it as the inability of the wound to maintain a balance between altered bioburden and an effective immune system,

indicated by an unexplained delay in healing but not necessarily a deterioration in the wound or other overt signs of clinical infection. However the concept of critical colonisation was dismissed by some clinicians who expressed the view that the wound is either infected or not without prodromal phase of infection (White and Cutting, 2006). Therefore it must be recognised that at present the term “critical colonisation” is theoretical. Additional research is required especially with regard to the diagnosis and appriopriate treatment of wounds (O’Brien, 2007).

1.6.1 Microorganisms isolated from diabetic foot ulcers

Diabetic foot infections are usually polymicrobial in nature and are attributed to aerobic bacteria such as *Staphylococcus aureus*, including MRSA strains, coagulase negative staphylococci, *Streptococcus* sp., Enterobacteriaceae and anaerobic flora such as *Bacteroides* sp., *Clostridium* sp., *Peptostreptococcus* sp., and fungi (Bowler *et al*., 2001; Dowd *et al*., 2008; Bansal *et al*., 2008).

Traditionally, studies of the wound microflora have concentrated on the role of the most common and easy to culture pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa.* These organisms are easy to grow and identify using traditional microbiological methods. Therefore, conventional methods are likely to overestimate the contribution of these species to the bioburden of chronic wounds. Nowadays, researchers and clinicians are beginning to realise that the diversity of microflora in chronic wounds may be an important contributor to the chronicity of wounds.

Wheat *et al.,* (1986) used an advanced approach to culturing bacteria, and discovered *Staphylococcus*, *Enterococcus*, and *Corynebacterium* sp. were the most common aerobic (or facultative) bacteria in foot ulcers. They also evaluated anaerobes in foot ulcers and identified common anaerobes such as *Peptostreptococcus* and

Bacteroides species. In other studies (MacFarlane *et al*., 1986; Ceilley, 1977) identified the importance of *Corynebacterium sp.* in foot ulcers. *Corynebacterium* species belong to the normal skin and mucous membrane microflora and have been considered to be non-pathogenic. However, bacteria such as *Staphylococcus epidermidis* had previously been mistakenly viewed as non-pathogenic for the same reason (Cogen *et al*., 2008). Although *Corynebacterium* may not be a common cause of acute infections, it appears to be a common (but overlooked) factor in chronic diabetic foot ulcer infections (Bessman *et al*., 1992). In the study by Dowd *et al*., 2008 *Corynebacterium* was the most predominant genus identified using the molecular approach. The most common species identified were *C. striatum*, *C. amycolatum*, *C. tuberculostearicum*, and *C. mucifaciens*. *Corynebacterium striatum* was identified in 22 of the 30 *Corynebacterium*-positive samples and this species has been associated with infections involving joints and open fracture wounds (von Graevenitz *et al*., 1998). Another study has associated *Corynebacterium* with diabetic foot osteomyelitis (Hartemann-Heurtier and Senneville, 2008) .

The impact of anaerobes in chronic wounds has also been well documented in the literature and anaerobic species are beginning to be recognized as a major population in chronic wound biofilms (Dowd *et al*., 2008; Bowler and Davies, 1999). The importance of anaerobes such as *Peptostreptococcus, Prevotella*, *Finegoldia* and *Peptoniphilus* sp. has been previously reported (Trengove *et al*., 1996). Anaerobes may be the most prevalent physiological type for a given wound or an individual wound type even though wounds are usually exposed to air. Bowler *et al.,* (1999) evaluated venous leg ulcers using cultural isolation techniques that included special considerations for the propagation of anaerobes. They reported that anaerobes represented 49% of the total microbial composition in such wounds. Dowd *et al.,* (2008), used a pyrosequencing approach to investigate the bacterial population of diabetic foot ulcers and reported that

30% of the sequences detected were anaerobes.

1.6.2 Epidemiology and characteristics of Methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus was first identified in the late 19th century and has since been recognised as part of the natural flora of humans. It frequents the face, hands and perineum, with the most common site being the nares (nostril) (Williams, 1963). Between 30% and 60% of the healthy population carry *Staphylococcus aureus*, of which between 10% and 20% are chronically colonised (ongoing, persistent population of *Staphylococcus aureus* on or in the body but in the absence of infection) (Foster, 2004).

MRSA is reported as the leading cause of wound infections in most parts of the world. The high prevalence of MRSA colonisation in diabetic foot ulcers is a consequence of antibiotic overuse and the selection of broad rather than narrow spectrum agents. In 2002, MRSA prevalence in skin and soft tissue infections was reported in the USA as 44.4%, in Italy as 41.8%, France as 34.7%, in Spain as 32.4% and in Germany as 12.4% (Lipski, 2004). MRSA is now endemic in both community and hospital environments and there has been a reported increase in hospital stays, increased cost, and increased morbidity and mortality associated with these infections compared to other diabetic foot ulcer infections (Lipski, 2004; Reiber *et al*., 1998). There is also evidence that MRSA colonisation of chronic ulcers is associated with delayed healing times (Bowling *et al*., 2007). Strategies to eliminate MRSA from colonised wounds are therefore essential and should include the use of simple, low-cost, effective treatments. MRSA mainly exists on the superficial part of the skin, is easy to detect and it is an excellent indicator organism that can be used to compare diagnostic methods and to demonstrate the ability of methods to assess the presence of microbial diversity. The number of deaths in England and Wales caused by MRSA decreased by

37% in 2009 to 781 from 1,230 in 2008. Deaths involving *Staphylococcus aureus* (including those which did not specify methicillin resistance) fell by 16% from 1,500 in 2008 to 1,253 in 2009. In 19% of deaths in 2009, MRSA infection was recorded as the underlying cause of death in UK. This figure varied between 17% and 36% over the 1993-2009 period (National Statistics. Deaths Involving MRSA: England and Wales, 2007 to 2011. Statistical Bulletin, August 2012).

1.6.2.1 Genotype and phenotype

MRSA is a Gram positive non-motile, non-facultative *coccus.* Optimum environmental conditions for growth are temperatures between 15ºC and 45ºC. High concentrations of sodium chloride do not alter growth, even when concentrations reach up to 15% (Parfentjev and Catelli, 1964). It is coagulase positive, making it distinguishable from the other Staphylococcaceae in laboratory testing and it is catalase positive which differentiates it from *Streptococci* species. MRSA produce a number of virulence factors which elicit suppurative infections and toxinosis. Surface proteins allow bacterial attachment to the extracellular matrix of the host, specifically the proteins laminin and fibronectin, found in epithelial and endothelial tissue (Lowy, 1998). Toxins produced by the bacteria damage host cell membranes and allow cell invasion. Alpha toxin is produced as a monomer that binds to the membrane of the susceptible cell. Sub-units then combine to form heptameric rings with a central pore, through which the cellular contents leak. In humans, platelets and monocytes are particularly sensitive to alpha toxin, thus inducing the release of inflammatory mediators (Menzies and Kourteva, 2000). Theta toxin is a small peptide toxin produced by most strains of *Staphylococcus aureus* (Dinges *et al*., 2000). Leukocidin toxin is a haemolytic multi-component protein which forms a hetero-oligomeric, transmembrane pore, composed of sub-units, to create an octameric pore in the membrane (Miles *et al*.,

2002). Panton Valentine leukocidin is an exotoxin and is also responsible for pore formation within host membranes (Lina and Piemont, 1999). The majority of strains of *Staphylococcus aureus* express a surface polysaccharide or microcapsule (type 5 or 8), which is thought to interfere with phagocytosis. Protein A is another surface protein which binds with IgG molecules (Foster, 1998).

The wide range of toxins associated with *Staphylococcus aureus* have been identified as causing a range of illnesses, including vomiting, sepsis and toxic shock syndrome (McCormick *et al*., 2001). Others include skin and soft tissue infections, such as cellulitis and abscesses, acute bacterial endocarditis, bacteraemia, infections associated with intravenous cannula sites, central venous access sites, osteomyelitis and post-operative wound infections.

It is, therefore, of little surprise that *Staphylococcus aureus* infections were associated with a mortality rate of almost 80% during the pre-antibiotic era. By 1942 this had reduced significantly with the introduction of penicillin (Smith and Bradshaw, 2008). The cessation was short-lived, however, when strains emerged that expressed inhibitory penicillinase. Towards the end of the 1950s, methicillin was introduced as a synthetic penicillin alternative. In 1961 the first MRSA isolates were reported in a British study (Jevons, 1961) and between 1961-1967 there were infrequent hospital outbreaks in Western Europe and Australia (MRSA Research Centre. MRSA History Timeline 1959-2012. Univeristy of Chicago Medicine available at http://mrsa-research-center.bsd.uchicago.edu/timeline.html).

By the 1980s epidemic strains of MRSA were being isolated from hospitals in the north east Thames and Essex regions, which were resistant to a range of antibiotics (Duckworth *et al*., 1988). Over the following ten years, strains of Epidemic Methicillin

Resistant *Staphylococcus aureus* (EMRSA) continued to emerge and were numbered EMRSA 1-14. Early in the 1990s, EMRSA-15 and later EMRSA-16 were isolated from UK hospitals (Johnson *et al*., 2001). These two identified strains have developed into the most successful and most resistant and continue to be isolated from hospitals around the world. EMRSA-17 appeared in 2002 (Aucken, 2002) and has an expanding resistance profile. Phenotypical-resistance to beta lactam antibiotics in MRSA is derived in part from the production of beta-lactamase, which destroys the beta-lactam ring of this group of antibiotics and also its penicillin-binding protein (PBP2a). The PBP is responsible for the integration and regeneration of cell wall components. Methicillin belongs to the beta-lactam group of antibiotics whose mode of action depends on their tendency and high affinity for combining with the PBP of the target bacteria. MRSA has PBP2a which has a low affinity for the beta-lactam antibiotics, thus maintaining cell wall synthesis, despite PBPs 1 through 4 being bound and inactivated by the drug (Richmond, 2002). Genetic expression of resistance to beta lactam antibiotics is determined by the *mec*A gene housed on the Staphylococcal cassette chromosome (SCC), which functions as a mobile genetic island that inserts and integrates with the *S. aureus* chromosome. The SCC can also detach from the chromosome and this freedom of movement allows for the horizontal transfer of SCC*mec*. The *mec*A gene itself is thought to be regulated by a number of other genes, constituting a *mec* complex, in which at least six different classes have been identified (class I through to class VI) (Berglund *et al*., 2009). These differ slightly in terms of the *mec* gene itself and also in the number or type of genes assisting *mec* in its insertion and excision from the chromosome. The *mec* gene is comprised of 2 regulatory components (*mec* and *bla*) and 5 auxiliary genes (Fem A-E). The regulatory component *Mec*R1 and *mec*1 are responsible for suppressing *mec*A transcription, and the *bla* genes play important roles both in controlling β-lactamase

expression and suppressing *mec*A gene expression (Inglis *et al*., 1988; Arede *et al*., 2012).

Typing of strains of MRSA through the SCC*mec* has revealed that nosocomial strains are mainly clonal in origin. For example, EMRSA-15 and EMRSA-16 are spread internationally and remain responsible for a high number of UK cases. There are currently 17 strains of EMRSA in the UK (Aucken, 2002).

A study by Enright in 2002 examined 359 MRSA isolates that were distributed worldwide and identified five lineages or clonal complexes, i.e. the vast majority of these isolates shared intimate genetic characteristics with each other (Enright *et al*., 2002). Lim *et al.,* (2002) examined 35 EMRSA from England and Australia and found distinct characteristics that allowed grouping of isolates according to genetic similarities. For example, a group of recent isolates were identified as sharing the same *mec*A complex as strains from America and New Zealand isolated in the 1980s (Lim *et al*., 2002).

Community MRSA (CA-MRSA) is now well recognised and was initially thought to be identical to nosocomial MRSA apart from the environment in which it flourished. When cases began to emerge in populations lacking the usual risk factors for MRSA, further investigation followed. One strain in the US resulted in four paediatric deaths (Gillet *et al*., 2002) and, during analysis, was found to be genetically distinct from nosocomial forms, due to a different *mec* complex (SCC*mec* IV) and the presence of a gene for Panton-Valentine leukocidin toxin. This particular CA-MRSA has demonstrated signs of global spread (Vandenesch *et al*., 2003), but there is a need for further testing using a variety of mechanisms, to reach agreement as to whether community and hospital strains are totally separate entities. There is also a scarcity of

data regarding genetic similarities or differences between methicillin sensitive *Staphylococcus aureus* (MSSA) and MRSA. Essentially, MRSA is so flexible and adaptable that there is huge potential for any number of genetic combinations.

1.6.2.2 MRSA prevalence

Between 1997 and 1999 the SENTRY Antimicrobial Surveillance Programme reported the prevalence of MRSA in isolates from all sites of infection (Diekema *et al.*, 2001). In Hong Kong 73% of isolates were positive for MRSA, the US 34.2% and in Europe 26.3%.

By 2001 a report from the European Antimicrobial Resistance Surveillance System identified prevalence for MRSA in the UK as 45% and in both Italy and Greece as 40% (European Antimicrobial Resistance Surveillance System. Annual Report, 2001). In a study of elderly hospital in-patients, at three weeks post admission, the prevalence of MRSA was 158/1000, which is 15.8% (Hori *et al.*, 2002). Grundmann *et al.*, (2002) sampled the prevalence of MRSA carriage in the normal elderly population living in their own homes. They found that those between the ages of 65 and 74 years had a prevalence of 2%, which increased to 6% as the age range increased to 75 years of age (Grundmann *et al.*, 2002).

1.6.2.3 Transmission

MRSA can be maintained and transmitted in any number of ways, but the most common is through human to human contamination, more specifically through healthcare worker to patient. A colonised staff member can easily assist in dissemination, throughout the course of a shift, by either acting as a carrier between patients or between contaminated inanimate objects and patients (Aucken, 2002).

MRSA has been isolated from computer keyboards, door knobs and stainless steel surfaces (Noyce *et al*., 2006), in addition to the more obvious sites such as hands, uniforms and watches. Multiple modes of transmission have led to MRSA spreading around the globe**.** Within the UK a study analysing 129 isolates of EMRSA-16 from 52 hospitals showed the dissemination of a strain originating in one hospital. Beginning in Kettering, it first spread to neighbouring counties with 15 hospitals. The strain then appeared in 21 hospitals in London and, over the course of three years, it was isolated from every region in the UK. Intercontinental spread has also been identified during analysis of a Canadian strain, which was later found to originate in India (Roman *et al*., 1997).

1.6.2.4 Risk factors for MRSA

Initial studies of risk factors for MRSA infection examined patients in ICU and burns units, as these groups were associated with high infection rates (Pujol *et al*., 1996). Patients with surgical wounds, enteral feeding tubes and intravenous or urinary catheterisation all increase risk (Coello *et al*., 1997). Also, colonisation with MRSA is a risk factor for the development of infection. In a study following 209 colonised patients, 29% developed infections over an 18 month period (Huang *et al*., 2003). Davies *et al*., (2004) studied infection rates following nasal colonisation and found that 19% went on to develop infection, compared with only 2% of the non-colonised group. Similarly, a study of colonised chronic ulcers showed a prevalence of 3.9% for developing MRSA bacteremia (Roghmann *et al*., 2001). Within the elderly hospital population risk factors identified are increasing age and length of stay, while their counterparts in the community were found to be at risk due to previous hospital admission and diabetes (Hori *et al*., 2002; Grundmann *et al*., 2002). Previous antibiotic use is an acknowledged risk factor and a recent meta-analysis of a study population of 4,364 MRSA-positive

and 19,865 control patients showed that those with recent exposure to antibiotics had a two-fold increase in risk for MRSA acquisition, versus their non-treated counterparts. The risk is even higher if previous treatment included quinolones or glycopeptides (Tacconelli *et al*., 2008), ciprofloxacin and cephalosporin (Hill *et al*., 1998) or cephalosporins and fluoroquinolones (Graffunder and Venezia, 2002).

1.6.3 Wound infection

Infection occurs when host defences are successfully evaded by micro-organisms and results in destructive changes in the host (Cooper, 2005). Infection in acute or surgical wounds in healthy patients is usually obvious. In chronic wounds and debilitated patients, diagnosis may rely on recognition of subtle or non-specific general signs (such as loss of appetite, malaise, or deterioration of glycaemic control in diabetic patients). The presence of infection delays the healing process, but also can lead to necrosis, which increases the size of the wound. Rapid recognition of the signs and symptoms of infection are crucial and reduces the risk of cross-infection (White, 2002).

Different types of wounds have different environments. Intact skin, acute and chronic wounds provide distinctly different environments for microflora (Mertz and Ovington, 1993). The intact skin contains natural microflora, which metabolize substances secreted onto the skin and produce fatty acids preventing the colonization of skin by pathogens. Also, the sweat contains antimicrobial substances such as lysosyme. Acute wound flora is similar to that of intact skin with aerobic and some anaerobic species, whereas traumatic tissue provides an environment suitable for microbe adherence, multiplication and point of entry to surrounding tissue, making it more prone to infection (Mertz and Ovington, 1993). Necrotic tissue and slough provide good medium for bacterial multiplication and according to many studies, almost all bacterial types have been implicated in the pathogenesis of wound infection at some time. Necrotic tissue and large amounts of exudate will encourage microbial proliferation,

while a dry wound environment hinders tissue repair. Infection involves cellulitis and with inappropriate treatment it can delay wound healing and may lead to septicaemia (Grey, 1998).

1.6.4 Biofilms and their role

Biofilms are found widely in nature and they are biologically and physically diverse dynamic communities usually comprising a mixed population of different microorganisms. A secretion of extracellular polymeric substance (EPS), a "glue", protects individual bacteria from environmental stresses, provides shelter for the unique heterogenous micro-niches inside the biofilm and holds the community together (Wolcott and Rhoad, 2008). The microcolony of the biofilm achieves a critical density of bacteria (a quorum) through the release of signalling molecules and permits differentiation into a true biofilm society (Stoodley *et al*., 2002).

Over the years, studies regarding bacterial structure and behaviour have used planktonic cells that are cultivated in liquid or solid media. However, recent studies have shown that naturally most bacteria are attached to surfaces as a sessile form especially in biofilm (Costerton, 2005). Biofilm formation comprises three main stages: reversible and irreversible attachment, microcolony formation and detachment of biofilm. Biofilm formation starts from attachment of a pioneer bacterium onto the surface. Initial attachment is mainly dependent on electrostatic attraction and physical forces, rather than the chemical attraction (Postollec *et al*., 2006). There are two types of attachment during biofilm formation, reversible and irreversible attachment. Reversible attachment happens when bacteria attach to the skin for a while and then desorb due to biological, chemical and physical factors. Some bacteria that finally form biofilm become irreversibly attached and produce a matrix composed of polymeric sugars, proteins and/or DNA (Wolcott *et al*., 2008). This matrix helps the bacterium to secure

itself to the surface and helps to protect the colonising microbiota from the environment and host immune defences. As the bacteria begin to grow and multiply they form an aggregate of cells called a microcolony. Bacteria within the microcolony continue to divide until a critical density of bacteria or quorum forms that allows the microcolony to develop further. Biofilm bacteria continue to produce extracellular polymeric substances (EPS), which traps scarce nutrients and protects bacteria from chemical substances such as antimicrobials and antibodies. The bacteria within biofilm multiply and the daughter cells extend outwards. The EPS also acts as an ion-exchange system because it consists of charged and neutral polysaccharide groups, which trap iron and concentrate trace nutrients from surroundings.

In a mature biofilm most of the content is water which is usually (75-95%). The remaining fraction (5-25%) are microorganisms (Geesey, 1994). Biofilm formation is controlled by quorum sensing molecules, which are also important to biofilm detachment as a means of regulating the biofilm population (Stoodley *et al*., 2001; Davies *et al*., 1998). In response to the signalling molecules some biofilm colonisers transform to a planktonic form. These transformed planktonic cells have the ability to later transform back to the sessile form to restart another biofilm in a different location. An enzyme often produced by a biofilm organism which aids dispersion is alginate lyase. It breaks the matrix and the organism disperses to other locations to initiate new biofilm (Rice *at al*., 2005). The mature biofilm is a fully functional system made up of different bacterial species and genera. Within a biofilm there are circulatory systems such as water channels for the transportation of nutrients for metabolic process of biofilm organisms and exchange and the disposition of waste products (de Beer, 1995).

A biofilm can form within hours of colonisation or may take several weeks depending on the particular organism and environmental factors (Mittelman, 1985). Biofilm structures are inherently resistant to antimicrobial challenge and are difficult to

eradicate from the infected host. Susceptibilities towards antimicrobials range from 10 to 1000 times less than equivalent populations of free-floating planktonic cells (Potera, 1999; Donlan, 2001; Gilbert *et al.*, 2002; Parsek & Fuqua, 2004). This resistance is a result of adaptation strategies developed over a million years. Wound biofilms are highly resistant to antibiotics and host defences and many clinicians struggle to successfully manage chronic wounds. Once established, a biofilm can maintain its integrity and cause major problems in the wound healing process. However, laboratory studies have shown that following physical disruption, it takes biofilm about 24 hours to re-establish the biomass (Stoodley *et al*., 2002). There are some topical agents, such as silver, honey or iodine that provide evidence of their value in managing biofilm (Chaw *et al*., 2005, Okhiria *et al*., 2004; Cooper, 2007). In chronic wounds use of antibiotics as a single agent struggles to suppress biofilm, but when used in conjunction with debridement and other topical agents, antibiotics can be more successful (Wolcott and Rhoads, 2008).

1.7 Treatment of chronic wounds

The management of a chronic wound depends on the type of the wound. An appropriate treatment should be focused on problems at the root of chronic wounds, including ischaemia, bacterial load, and imbalance of proteases. Various methods exist to improve these problems such as wound debridement, use of antibiotics, irrigation, warming, moist wound healing, vacuum-assisted closure, oxygenation and removing mechanical stress (Velander *et al*., 2004).

An appropriate level of moisture is important for speeding the healing of chronic wounds (Thomas *et al*., 2005). Topical antimicrobials lower the number of bacteria in wounds and they can also help by keeping the wound environment moist. Topical antimicrobials are in the semisolid composition of cream or ointment which provide a

moist environment for a wound and reduces skin contraction (Brem *et al*., 2004; Patel *et al*., 2000). Some studies show that using tea tree oil have also anti- inflammatory effects (Halcón and Milkus, 2004). Disinfectants have been shown to be ineffective because they damage tissues and delay wound contraction (Patel *et al*., 2000). However, some studies show that use of antiseptics such as polyhexanide, octenidine, chlorhexidine, triclosan and PVP-iodine can be beneficial to lower bacterial level in wounds and speed the rate of healing (Koburger *et al*., 2010). An excess of exudate and the presence of necrotic tissue provide the nutrients for bacterial growth and increase the likelihood of infection (Mustoe, 2004). Since bacteria can thrive on dead tissue, wounds are often surgically debrided to remove the devitalised tissue (Brem *et al*., 2004). Debridement and drainage of wound fluid are an important part of the treatment for diabetic ulcers that may create the need for amputation if infection gets out of control. Mechanical removal of bacteria and devitalized tissue is also the concept behind wound irrigation, which is accomplished using pulsed lavage. Removing necrotic or devitalised tissue is also the aim of larvae therapy, the intentional introduction by a health care practitioner of live, disinfected maggots into non-healing wounds. Maggots stimulate wound healing by dissolving only necrotic, infected tissue and disinfecting the wound by killing bacteria. Larvae therapy has been shown to accelerate debridement of necrotic wounds and reduce the bacterial load of the wound, leading to earlier healing, reduced wound odour and less pain. The combination and interactions of these actions make maggots an extremely potent tool in chronic wound care (Bowling *et al.,* 2007*).*

To improve ischaemic tissue and remove fluid, negative pressure wound therapy (NPWT) is widely used. This therapy, also known as vacuum-assisted closure, reduces swelling in tissues, which brings more blood and nutrients to the area, as does the negative pressure itself (Moreo, 2005). The treatment also decompresses tissues and

alters the shape of cells, causes them to express different mRNAs and to proliferate and produce ECM molecules (Snyder, 2005).

Blood vessels constrict in cold and dilate in warm tissue, altering blood flow to the area. To prevent infection and ischaemia, tissue should be kept warm (Thomas *et al*., 2005). Some healthcare professionals use 'radiant bandages' to keep the area warm. During surgical procedures special care must be taken to prevent hypothermia, which increases rates of post-surgical infection (Mustoe, 2004). Surgical arterial revascularisation in diabetic patients is used to treat underlying ischaemia. Surgery can also help patients with venous ulcers to correct vein dysfunction. There are methods used to increase tissue oxygenation such as Hyperbaric Oxygen Therapy (HBOT) in patients that are not candidates for surgery. HBOT can compensate for limitations of blood supply and correct hypoxia (Alleva *et al*., 2005; Kranke *et al*., 2004). Higher oxygen levels in tissues increase the level of growth factors, kills bacteria and speeds angiogenesis. However, increased oxygen levels also means increased production of Reactive Oxygen Species (ROS) (Alleva *et al*., 2005). Antioxidants, molecules that can lose an electron to free radicals without themselves becoming radicals, can lower levels of oxidants in the body and have been used with some success in wound healing (Schönfelder *et al*., 2005).

Chronic wounds are found to under express growth factors necessary for healing. Therefore, wound healing may be improved by stimulating or replacing growth factors (GF) and also preventing overproduction of proteases that break them down (Edwards *et al*., 2004). There are several ways to increase the concentration of growth factors in wounds. Direct application of GF is commonly used, however it requires large amounts of the factors and many repetitions (Schönfelder *et al*., 2005). Spreading a gel containing the patient's own blood platelets is another way of increasing GF levels.

Platelets secrete growth factors such as insulin-like growth factor 1–2 (IGF) vascular endothelial growth factor (VEGF), platelets derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor-β (TGF-β) (Crovetti *et al.*, 2004). Other possible therapies involve culturing and implanting fibroblasts into the wounds or implanting keratinocytes to speed epithelialisation (Brem *et al.*, 2004). Another alternative for treatment are artificial skin substitutes containing fibroblasts and keratinocytes in the collagen matrix to replicate skin and release growth factors.

Cellular dressings are also widely used. They have a great ability to keep the wound moist and absorb the excess of exudate. They provide a matrix for cellular proliferation and migration (Schönfelder *et al.*, 2005).

Some researchers are seeking ways to improve healing by using protease inhibitors such as secretory leukocyte protease inhibitor (SLPI), which are lowered in chronic wounds. SLPI inhibits proteases and also inflammation and microorganisms like bacteria, viruses and fungi and may prove to be an effective treatment (Lai *et al.*, 2005). Oestrogen has shown to improve wound healing in animals with removed ovaries and also in elderly patients, possibly by preventing neutrophils from releasing elastase and entering the wound (Kanda and Watanabe, 2005).

Nowadays there are a number of bioengineered skin products or skin equivalents available for the treatment of burns as well as acute and chronic wounds. Several complex products have been developed and tested in human wounds since the initial use of keratinocyte sheets (Leigh *et al.*, 1991; Gallico, 1990). There are skin equivalents containing living fibroblasts or keratinocytes, or both (Sabolinski *et al.*, 1996; Hansbrough, 1992) or cellular materials or extracts of living cells currently available (Margolis and Lewis, 1995; Phillips, 1993). Clinical trials on these products showed that the effect was 15-20 percent better than conventional 'control' therapy, however there is

debate over what constitutes an appropriate control. Off-loading and saline-soaked gauze have been accepted by the Food and Drug Administration as the control in US clinical trials. The wound dressings to be used and method of off-loading differ between the countries and is also a subject to controversy (Falanga, 2005).

Living cells, known as a "smart material", are capable of adapting to their environment, hence bioengineered skin may work by delivering these cells. Research has shown that some of these living constructs are able to release growth factors and cytokines (Mansbridge *et al*., 1998). Gene therapy in wound healing is a new and emerging technology, which introduces certain genes into wounds by a variety of biological vectors (viruses) or physical means. *Ex vivo* approaches based on cells manipulation before re-introduction into the wound have been developed. There are also *in vivo* techniques relaying on a simple injection or the use of a gene gun (Slama *et al*., 2001; Badiavas and Falanaga 1999). Most of the gene therapy studies in relation to wounds are still in the experimental stage and have been done only in animal models, however there are promising indications that certain approaches may work in humans. Isner *et al*., (1998) introduced a naked plasmid DNA encoding the gene for vascular endothelial growth (VEGF). They reported improved healing and angiogenesis in selected patients with ulcers resulting from arterial insufficiency (Isner *et al*., 1998).

In the last two decades research has provided new approaches in the use of stem cells in the treatment of burns as well as acute and chronic wounds. Pluripotent stem cells (PSCs), are able to differentiate into a variety of cells such as keratinocytes, fibroblasts and endothelial cells, which are essential in the wound healing process. Most of the pluripotent stem cells are derived from embryos thus it is the subject of much controversy. Pluripotential mesenchymal stem cells, which are the source of new

connective tissue, are present in bone marrow and have been proved to differentiate into various mesenchymal cell types (Quesenberry *et al*., 2002; Mulder *et al*., 2010).

Direct application of autologous bone marrow and its cultured cells may accelerate the healing of non-healing chronic wounds (Badiavas and Falanga, 2003). These findings were reported in an uncontrolled clinical trial and need to be confirmed in a larger controlled study. However, there is the potential that stem cells may reconstitute dermal, vascular and other components required for optimal healing, when pathophysiological abnormalities in chronic wounds are considered (Falanga, 2005).

1.8 Laboratory and clinical diagnosis of wound infection

1.8.1 Clinical diagnosis of wound infection

To diagnose infection, laboratory results must be evaluated in conjunction with clinical assessment of the wound and patient. There are several traditional criteria of diagnosing local infection such as redness, swelling, heat, increased exudate, odour, delayed healing, friable tissue, pain and wound breakdown. The infection can develop from local to systemic with additional signs such as raised white cell count and presence of serum C-reactive protein and sepsis with fever, rigors, chills, hypotension, multi-organ failure, and death (White, 2001).

1.8.2 Laboratory diagnosis of wound infection

1.8.2.1 Conventional microbiology

The methodology used for wound culture has been prone to controversy for many years and there are many questions regarding almost every aspect of it. The first question applies to the timing of microbiological asssessment of the chronic wound, as in much of medical practice, the timing of clinical action is sometimes more important than the action itself. Clinical diagnosis of infection is crucial as 100% of wounds are

contaminated at the time of wounding and soon after 100% of the wounds became colonised (Bowler *et al*., 2001). When the wound infection is confirmed there are other questions such as what type of technique to use to sample the wound. Qualitative and quantitative microbiology of wounds can be investigated by sampling wound tissue or wound fluid. Collecting a deep tissue biopsy has been recognised by most of the practitioners as the most useful method for years (Bowler *et al*., 2001). Superficial, devitalised tissue removed by curettage, which is often used in the management of diabetic foot ulcers, may also be used for evaluation of microbial content (Pallua *et al*., 1999). Pallua *et al.,* (1999) described the technique involving dermabrasion, which enables the acquisition of deeper tissue without being as invasive as the biopsy method. When the wound produces fluid, sampling by needle aspiration can be applied and this technique may also be used to sample deeper pockets of fluid beneath superficial debris. A variety of other techniques, including the dry and pre-soaked velvet pad, filter paper discs and cylinder scrubbing have also been used to sample a superficial wound fluid. However, the most common method of sampling is wound swabbing. This technique has been practiced for more than 100 years and involves sampling superficial wound fluid and tissue debris and enables a quantitative and qualitative analysis of wound microflora (Clinical Laboratory Standard Institution, M40- A standard, 2004). There are many aspects, which need to be considered when using the superficial swab technique including what type of swab should be used and if the swab should be dry or pre-moistened, whether superficial swabbing reflects deeper tissue cultures or whether the swab recovers fastidious microorganisms? Fatty acids contained in cotton swabs can inhibit bacterial growth. For this reason alginate, rayon or polyurethane swabs have been recommended (Clinical Laboratory Standard Institution, M40- A standard, 2004). The most important aspect of obtaining the swab culture is wound bed preparation and this is also a subject of much discussion. There are some questions regarding how the

swab should be rolled across the wound surface and if quantitative microbiology is accurate. Most clinicians recommend that samples from an ulcer should be cultured by simultaneously rotating and zig zagging a swab across the wound to cover as much surface as possible. Others prefer sampling the part of the wound with the most dramatic signs of infection -Levine's technique (Levine *et al*., 1976; Cooper and Lawrence, 1996).

Quantitative microbiology has been proposed as a potential technique to diagnose wound infection, because local and even systemic signs of infection can be subtle or misleading. Quantitative biopsy has been recognised as a gold standard in the diagnosis of wound infection (Robson, 1997). There is a strong association between the ability of wound to heal and the number of organisms recovered (Bowler *et al*., 2001; Xu *et al*., 2007), but these findings must be considered with balanced perspective. At least 20% of heavily colonised wounds with bacterial numbers greater than 10^5 cfu / g of tissue will still heal (Sibbald *et al*., 2003). Also, most infections in chronic wounds are polymicrobial and there are significant and dynamic interactions between multiple bacterial populations within the wound. Growing evidence suggests that the quantitative swab culture may adequately approximate qualitative findings obtained from tissue biopsy (Bill *et al*., 2001; Bozkurt *et al*., 2011; Pellizzer *et al*., 2011). Processing quantitative swabs and biopsies is a complex process requiring several steps and many routine laboratories do not have the capabilities to perform the time consuming procedures. The swab culture is a non-invasive method but it gives no information on bacterial contamination of the deeper layers of the wound (Buchanan *et al*., 1986; Pallua *et al*., 1999). The biopsy method, on the other hand, reveals the bacterial situation of the whole thickness of the wound, but has the disadvantage of being invasive. In contrast, processing semi-quantitative swab cultures is routine in most laboratories (Uppal *et al*., 2007).

1.8.2.2 Swab transport systems

Different swab systems are used to transport a variety of specimen types to the diagnostic laboratory and these systems often differ depending upon the category of organism being investigated e.g. bacteria, viruses or fungi and the method which is going to be used (conventional or molecular identification). The ideal swab system must absorb organisms from the infection site, maintain viability during transport and allow release of organisms from the swab to the appropriate media during cultural techniques. Liquid and gel-based swab systems have been used for many years, but have limitations as the specimen is diluted by immersion within the liquid or gel. Three quantifiable parameters influence the performance of specimen transport: time, temperature and quality of transport swab. Additionally, during wound surface swabbing it is likely that nutrients (bodily fluids and skin cells) as well as bacteria will be transferred to swab causing overgrowth during transport.

Before the production of the United States National Committee for Clinical Laboratory Standards (NCCLS) M40-A standard in 2004, there had been no recognised standard for the performance of swab transport systems. The standard provides a method of quality control testing, together with acceptance criteria for viability and overgrowth of bacteria. In the absence of a standard procedure for determining the effectiveness of swab transport systems, previous papers on this subject have only been able to provide comparative data. The new standard resolves this by defining whether a product is acceptable in terms of bacterial survival (Clinical Laboratory Standard Institution, M40-A standard, 2004).

1.8.2.3 Molecular techniques for bacterial identification

The structure of DNA was described in the late 1960s, however it was not routinely used in research until restriction enzyme and recombinant DNA techniques were discovered in the 1970s. Over the past 20 years, molecular techniques have been developed extensively. Nucleic acid amplification technology has revolutionised microbial detection and identification. Molecular detection methods, especially PCR-based methods, have become important methods for detection of microorganisms in clinical diagnosis laboratory settings (Millar *et al*., 2002).

Identification of microorganisms using conventional culture methods may take several days to allow the sufficient growth of an organism required for an accurate detection. Often in diagnostic microbiology, a delay in obtaining the correct results leads to patients being managed empirically and occasionally inappropriately. In order to improve the diagnosis of wound infection and treatment in patients, molecular approaches should be considered. Molecular approaches should also be considered in cases when conventional culture fails to identify the causal organism prior to antibiotic therapy; where the organism is fastidious in nature; in the cases of endocarditis or where the organism is slow growing or specialised cell culture techniques are required (Millar *et al*., 2007).

Recently antibiotic resistance in bacterial pathogens has become an important issue and the control of infections is subsequently extremely difficult. Consequently, there has been great interest in being able to detect antibiotic resistant microorganisms using molecular methods, particularly when the microorganism is fastidious or non-culturable. There is a widespread concern with the occurrence of MRSA and also glycopeptide resistant enterococci (GRE), particulary on surgical wards. There are some studies employing molecular methods to detect MRSA through a simple PCR assay, targeting the *mec*A gene locus (Kobayashi *et al*., 1994; Towner *et al*., 1998). Other

studies use multiplex PCR for MRSA identification. However, there are not many studies applying quantitative molecular methods which provide an estimation of bacterial numbers.

Most molecular assays rely on three basic components, including nucleic acid extraction, amplification/analysis and identification of an amplified product. There are several factors which help determine which type of assay to employ. If speed is an important factor of the assay, employment of real-time assays should be adopted. Where numerous targets are important, multiplex PCR formats should be employed.

1.8.2.3.1 Use of Real-Time PCR assay in diagnostic microbiology

The introduction of RT- PCR assays in clinical laboratories revolutionized the diagnosis of many microbial infections. RT-PCR is based on Polymerase Chain Reaction chemistry with the incorporation of fluorescent probes, that allows for the detection and quantification of the amplified product in the same reaction. Detection of the PCR product is completed in a short period of time (1-2h). This method has a high specificity and sensitivity alternative to culture or immunoassay-based testing methods (Espy *et al*., 2006).

Recent studies have demonstrated the advantage of RT-PCR assays in identification of many important pathogens that traditionally have been detected by direct immunoassay techniques (antigen testing methods group A Streptococcus from throat swabs, Verotoxin, *Escherichia coli* 0157:H7 from faeces or *Clostridium difficile* toxin from faeces).

Some studies have used RT-PCR for the identification of microorganisms for which the routine culture method was focused on the detection of a single pathogen from a sample (group A Streptococcus from throat swabs, group B Streptococcus from vaginal/anal swabs) (Uhl *et al*., 2003).

The RT-PCR assay has been also developed for the identification of fastidious and slow growing microorganisms such as *Anaplasma phagocytophila*, *Legionella* spp, *Mycoplasma pneumoniae, Bordetella pertussis*, *Chlamydophila pneumoniae* or for which culture methods did not exist for example *Tropheryma whipplei.*

There are also some studies applying RT-PCR for testing the microorganisms responsible for community-acquired pneumonia such as *Chlamydophila pneumonia*, *Mycoplasma pneumoniae* and *Legionella* spp and *Streptococcus pneumoniae* (Murdoch, 2003; Ramirez *et al*., 1996). These organisms are very slow growing and difficult to recover using conventional culture methods due to their special requirements. In a recent study quantitative RT-PCR demonstrated that the numbers of *Streptococcus pneumoniae* organisms detected by real-time PCR in nasopharyngeal secretions correlated with the numbers detected by semi-quantitative cultures (Grisold *et al.,* 2002). However, in order to support these findings other prospective clinical studies are required. It could be debatable that patients colonized but not infected with group A Streptococcus can be identified. However, a study by Uhl *et al.,* (2003) showed that all patients with group A Streptococcus detected by real-time PCR had clinical criteria for streptococcal pharyngitis. Following these findings quantitative RT-PCR was implemented and correlated with traditional quantitative culture methods for the purpose of diagnosing or predicting the possibility of developing MRSA wound infections (Chapter 4).

MRSA infections have worse outcomes and higher associated costs than infections caused by Methicillin Sensitive *Staphylococcus aureus* (Cockerill, 2003). The development of rapid and accurate MRSA identification methods has important implications for the treatment and management of colonised and infected patients. There are many molecular approaches developed to date which decrease the time it takes to identify MRSA. However, most of the assays are based on the detection of the

Staphylococcus aureus specific *mec*A gene, which is also present in some Coagulase Negative *Staphylococcus* species. Therefore these methods can not be applied to nonsterile nasal samples or chronic wound specimens, without previous isolation, capture or enrichment of MRSA. These samples often contain both coagulase- negative staphylococci and *Staphylococcus aureus*, either of which carry *mecA* gene.

Hulesky *et al.,* (2004) described a RT-PCR multiplex assay useful for the detection of MRSA directly from nasal specimens containing a mixture of staphylococci. Five primers specific to the various SCC*mec* right extremity sequences, including three new sequences, were used in combination with a primer and three molecular beacon probes specific to the *Staphylococcus aureus* chromosomal *orf*X gene located to the right of the SCCmec integration site. Out of 1657 MRSA isolates tested, 98.7% strains were detected with the PCR assay, whereas 26 of 569 (4.6%) MSSA strains were misidentified as MRSA. None of the 62 nonstaphylooccal species or the 212 methicillin resistant or methicillin susceptible coagulase negative staphylococci were detected by the assay.

Kolman *et al*., (2010) evaluated several published RT-PCR assays for MRSA detection, before introducing direct swab molecular detection to the MRSA surveillance program. The previously mentioned Huletsky's *et al*., (2004) assay was evaluated and compared with Cuny's assay based on one forward primer of which at least five of its 3' nucleotides are within the *S. aureus orf*X gene (present in MSSA) and an *S. aureus* orfX specific reverse primer. In contrast to the high accuracy observed by Huletsky's when applied on pure cultures, Kolman *et al*., (2010) observed a high percentage of false positives (specificity = 86% and positive predictive value = 77.8%) when applying Cuny's assay with MSSA strains. This is probably due to the design of Cuny's forward primer, which overlaps five of its 3' nucleotides with *S. aureus orf*X gene (present in

MSSA). The high false positive rate for MSSA's (>25%) reduces the likelihood of its use.

1.8.2.3.2 PCR- Denaturing Gradient Gel Electrophoresis (DGGE) sequencing in diagnostic microbiology

DGGE is a technique used to generate a pattern of genetic diversity in complex microbial ecosystems such as soil, sediments, rivers, deep seas, the gastrointestinal tract (GI) and many different biofilms (Muzyer *et al.*, 1993; Muyzer *et al.*, 1998). This molecular approach generates a genetic profile or “fingerprint” of the microbial community within a sample. Samples containing multiple organisms are amplified using PCR. The amplification product usually contains sequences that are well conserved and passed between microorganisms (for example 16S rDNA). These fragments are separated on a gel during the DGGE procedure (Creighton, 1999). Negatively charged DNA is attracted to the positive electrode and forced to migrate through the polyacrylamide gel pores. DNA melts once it reaches the concentration of the denaturing reagents at which the double strand unwinds. To prevent the complete melting of DNA, GC-clamp (stretch of GC-rich sequences) is commonly added to one-end of the DNA sequences (Muyzer *et al.,* 1993).

Different sequences of DNA (from different bacteria) will denature at different concentrations resulting in a pattern of bands. Each band theoretically represents a different bacterial population present in community. Individual DNA sequences or “bands” from this profile can be excised and sequenced to identify the dominant members of the microbial population (Muzyer *et al.,* 1998; Sheffield *et al.*, 1989).

There are many applications of DGGE, especially in environmental and clinical microbiology. For example, DGGE allowed the identification of over 65 *Mycoplasma* species of human and veterinary origins in less than 24h (McAuliffe *et al.*, 2005).

Mycoplasma cause various diseases associated with pneumonia, arthritis, conjunctivitis and infertility. They are fastidious and slow growing organisms and they also require serological tests to be identified (McAuliffe *et al.,* 2005). PCR- DGGE proved to be successful in the detection of numerous gene mutations (van der Hout *et al.*, 2006). This method is also useful in studying complex microbial communities such as in the gastrointestinal tract of food producing animals (Al-Soud *et al*., 2003; Gong *et al*., 2002). DGGE also proved to be a potential method for screening large numbers of patients for the rapid and reliable identification of changes in both breast cancer genes BRCA 1 and BRCA 2 (van der Hout *et al.*, 2006).

The importance of pathogenic biofilms in chronic wounds is only now beginning to be realised and researched. James *et al*., (2008) investigated chronic wound specimens obtained from 77 patients and acute wound specimens obtained from 16 patients. 60% of chronic wounds evaluated by microscopy were characterized as containing biofilm, whereas only one of the 16 acute wound specimens was characterized as containing biofilms (6%). Molecular analyses of chronic wound samples were performed using DGGE methods and revealed diverse polymicrobial communities and the presence of bacteria, including strictly anaerobic bacteria, not revealed by traditional techniques (James *et al*., 2008).

Davies *et al*., (2004) used a combination of conventional cultural analysis and 16S rDNA PCR- DGGE to compare the bacterial community of 8 healing and 10 non-healing chronic venous leg ulcers. The majority of healing and non-healing wounds contained the aerobes *Staphylococcus* and *Pseudomonas* spp. (89% and 80%, respectively), when identified using conventional cultural analysis. DGGE- sequencing allowed the detection of strains that were not identified by cultural means. More than 40% of the sequences represented organisms not cultured from the wound from which they were amplified. DGGE profiles also showed that all of the wounds possessed one

apparently common band, identified by sequencing as *Pseudomonas* sp. The intensity of this PCR signal suggested that the bacterial load of non-healing wounds was much higher for pseudomonads compared to healing wounds and that it may have been significantly underestimated by cultural analysis (Davies *et al*., 2004).

Price *et al*., (2009) used 16S rRNA gene-based pyrosequencing methods to identify bacteria in chronic wounds. They additionally assessed the impact of diabetes and antibiotics on chronic wound microbiota. The diversity of microflora was significantly higher when determined by 16S rRNA gene-based pyrosequencing analysis as compared to the culture-based analyses. The limitations of culture-based methods to characterize diverse bacterial communities from environmental and clinical samples have been reported previously. However, many organisms missed by culture-based methods in the Price study were theoretically culturable using conventional methods. Some of the organisms that were missed by culture-based methods were proportionally rare and may have been masked by more dominant organisms in the culture media.

Dowd *et al*., (2008) investigated pathogenic biofilms in diabetic foot ulcers, pressure ulcers and venous leg ulcers by using 3 separate 16S-based rDNA molecular amplifications followed by pyrosequencing, shotgun or Sanger sequencing, and denaturing gradient gel electrophoresis. The most common pathogens present in the biofilms of all chronic wounds were *Staphylococcus*, *Pseudomonas*, *Peptoniphilus*, *Enterobacter*, *Stenotrophomonas*, *Finegoldia*, and *Serratia* spp. There was a significant difference in bacterial populations between different types of wounds noted. In pressure ulcers, 62% of the populations were identified as obligate anaerobes. Some wounds were identified with bacteria not recognised as pathogenic such as *Rhodopseudomonas* and *Abiotrophia para-adiacens* spp. In addition, the results of molecular analyses were compared with conventional culture approaches, where in only one wound type the

primary bacterial population was correctly identified. These results indicate the need for improvement of diagnostic methods.

The DGGE method can give valuable additional information about chronic wound microflora that is not apparent from cultural analysis alone. A better understanding of wound ecology will help clinicians to better manage the wound. Furthermore, the comparison of normal skin and wound microflora has not been investigated sufficiently so far and it may give important information with regards to the impact of certain bacteria on wound healing, their pathogenicity and role in biofilms.

1.9 Aim and Objectives of the study

The aim of the study was to compare the diagnostic validity of conventional culture methods with molecular approaches for bacteria identification and quantification in chronic wounds in terms of sample collection, transportation and processing in the laboratory.

Objectives:

- To establish which is the most appropriate swab transport device for futher *in vivo* studies on patients with chronic wounds and healthy volunteers

- To compare semi-quantitative swab and biopsy cultures with quantitative methods in order to test the diagnostic validity of semi-quantitative swab culture

- To test the hypothesis that wound infection exists when the bacterial load is 10^5 cfu/gram of tissue or greater

- To develop a quantitative RT-PCR assay for the direct detection of MRSA from wound swabs and to compare this method with quantitative conventional tests.

- To develop and apply the use of DGGE sequencing to the analysis of the diversity of microflora in chronic wounds and on healthy skin. The results of the study will help to select the panel of the most common and clinically important bacteria for future development of multiplex RT-PCR for chronic wounds.

CHAPTER 2

Investigation of swab transport systems for bacterial recovery and performance with DNA extraction and PCR

2.1 Introduction

In clinical diagnostics, successful sampling and transport of microorganisms to the laboratory is crucial for an accurate diagnosis and treatment of the patient. Swabs are commonly used in sampling and the swab material and transport medium play a major, but often overlooked role in sampling. Within the hospital setting, the use of transport devices for various routine microbiology cultures began as researchers noticed the difference in bacterial variability from specimens plated at the bedside compared to those transported to the laboratory (Stuart, 1946). Nowadays, a number of factors contribute to the increasing importance of the use of transport devices to maintain specimens for microbiological testing. These factors include the increased use of outpatient treatment that has accompanied shortened hospital stay, and the centralisation of laboratory services (Human and Jones, 2004). The ideal swab system must absorb organisms from the infection site, maintain viability during transport without allowing growth and allow the release of organisms from the swab to the appropriate media during cultural techniques. These are the most important aspects to be considered when choosing the appropriate collection device. Poorly collected or transported specimens may fail to isolate causative microorganisms and may recover contaminants or normal microflora.

Agar gel and liquid transport systems have been used for many years. The first bacteriological transport medium was introduced in 1946 by Stuart, who proposed the first simple semi-solid, non nutrient medium containing agar, calcium chloride, sodium glycerophosphate, thioglycollate and methylene blue for transporting clinical swab samples (Stuart, 1946). Further developments occurred in 1964, when a modification of the Stuart medium – the Cary-Blair medium was proposed. The Cary-Blair medium has

an improved buffering system due to the replacement of sodium glycerophosphate by inorganic phosphates. The improved formulation prevents overgrowth of Enterobacteriaceae and is recommended for the transportation of rectal and faecal specimens (Cary and Blair, 1964). In 1967 the Amies swab was introduced as another modification of Stuart's medium. In the Amies medium the glycerophosphate was replaced for an inorganic phosphate buffer to improve bacteria recovery and prevent overgrowth (Amies, 1967). Commercially manufactured swab transport kits appeared on the market in the mid-1970s. The Cary-Blair medium is almost exclusively associated with collection and transport of enteric bacteria, however Stuart and Amies media have been widely used for the transportation of a broad range of clinical samples including wound specimens (History of Transport Systems. Copan, available at http://copanitalia.com/index-56.htm).

Transport media protect swabs from ambient air and provide a moist, balanced pH environment for microorganisms during transport and storage, however they do have limitations including specimen dilution by immersion within the liquid or gel. Also, some gel and liquid media transport swabs are not suitable for molecular testing as their components have been found to interfere with diagnostic detection tests using molecular methods (Poddar *et al*., 1998). Medium free transport systems do not dilute the sample and they do not provide potential nutrients for organism growth. They also do not interfere with the Gram staining (Stuczen and Edwards-Jones, 2010; unpublished data). The swab tip and the transport medium must be made of materials that are sufficiently non-toxic or non-inhibitory to maintain microorganism viability throughout the collection and transport process. Cotton-tipped swabs have been shown to be inhibitory to some microorganisms, hence, cotton-tipped swabs should not routinely be used for specimen collection for culture (Mandler and Sfondrini, 1977). Compressed cellulose sponge material in the liquid transport systems may contain sulphur compounds and can

inhibit certain microorganisms. The glue used by some swab manufacturers may be inhibitory to certain bacteria and may also be extracted during specimen preparation (such as enzymatic digestion or chemical extraction process) and interfere with molecular detection methods (Lauer and Masters, 1988). Wire/metal shafts may also contribute to interference problems if the shaft comes in contact with extracting reagents in molecular testing (Wadowski *et al*., 1994). The most commonly used swab transport device in wound care in UK is the Amies gel swab, however this swab is not recommended for molecular methods as it contains agar. Gibb and Wong (1998) observed inhibition of PCR in swabs submitted in routine bacteriological transport media containing agar. No inhibitory effect was observed with a transport medium which did not contain agar. In recent years there has been a significant increase in the use of molecular techniques in bacterial identification hence even more is required of the transport medium. It is not likely to be practical to collect two swabs per patient, i.e., one for culture and one synthetic for PCR, as suggested by Wadowsky *et al*., (1994). For this reason the swab should be suitable for both conventional and molecular testing.

There had been no recognised standard for the performance of Swab Transport Systems (STSs) before the production of the United States National Committee for Clinical Laboratory Standard (NCCLS) M40-A standard in 2003. In the absence of a standard procedure for determining the effectiveness of STSs, previous papers on this subject have only been able to provide comparative data. The new standard resolves this by defining whether a product is acceptable in terms of bacterial survival. The M40-A standard provides a standardised quantitative method (swab elution) and qualitative method (roll-plate) to be utilised by laboratories and manufacturers to measure the performance characteristics of swab transport systems. It provides a method of quality control testing, together with acceptance criteria not only for viability but also for

overgrowth of bacteria. There are 10 control aerobic, anaerobic and facultative anaerobic bacterial strains such as *Haemophilus influenzae* ATCC 10211, *Neisseria gonorrhoeae* ATCC 43069, *Pseudomonas aeruginosa* ATCC BAA-427, *Streptococcus pneumoniae* ATCC 6305, *Streptococcus pyogenes* ATCC 19615, *Bacteroides fragilis* ATCC 25285, *Fusobacterium nucleatum* ATCC 25586, *Peptostreptococcus anaerobius* ATCC 27337, *Prevotella melaninogenica* ATCC 25845, and *Propionibacterium acnes* ATCC 6919 used. These strains are the minimum that must be included to evaluate new swab transport devices before they are approved for patients. *Pseudomonas aeruginosa* is included in the M40-A standard as an indicator for overgrowth and is normally only tested at 4˚C. *Streptococcus pyogenes* is an important human pathogen and this is the reason why it is included in the M40-A standard. It is a cause of many important human diseases ranging from mild superficial skin infections to life-threatening systemic diseases. Additional strains may be employed when required for intended use.

The CLSI M40-A method was used recently to evaluate several swab collection and transport devices for the maintenance of bacterial viability (Graver and Wade, 2004; Human and Jones, 2004; Morosini *et al*., 2006; Van Horn and Rankin, 2007). In the recent study four different swab transport systems were evaluated – a new dry Sigma Swab (Medical Wire & Equipment Ltd.), a gel Amies HealthLink Transporter (Copan Diagnostics), a liquid Amies Sigma Transwab (Medical Wire &Equipment Ltd) and the E-Swab (Copan Diagnostics). The Sigma Swab is a medium free transport system with a polyurethane foam bud. The HealthLink Transport swab contains plain gel Amies medium and a rayon bud. The Sigma Transwab is a new swab transport system containing liquid Amies transport medium and a soft polyurethane foam bud, which is highly absorbent and has an open cell structure which allows a complete flow through of medium and reagents with a maximum release of microorganisms into the liquid medium. The Copan Diagnostics E-Swab is a Nylon Flocked Swab with modified

Amies liquid transport medium. Each tested swab is commercially available and recommended for wound sampling and has a different structure of the bud and different media formulation.

2.2 Aim of the study

The aim of the study was to establish the most appropriate swab transport device (suitable for both conventional and molecular testing) for further *in vivo* studies on patients with chronic wounds and healthy volunteers. Four different swab transport systems were evaluated – a new dry Sigma Swab (Medical Wire & Equipment Ltd.), a gel Amies HealthLink Transporter (Copan Diagnostics), a liquid Amies Sigma Transwab (Medical Wire &Equipment Ltd) and the E-Swab (Copan Diagnostics). Medical Wire's dry Sigma-Swab, Copan's HealthLink Transporter (HLT) swab and Medical Wire Sigma Transwab were evaluated for their ability to maintain viability of *Escherichia coli*, *Staphylococcus aureus* and *Bacteroides fragilis*. The E-Swab has been previously tested with *S. aureus*, *E. coli* and *B. fragilis* and has met acceptance criteria for all isolates therefore was not included in conventional testing (Nys *et al*., 2010; Saegeman *et al*., 2010). The Sigma dry swab, E-Swab and Sigma Transwab were also tested for their performance with molecular methods and the impact of the transport medium components on DNA extraction and PCR. The HealthLink Transporter was not included in molecular testing because gel swabs are not recommended for molecular methods due to interference with PCR (Gibb and Wong, 1998).

All swabs were previously tested with all bacteria required by the standard M40-A method (Quality Control Standard for testing Swab Transport Systems) and met acceptance criteria for most of the aerobic and anaerobic microorganisms (Van Horn *et al*., 2008; Van Horn and Rankin, 2008; Rishmawi, 2007).

2.3 Materials and Methods

2.3.1 Materials for the evaluation of swabs according to the standard M40-A protocol

2.3.1.1 Swab Transport Systems used in the study

- Sigma dry Swab MW941 (Medical Wire & Equipment Ltd, Corsham, UK)

- Sigma Transwab MW176S (Medical Wire & Equipment Ltd, Corsham, UK)

- HealthLink Transporter Swab 4140 (Copan Diagnostics Ltd, Brescia, Italy)

2.3.1.2 Media used in the study

- Nutrient Agar BO0336 (Oxoid, Hampshire,UK)

- Brain Heart Infusion Agar CM1136 (Oxoid, Hampshire, UK)

The media were prepared according to the manufacturer's instructions, sterilised at 121ºC for 15 min and dispensed in 25 ml volumes into sterile Petri dishes.

2.3.1.3 Bacterial strains used in the study

Table 2.1 Bacterial strains, media and incubation conditions used in the recovery studies.

Species	Strain	Plate media	Incubation atmosphere	Testing Time
Staphylococcus aureus	NCTC 6571	Nutrient agar	Aerobic	48h
Escherichia coli	ATCC 8739	Nutrient agar	Aerobic	48h
Bacteroides fragilis	ATCC 25285	Brain-heart infusion agar	Anaerobic	48h

2.3.2 Methods for the evaluation of swabs according to the standard M40-A protocol

The efficacy of swab transport systems was assessed according to M40-A CLSI standard (Clinical Laboratory Standards Institution).

An isolate of each strain was freshly grown on solid media (Table 2.1) and suspension was prepared in sterile saline to a concentration of approximately 1.5×10^8 cfu/ml (equivalent to 0.5 McFarland standard) and diluted 1:10. Serial 10-fold dilutions were prepared from the suspension and plated using a spiral plater (Don Whitley Scientific, BS5687) onto appropriate agar (Table 2.1). The plates were incubated at 37°C for 24 h, and colony forming units counted to confirm inoculum concentration. All swabs were inoculated with bacteria by dipping the swab for 10 seconds in the well containing 50 µl of inoculum suspension and allowing the fluid to absorb. The swabs were inserted back into the transport device and incubated at room temperature (20°C) and at 4°C for 0 h, 24 h and 48 h (as required for M40-A) to determine the survival or overgrowth of bacteria on swab devices at two different ranges of temperature. After the appropriate incubation period serial dilutions were prepared from the liquid transport medium of each swab. The Sigma dry swab was placed into 1 ml of sterile saline, vortexed and serial dilutions prepared. Serial dilutions were inoculated onto the appropriate agar using a spiral plater (Don Whitley Scientific).

All plates were incubated at 37°C for 48 h in the appropriate conditions. After incubation, a quantitative count was performed using Acolyte counter (Don Whitley Scientific). All experiments were carried out in triplicate (Figure 2.1).

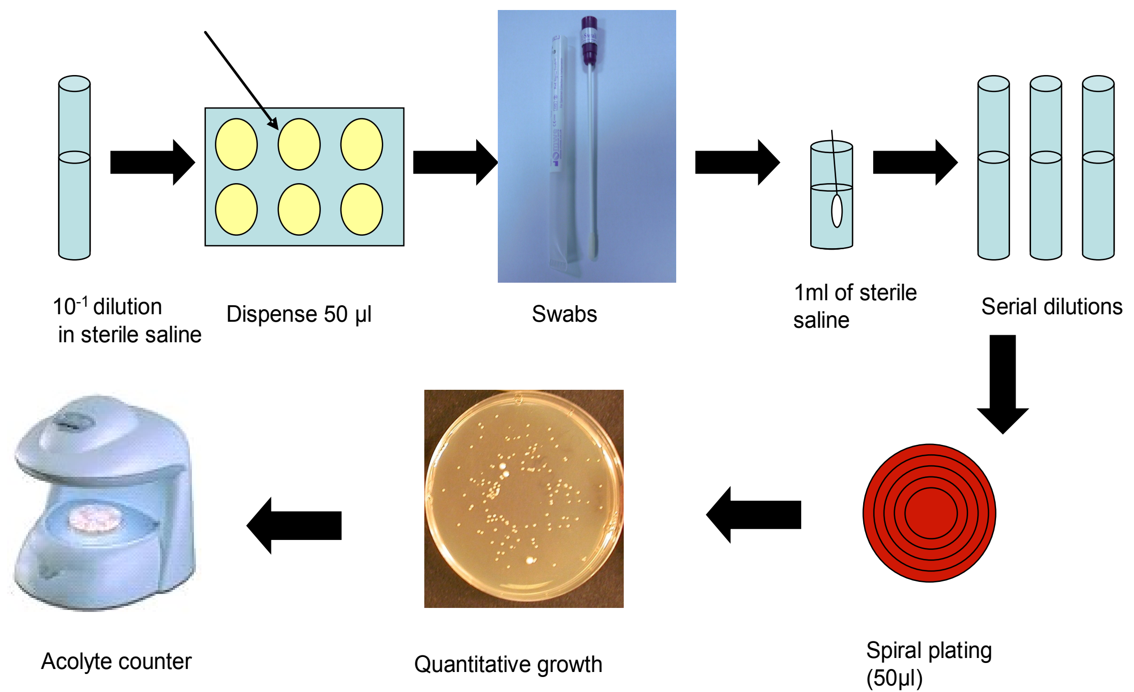


Figure 2.1 Method of processing the swab for evaluation of bacterial viability.

2.3.3 Materials for the evaluation of the performance of the swabs with DNA extraction and PCR

2.3.3.1 Swab Transport Systems used in the study

- Sigma dry Swab MW941 (Medical Wire & Equipment Ltd, Corsham, UK)

- Sigma Transwab MW176S (Medical Wire & Equipment Ltd, Corsham, UK)

- E-Swab 480C (Copan Diagnostics Ltd, Brescia, Italy)

2.3.3.2 Bacterial strains used in the study

- *Staphylococcus aureus* NCTC 6571

- *Escherichia coli* ATCC 8739

2.3.3.3 DNA extraction

Swabs were placed into a vial containing 180μl of molecular grade water, vortexed and DNA extracted from the suspension using a DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.3.3.4 Polymerase Chain Reaction Reagents

Stock concentrations:

- 10xNH_4 buffer (Bioline, London, UK)

- $MgCl_2$ - 50 mM (Bioline, London, UK)

- dNTPs – 100 mM (Bioline, London, UK)

- 16s rDNA primers in final concentration 1μM each (Integrated DNA Technologies, Leuven, Belgium):

518R- 5' -CGT ATT ACC GCG GCT GCT GG-3'

101F –GC clamp - 5' - CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG (Liu *et al.,* 1997)

- Molecular grade H_2O

- BioTaq polymerase 5 U/μl (Bioline, London, UK)

2.3.3.5 Horizontal Gel Electrophoresis

Reagents:

- Agarose (Bioline, London, UK)

- 1xTAE buffer prepared from the concentration of 50x TAE buffer. Volume adjusted to 1l with additional distilled H_2O. 50x TAE was prepared by adding 242g of Tris base, 57.1 ml of Glacial Acetic Acid and 18.6 g of EDTA to 900 ml of distilled water and adjusting volume to 1 litre with additional distilled H_2O.

- Ethidium bromide (1mg/ml)

- Crystal 5xDNA Loading Buffer Blue (Bioline, London, UK)

- Hyperladder IV – separation range 100 – 1013 base pairs (Bioline, London, UK)

To prepare the mini-gels, 0.5g agarose was added to 50ml 1xTAE buffer. He agarose and TAE buffer were mixed and heated in the microwave for 30 seconds. When the agarose cooled down slightly, 1 µl of ethidium bromide (1mg/ml stock) was added and swirled gently. Agarose was poured into a previously prepared gel plate with a comb and cooled for 30-45min. 1xTAE was poured to about 4mm above the gel and the comb was removed carefully. 5µl of DNA was added to 2µl of DNA loading buffer and loaded onto a 1% agarose gel**.** The size of the PCR products was determined by loading 2µl of loading buffer to 5 µl of HyperLadder IV (Bioline, London, UK). After the sample run, bands were visualised under UV light using a GelDoc UV Imaging System (Bio-Rad, Hertfordshire,UK).

2.4 Methods for the performance of the swabs with DNA extraction and PCR

All swabs were inoculated in duplicate with the same concentration of bacterial strain (Section 2.3.4.2) and DNA was extracted using the Qiagen Kit according to the manufacturer's instructions. The experiment was repeated three times to improve the accuracy of the results. After DNA extraction, samples were run on an agarose gel for 45 minutes at 95 volts to look for visible bands. By running DNA through an EtBr-treated gel and visualising it with UV light, any band containing more than ~ 20 ng DNA becomes distinctly visible. Hyperladder IV was used to size DNA fragments. To ensure that the samples which did not produce bands still contain DNA (less than 20 ng) they were used as templates for PCR (using 16S rDNA primers). PCR reactions were carried out in PTC – 200 Thermocycler (GRI) in a 25 µl reaction volume each mixture containing a final concentration of 1xNH_4 reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM total dNTPs, 0.25U Taq, 1 µM of each primer and 0.5 µl of template DNA. PCR conditions for universal 16S primers are described in Table 2.2.

Table 2.2 PCR conditions for universal 16S rDNA primers (Linton *et al*., 2007).

Temperature	Time of hold	Number of cycles
92°C	2 min	1
94°C	30 sec	5
40°C	1 min	
94°C	30 sec	30
50°C	1 min	
72°C	3 min	

2.5 Results

2.5.1 Evaluation of the dry Sigma Swab, Amies HealthLink Transporter and the Sigma Transwab for the recovery of *Staphylococcus aureus*, *Escherichia coli* and *Bacteroides fragilis*

The number of viable cells of *S. aureus* recovered from the dry Sigma swab remained stable for up to 48 h for specimens incubated at 4ºC (Figure 2.1). For Amies HealthLink specimens (stored at 4°C) the number of viable organisms of *S. aureus* decreased by 0.2 log after 48 h of incubation. The number of viable cells of *S. aureus* recovered from the Sigma Transwab increased by 0.2 log after 48 h incubation for swabs held at 4ºC (Figure 2.2).

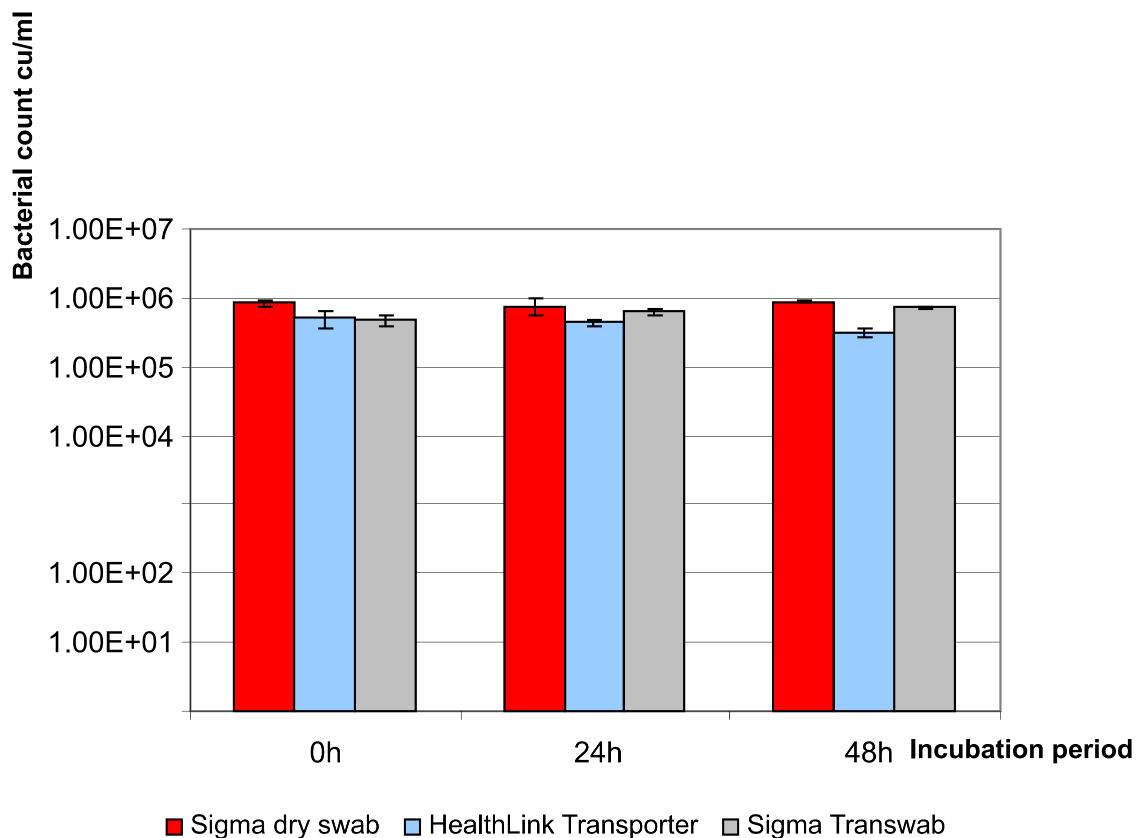


Figure 2.2 The recovery of *Staphylococcus aureus* from the Sigma dry Swab, Amies HealthLink Transporter and Sigma Transwab demonstrating the viability of *S. aureus* over 48 h of incubation at 4˚C (the number of viable cells remained stable for Sigma dry swab; ↓0.2 log for HealthLink Transporter; ↑0.2 log for Sigma Transwab).

For the dry Sigma swab specimens held at room temperature, there was a 0.3 log increase in numbers of *S. aureus.* There was a 0.6 log increase in numbers of *S. aureus* for Amies HealthLink Transporter specimens held at room temperature. The number of viable cells of *S. aureus* remained stable for 48 h for Sigma Transwab (Figure 2.3).

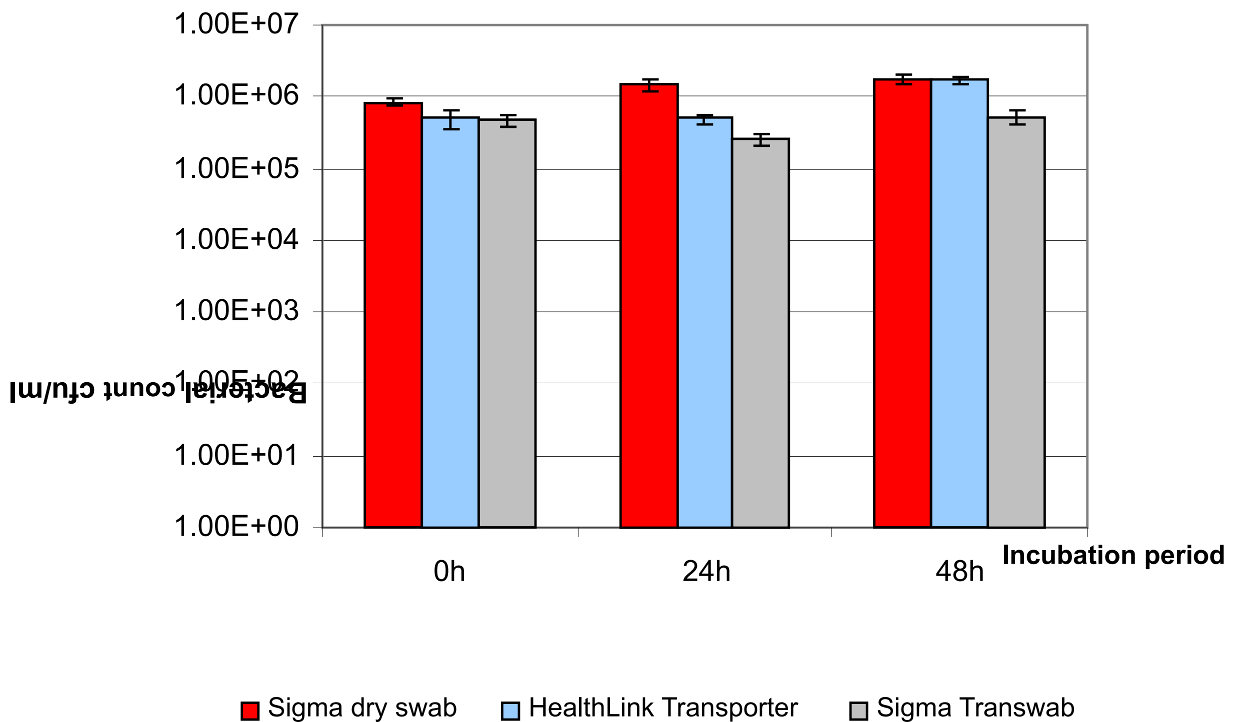


Figure 2.3 The recovery of *Staphylococcus aureus* from the Sigma dry swab, Amies HealthLink Transporter and Sigma Transwab demonstrating the viability of *S. aureus* over 48 h of incubation at room temperature (↑0.3 log for Sigma dry swab; ↑0.6 log for Amies; the number of viable cells remained stable for Sigma Transwab).

The number of viable cells of *E. coli* recovered from the Sigma dry swab was stable for up to 24 h and increased only by 0.2 log after 48 h of incubation at 4ºC. There was an increase in numbers of *E. coli* by 0.5 log for HealthLink transporter. The number of viable cells of *E. coli* recovered from the Sigma Transwab decreased by 0.1 log after 24h of incubation and then increased by 0.3 log after 48 h incubation for swabs held at 4ºC (Figure 2.4).

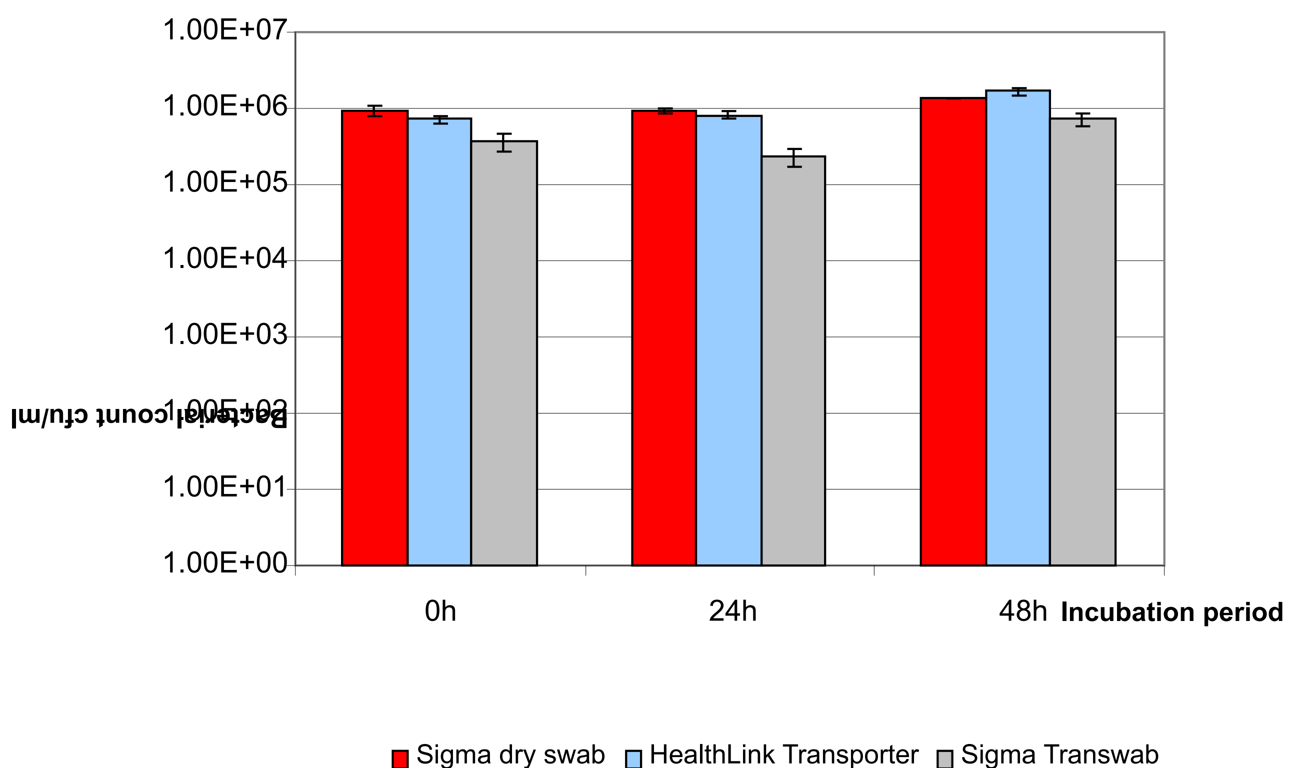


Figure 2.4 The recovery of *Escherichia coli* from the Sigma dry Swab, Amies HealthLink Transporter and Sigma Transwab demonstrating viability of *E. coli* over 48 h of incubation at 4˚C (↑0.2 log for Sigma dry swab; ↑0.5 log HealthLink Transporter; ↑0.2 log Sigma Transwab).

For the Sigma dry swab samples held at room temperature, there was a 0.4 log increase in numbers of *E. coli* after 48 h of incubation. There was a 0.7 log increase in *E. coli* for Amies HealhLink Transporter specimens held at room temperature. For the Sigma Transwab the number of viable cells of *E. coli* remained stable for 48 h (Figure 2.5).

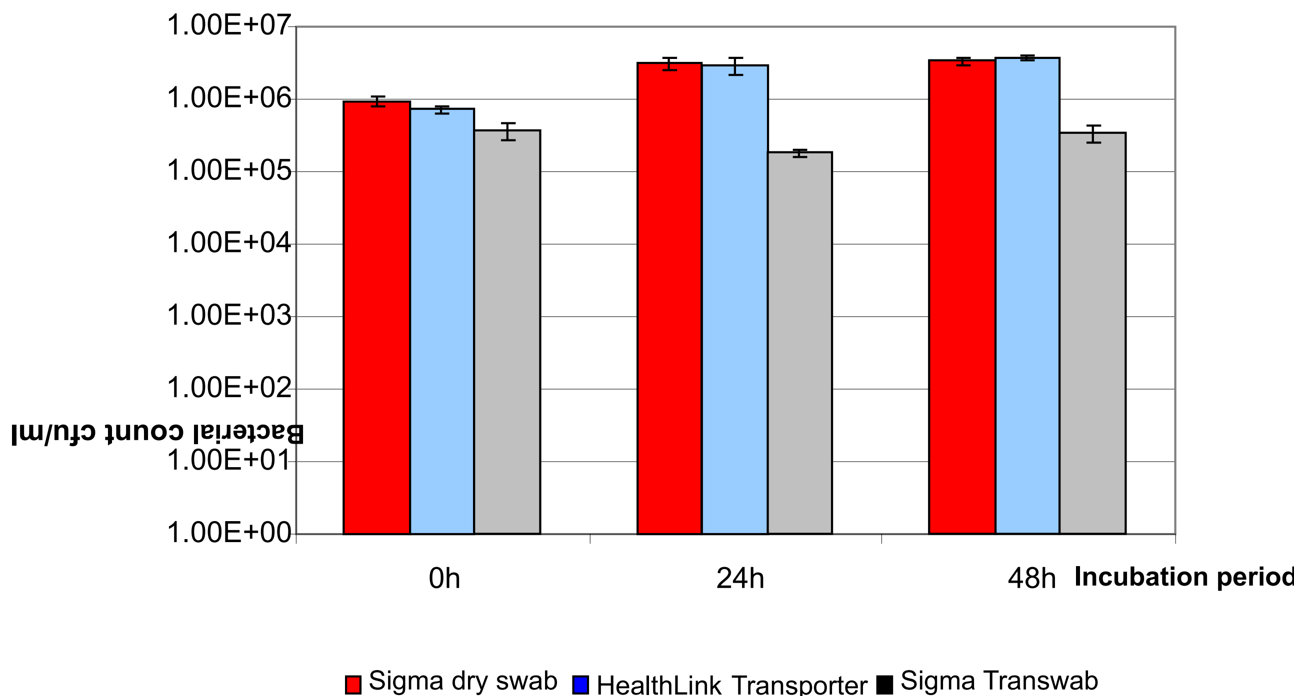


Figure 2.5 The recovery of *Escherichia coli* from the Sigma dry swab, Amies HealthLink Transporter and Sigma Transwab demonstrating viability of *E. coli* over 48 h of incubation at room temperature (↑0.4 log Sigma dry swab; ↑0.7 log HealthLink Transporter; the number of viable cells remained stable for Sigma Transwab).

The number of viable cells of *Bacteroides fragilis* remained stable for the Sigma dry swabs held at 4ºC for 48 h. For the HealthLink Transporter swabs held in the same conditions, the number of viable cells decreased by 0.4 log and for the Sigma Transwab there was 0.5 log increase in *Bacteroides fragilis* count (Figure 2.6).

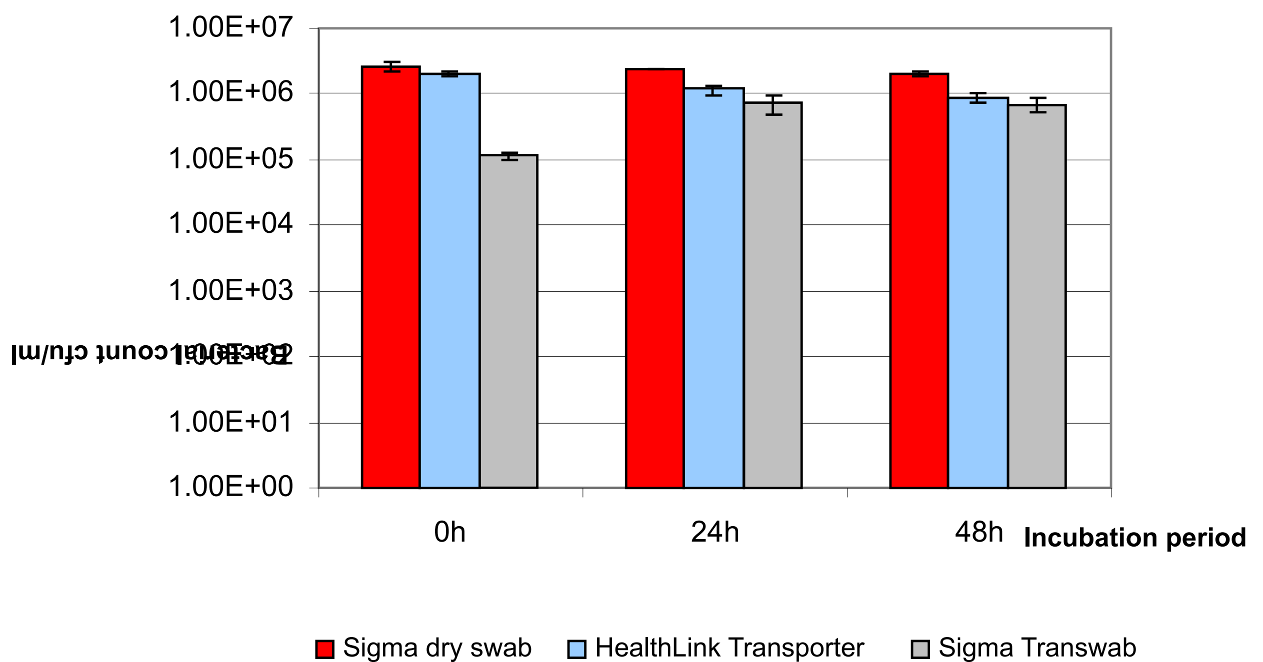


Figure 2.6 The recovery of *Bacteroides fragilis* from the Sigma dry swab, Amies HealthLink Transporter and Sigma Transwab demonstrating viability of *B. fragilis* over 48 h of incubation at 4˚C (the number of viable cells remained stable for Sigma dry swab; ↓0.4 log HealthLink Transporter; ↑0.5 log for Sigma Transwab).

The Sigma dry swab maintained the viability of *Bacteroides fragilis* for up to 48 h at room temperature with no loss of viable cells. With the two other swab systems there were 0.1 log reduction and 0.4 log increase in number of viable cells observed in the HealthLink swab and the Sigma Transwab respectively (Figure 2.7).

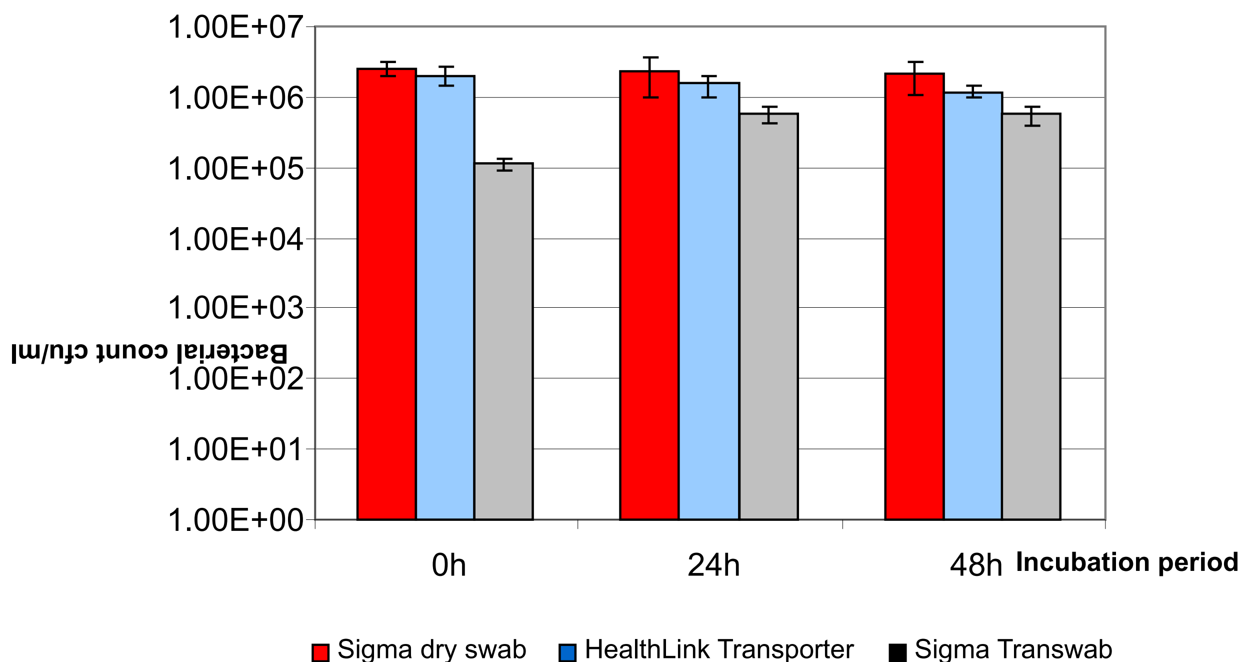


Figure 2.7 The recovery of *Bacteroides fragilis* from the Sigma dry swab, Amies HealthLink Transporter and Sigma Transwab demonstrating viability of *B. fragilis* over 48 h of incubation at room temperature (the number of viable cells remained stable for Sigma dry swab; ↓0.1 log for HealthLink Transporter; ↑0.4 log for Sigma Transwab).

To be considered acceptable, for specimens held at 4º C, there should be no more than a 1 log increase in cfu and no more than a 3 log decline in cfu between the zero-time cfu and cfu after the specified holding period. For specimens held at room temperature, there should be no more than a 3 log decline in cfu between zero-time cfu count and the cfu of the swabs that were stored.

All tested transport swabs met acceptance criteria at both storage temperatures for each isolate tested, but the Sigma dry swab and the Sigma Transwab maintained bacterial viability better than the Amies HealthLink Transporter.

2.5.2 The performance of the Sigma dry swab, Sigma Transwab and Copan E-swab with DNA extraction and PCR

All swabs were tested with *Staphylococcus aureus* and *Escherichia coli* for their performance with DNA extraction. They were inoculated in duplicate with the same concentration of microorganisms (approximately 1.5 x 10^6 cfu/ml) and DNA was extracted using the Qiagen kit (Section 2.3.3.3). DNA was separated using gel electrophoresis in all swabs inoculated with *E. coli* (Figure 2.8 a, b) and only from the Sigma dry swab for swabs inoculated with *Staphylococcus aureus* (Figure 2.9 a, b). However, for swabs inoculated with *E. coli*, the Sigma dry swab showed more distinct bands than the Sigma Transwab and the E-swab. Running DNA through an EtBr-treated agarose gel and visualizing it with UV light results in any band containing more than ~20 ng DNA becoming distinctly visible. There were no bands detected from the Sigma Transwab and E-swab for swabs inoculated with *S. aureus,* which suggested that there was less than 20 ng DNA extracted from the samples. All samples with DNA extracted from *S. aureus* were additionally used as template DNA for PCR (using 16S rDNA standard primers) and the DNA was successfully amplified and detected from all specimens (Figure 2.10).

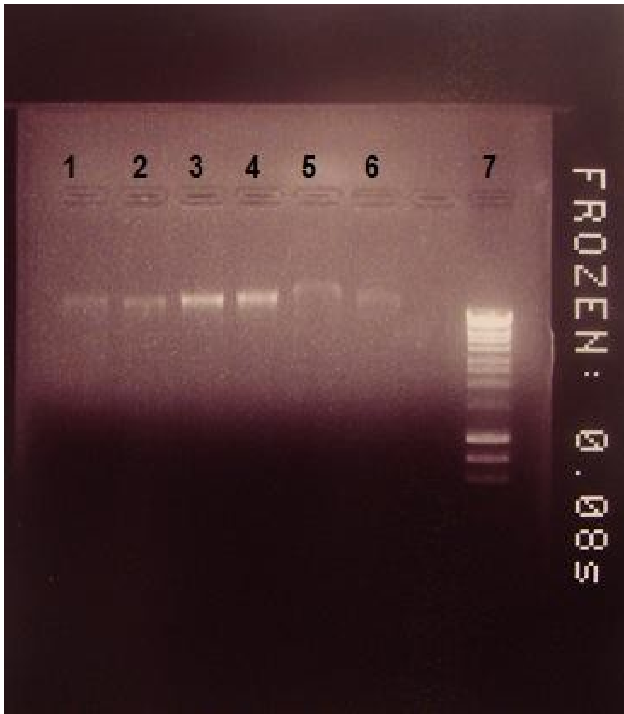


Figure 2.8 (a, b – repeated experiment) DNA extraction of *E. coli* from the Sigma Transwab swab (1, 2), Sigma dry swab (3, 4) and E-Swab (5, 6) demonstrating successful extraction for all swabs tested. From visual observation more distinct bands were achieved with the Sigma dry swab.

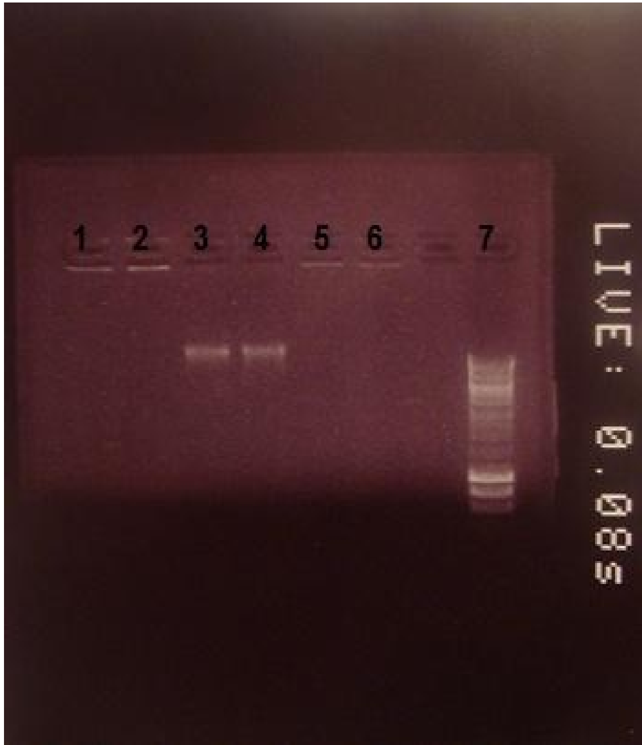
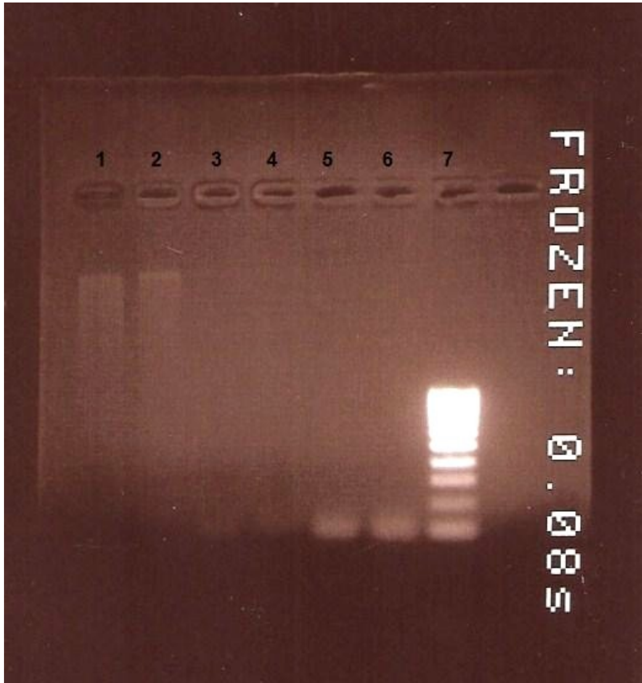


Figure 2.9 a) DNA extraction of *S. sureus* from the Sigma Transwab swab (1, 2), Sigma dry swab (3, 4) and E-Swab (5, 6);
b) DNA extraction of *S. sureus* from the Sigma dry swab (1, 2), Sigma Transwab swab (3, 4) and E-Swab (5, 6) demonstrating successful DNA extraction achieved only from the Sigma dry swab.



Size (bp)

1000
900
800
700
600
500
400
300
200
100

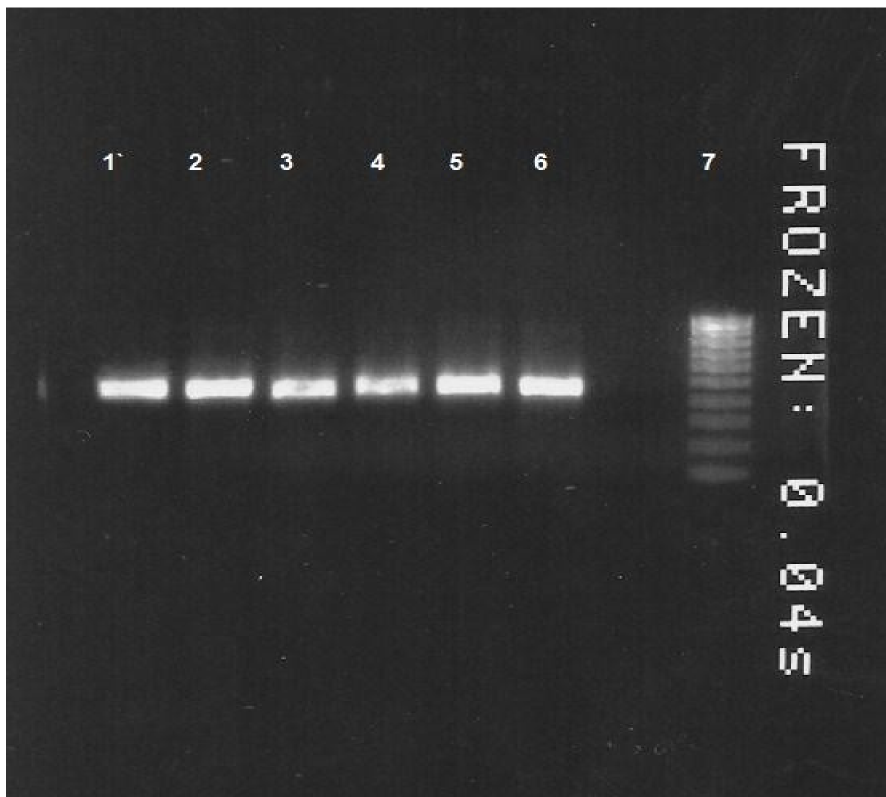


Figure 2.10 Agarose gel electrophoresis of the PCR products – the Sigma dry Swab (1, 2), Sigma Transwab (3, 4) and E-Swab (5, 6) demonstrating successful amplification of DNA from all Swab Transport Systems inoculated with *S. aureus.* Hyperladder IV (Bioline) was used to assess the size of the amplicon (Sample 7).

2.6 Discussion

Loss of microbial viability during transport may have a negative effect on bacterial culture results, especially when present in low numbers. The perfect transport device should maintain the viability of bacteria, prevent overgrowth and release microorganisms to the media during cultural techniques. The Sigma dry swab, Amies HealthLink Transporter, Sigma Transwab and E-swab were previously evaluated with 10 strains required by the M40-A standard and met acceptance criteria for most of isolates tested (Stuczen, 2011, unpublished data; Van Horn *et al*., 2008; Van Horn and Rankin, 2008; Rishmawi, 2007). The aim of this study was to establish the most appropriate device (suitable for conventional and molecular methods) for further testing on healthy skin and patients with wounds. In order to validate the swabs for their ability to recover the most common Gram positive and Gram negative bacteria found in wounds additional strains of *S. aureus* and *E. coli* were employed. *Bacteroides fragilis* is included in the M40-A standard, however the swabs were validated again to confirm the recovery of anaerobes. The performance of the swabs with DNA extraction and PCR was also investigated to look at the possible interference of liquid media or the swab bud itself with molecular methods.

For the M40-A standard to be considered acceptable, specimens held at 4ºC, there should be no more than a 1 log increase in cfu and no more than 3 log decline in cfu between the zero-time cfu and after the specified holding period. For specimens held at room temperature, there should be no more than a 3 log decline in cfu between the zero-time cfu and the cfu of the swabs that were stored. Although no differences were detected between The Sigma Swab, Amies HealthLink Transporter and Sigma Transwab (increase or decline in cfu less than 1 log), the bacteria recovery rate was higher for the Sigma dry swab as compared to other two swabs. The number of viable cells of *S. aureus* recovered from the Sigma dry swab remained stable for 48 h at 4˚C and increased only by 0.2 log for *E. coli* after 48h. The number of viable cells of *B. fragilis*

recovered from Sigma dry swabs remained stable for 48h at both temperatures. From the literature searches conducted there are few peer-reviewed studies comparing the Sigma dry swab with other transport devices. The fact that the dry swab can absorb, maintain viability and release bacteria as well as swabs containing transport media is a big step forward. Liquid and gel based systems have limitations as the sample is diluted by immersion. The presence of bacteria in low numbers and additionally dilution within the liquid or gel can have a negative effect on microorganism detection and identification in the laboratory. Additionally, if the sample is polymicrobial the transport medium may cause overgrowth of other bacteria, especially Gram negative microorganisms and thereby mask the presence of causative pathogens.

There was a 0.2 log decrease in *S. aureus* count after 48 h incubation at 4˚C and a 0.6 log increase at room temperature for the Amies HealthLink Transporter. For samples inoculated with *E. coli* there was an increase in number by 0.5 log at 4°C and by 0.7 log at RT. There was 0.4 log decrease and 0.1 log decrease in viable cells of *B. fragilis* observed for the HealthLink Transporter (HLT) specimens held at 4˚C and room temperature respectively after 48 h incubation. The HealthLink transporter was previously evaluated for the recovery of *S. aureus* and *B. fragilis.* Van Horn and Rankin (2005) observed a 3 log overgrowth at room temperature for *S. aureus*. They tested HealthLink transporter with *P. aeruginosa* and also observed a 3 log overgrowth after 48h of incubation at RT. The same authors in 2007 evaluated HLT with 10 strains recommended by M40-A including *B. fragilis* and found that HLT met acceptance criteria for all isolates tested. In this study there was a higher recovery achieved with the HealthLink Transporter in comparison to a study by Van Horn and Rankin (2005), which can be explained by the fact that manufacturers continually improve their products and the manufacturing process to achieve the best results possible for bacterial

recovery. They are regularly comparing their products' performance in their own and third-party studies.

The number of viable cells of *S. aureus* recovered from the Sigma Transwab increased by 0.2 log and *E. coli* by 0.3 log after 48 h of incubation for swabs held at 4°C. For swabs held at RT the number of viable cells of *S. aureus* and *E. coli* remained stable for 48h. Rishmawi *et al* (2007) observed a 2 log increase in number of viable cells of *E. coli* after 48h of incubation for Sigma Transwab. The number of viable cells of *S. aureus* remained stable at RT for 48 h of incubation which is similar to my findings.

For Sigma Transwab in the recent study there was a 0.5 log increase in the *B. fragilis* count after 48 h incubation at 4˚C and a 0.4 log increase for samples held at room temperature. The full validation of Sigma Transwab was performed previously (Stuczen, 2011; unpublished data) and Sigma Transwab met acceptance criteria for all aerobic and facultative bacteria recommended by the M40-A standard at both temperatures over 48 h. The three batches of the Sigma Transwab were tested and recovered *Bacteroides fragilis, Peptostreptococcus anaerobius, Propionibacterium acnes*, and *Prevotella melaninogenica* for 48 hours at both 4°C and room temperature, and *Fusobacterium nucleatum* for 24 hours at both 4°C and room temperature. *Neisseria gonorrhoeae* was recovered from all 3 batches after both 24 h or 48 h at 4°C with only a 1 log reduction in recovery after 48 h.

The E-Swab has been previously tested according to the M40-A standard with 10 isolates recommended and with other additional strains employed (Van Horn *et al.,* 2008; Saegeman *et al*., 2011; De Silva *et al*., 2010; Nys *et al*., 2010). Van Horn *et al*., (2008) found that the E-swab met acceptance criteria for all five aerobic strains (*H. influenzae, S. pyogenes, S. pneumoniae, P. aeruginosa, N. gonorrhoeae*), however overgrowth was observed with *P. aeruginosa.* They also observed a 2 log overgrowth

with *S. pyogenes* at RT. At 4°C all aerobic strains were recovered at the acceptance level. For *B. fragilis* they observed a 1.4 log increase at 24 h and a 1.5 log decrease at 48 h at room temperature. At 4°C there was a 0.7 log decrease observed. Nys *et al.,* (2010) evaluated the E-swab for the recovery of *E. coli* and observed that the number of viable cells remained stable over 48 h at both temperatures tested. Saegeman *et al.,* (2011) tested the recovery of MRSA from the E-swab and compared it with a dry cotton swab and an Amies gel swab (Copan). They observed higher recovery of MRSA for the E-swab, which may lead to a higher detection rate.

There has been a significant increase in the use of immunological and molecular techniques in bacterial identification in recent years (Relman, 2002) thus even more is required of the transport medium. Swab transport systems used for sample collection and DNA extraction procedures need to be carefully considered and optimized for PCR due to the possible PCR inhibition caused by the presence of agar (Gibb and Wong, 1998), calcium alginate and aluminium swab shafts (Wadowsky *et al*., 1994) or by mucolytic agents (Deneer and Knight, 1994). It is important that transport swabs do not interfere with diagnostic tests. In this study the Sigma dry swab, the Sigma Transwab and E-Swab were compared for their performance with molecular methods. All swabs were inoculated with the same concentration (overnight broth) of the control strain of *S. aureus* and *E. coli.* DNA was extracted from transport swabs after 30 minutes of incubation at room temperature. DNA was detected using agar gel electrophoresis from all swabs inoculated with *E. coli.* For swabs inoculated with *S. aureus,* DNA was detected from the Sigma dry swab only. The amount of DNA extracted from the Sigma Transwab and E-swab was less than 20 ng as the bands were not visible on the electrophoresed gel.

The cell wall of Gram positive bacteria has a thicker and highly cross linked peptidoglycan layer than Gram negative bacteria and lacks the outer membrane. For that

reason, DNA extraction is more difficult and less efficient for Gram positive bacteria (Beveridge, 1999). The use of appropriate DNA extraction methods is critical for successful PCR on clinical samples. As the DNA extraction in this study was successful with the dry Sigma swab, the problem was related to swabs containing liquid media. The samples inoculated with *S. aureus* were additionally used as template DNA for PCR and the DNA was successfully amplified and detected from all specimens (Figure 2.10). This means that even though all swabs were inoculated with the same concentration of *S. aureus*, the DNA extraction was less efficient from swabs containing liquid medium compared to the Sigma dry swab.

There are many studies confirming the interference of gel and liquid transport media on DNA extraction and other molecular techniques. This is the first report comparing the performance of different swab transport systems including the Sigma dry swab with DNA extraction and PCR. Cloud *et al.,* (2002) compared several types of swabs to determine which single material could optimally be used for both culture and PCR. He found that calcium-alginate swabs inhibited the PCR reaction. However, dacron or rayon swabs provided acceptable results. The main component of calcium-alginate swabs responsible for PCR inhibition is reported to be the alginate, which is a crude extract from seaweed. Reports of whether or not components of the aluminium shaft are inhibitory have been conflicting. Wadowsky *et al.,* (1994) reported inhibition due to the aluminium shaft, whereas Rasmussen *et al.,* (1992) and He *et al.,* (1994) had success in PCR assays when using aluminium shafts. Gibb and Wong (1998) observed inhibition of PCR in throat swabs submitted in routine bacteriological transport media. Experimental studies showed that agar within the medium, that was co-extracted with DNA by DNAzol (Gibco Laboratories, USA, 2002), was the inhibitory agent. No inhibitory effect was observed with a transport medium that did not contain agar.

The Sigma dry swab was chosen as a collection and transport swab for the further studies on patients and healthy volunteers where two samples (one for conventional and one for molecular testing) were required (Chapter 4 and Chapter 5). For the accuracy of the results it was important to use the same swab transport system and sampling method in order to compare two techniques (conventional and molecular) for bacterial identification and quantification. The advantage of using dry swabs is that the sample is not diluted within the liquid medium and even small amounts of bacteria can be recovered. The recovery studies confirmed that this swab is able to maintain the viability of bacteria at 48h, which allowed transport delays to have minimal impact on organism recovery. Also, this swab is suitable for molecular testing as it does not contain medium, which may interfere with DNA extraction and other molecular methods. For studies where only conventional microbiology was investigated (Chapter 3) the Amies gel swab was used as it was the recommended swab in the hospitals and our findings confirmed that there was no significant difference in recovery of bacteria between an Amies swab and the other swab systems investigated.

2.7 Conclusions

All tested swabs (dry Sigma Swab, swabs containing liquid Amies medium- E-swab and Sigma Transwab, and gel Amies HealthLink Transporter) are suitable for the collection and transport of samples containing *S. aureus, E. coli* and *B. fragilis* to be processed using conventional methods. However, when tested for their performance with molecular methods, only the dry Sigma swab provided sufficient DNA extraction for both microorganisms tested – *E. coli* and *S. aureus.* Even though all swabs were inoculated with the same concentration of *S. aureus*, the E-swab and Sigma Transwab did not produce visible bands due to the very low concentration of DNA extracted (below 20ng). This is an important finding, especially for samples with low

concentrations of bacteria or samples for quantitative molecular analysis as it may lead to misdiagnosis and inaccurate results.

CHAPTER 3

Diagnostic validity of conventional microbiological culture methods for wound swabs and biopsies using MRSA as an indicator organism

3.1 Introduction

The most practical and widely used method for obtaining a specimen for wound culture is a superficial swab (Gardner *et al*., 2007). Swab cultures of wounds are non-invasive and most laboratories are capable of semi-quantitatively processing these specimens. The procedures used for processing a semi-quantitative swab are routine in most laboratories and the materials required are culture media, sterile loops and an incubator. Processing quantitative swabs requires several steps and many routine microbiological laboratories may not want to deal with a process that complex. Also it is debatable if the numbers of bacteria or the presence of a specific pathogen within the wound have a negative impact on wound healing (Ratliff, 2008).

Previous studies have suggested that superficial swab cultures may be comparable to tissue cultures in determining the bioburden of chronic wounds (Levine *et al*., 1976; Pellizzer *et al*., 2001; Bozkurt *et al*., 2011). However due to methodological issues and design of the studies, the ability to draw definitive conclusions from these studies has been problematic. The technique used to culture, isolate and identify organisms varied greatly from study to study (Gardner *et al.,* 2006). Some of the studies had a very small sample size (Levine *et al*., 1976; Herruzo-Cabrera *et al*., 1992; Bhabha *et al*., 2011), were performed on samples collected from acute wounds such as burns or surgical wounds (Buchanan *et al.,* 1986) or used wound models rather than clinical cases (Sullivan *et al*., 2008). Also it is difficult to compare the method of sample processing and bacterial identification (semi-quantitative *vs* quantitative or swab *vs* biopsy) without having an indicator microorganism. Chronic wounds are polymicrobial in nature and their microbial patterns may vary between samples from the same patient and vary from patient to patient. Without an indicator microorganism and by taking only total bacterial count into consideration when comparing the semi-quantitative and

quantitative methods we are not able to give information on the ability of the method to identify and quantify a specific pathogen. Most of studies failed to provide a definition of "positive culture" or define a positive culture as the growth of any organism. Therefore, it is impossible to determine whether these study findings were consistent with current understanding of wound microbiology, especially as all chronic wounds are contaminated with microorganisms although they are not necessarily infected (Gardner *et al*., 2006).

The most serious methodological problem in many studies was that specific techniques used to collect the swab specimens were not described (Sapico *et al*., 1986; Herruzo-Cabrera *et al*., 1992; Rudensky *et al*., 1992). Swabbing techniques vary greatly according to wound bed preparation (cleansing or no cleansing), area of the wound sampled and duration of sampling (Gardner *et al*., 2006).

For many years, the gold standard to determine wound bacterial bioburden has been quantitative tissue biopsy (Dow, 2003; Gardner *et al*., 2001; Kingsley *et al*., 2003; Robson, 1997). In today's healthcare settings tissue biopsies are not commonly used due to the potential damage to healing tissue and the lack of facilities to process tissue biopsies. Furthermore, the significant pain in sensate soft tissue and the increased expense with this modality are other contributing limitations of tissue biopsy collection (Bill *et al*., 2001). The limitations of a tissue biopsy have been reported by Bowler *et al.,* (2001). They suggest that many chronic wounds are not homogenous in the quantity and types of bacteria within wound tissue. Bill *et al.,* (2001) studied 38 patients with chronic wounds of various aetiologies to evaluate the correlation between quantitative wound biopsy and swab culture. They found 79% correlation between both methods and concluded that quantitative swab culture provides a valuable adjunct in the management of chronic wounds.

Bozkurt *et al.,* (2011) compared superficial swab cultures with deep tissue biopsy and evaluated the reliability of superficial swab cultures in diabetic foot infections. The patients were divided into two groups: those with osteomyelitis (33 patients) and with soft tissue infections (42 patients). The compatibility rate between two methods in patients with osteomyelitis was 58.7% whereas in patients with soft tissue infections it was 89.1%. They concluded that superficial swab cultures could be valuable to identify the pathogens in infected diabetic wounds without osteomyelitis. The accuracy of swab specimens diminishes when osteomyelitis develops and in this case tissue culture seems more reliable (Bozkurt *et al*., 2011). Pellizzer *et al.,* (2011) compared deep tissue biopsy and superficial swab culture in patients with limb-threatening diabetic foot infections and concluded that swabbing and deep tissue biopsy cultures appear to be equally reliable for the initial monitoring of antimicrobial treatment in severe diabetic foot infections. No statistical difference was observed between two procedures in terms of either species or frequency of isolation (Pellizzer *et al*., 2011).

The use of quantitative tissue/swab culture has been suggested for determining infection in the chronic wounds. Some scientists agree on the basis of work of Robson and Heggers (1970) that greater than 10^5 organisms per gram of tissue is diagnostic of acute or chronic infection and delayed wound healing. However, diagnosing wound infection is a clinical skill. The progression from colonisation to infection can not be predicted only by the presence of a specific type of microorganism or by a certain quantity of bacteria (Sibbald, 2003). This is because the host immune response plays a critical role in determining whether wound infection will arise. In a healthy host with strong immune response, the likelihood of infection will be reduced, as the host will be able to tolerate exposure to a higher number and greater variety of microorganisms (Patel, 2010). Exposure to the same quantity and variety of microorganisms in the

compromised host carries an increased risk of wound infection due to impaired immune response. Where wound infection is suspected, wound investigation such as swabbing can help to confirm whether any microorganisms are predominent and if direct antibiotic treatment is required. In chronic wounds, which are polymicrobial in nature and colonised with several microorganisms, wound infection may occur as consequence of synergy between the organisms. Synergy increases bacterial virulence through increased metabolic efficiency, substrate accessibility, enhanced resistance to environmental stress and inhibitors and an increased ability to cause infection even though individually the organisms are of low virulence (Patel, 2010; Percival and Bowler, 2004). Mechanisms that create pathogenic synergy include protection from phagocytosis and intracellular killing, release of growth factors, modification of the local environment and the protection of sensitive species by the inactivation of inhibitors. This protection has been named "indirect pathogenicity" (Brook, 1989). In many animal models combinations of aerobic and anaerobic bacteria have been shown to produce levels of sepsis or disease that could not be induced by individual species (Brook, 1987). Such synergy has been demonstrated in wound microorganisms including: *Klebsiella pneumoniae* and *Porphyromonas asaccharolytica* (Bowler *et al*., 2001; Mayrand and McBride, 1980); *Escherichia coli* and *Bacteriodes fragilis* (Bowler *et al.,* 2001; Rotstein and Kao, 1988) and *Staphylococcus aureus* (Bowler *et al.,* 2001).

Taking and processing a wound tissue biopsy is expensive, invasive, labour intensive, is a painful method and disrupts the wound bed from healing. It also requires trained personel to perform the procedure (Fleck, 2006). It is not standard practice in the majority of healthcare facilities and wound swabbing remains the most frequently used method of collecting a wound sample. For this reason the diagnostic validity of semi-quantitative and quantitative swabs from chronic wounds should be investigated and established.

3.2 Aims and objectives

The aim of this study was to examine and compare the diagnostic validity of semi-quantitative swab culture methods routinely used in the laboratories with quantitative methods of sample processing to establish the best method of culturing. This study addressed the limitations of previous studies by delineating and fully describing the swab and biopsy technique, applying a standard, research-based definition of positive culture, describing microbiological procedures to enhance recovery and quantification of organisms consistent with this definition and using an indicator microorganism (MRSA) to compare the diagnostic validity of the semi-quantitative method. As the patients did not have clinical signs of infection, MRSA was a good organism to use to assess microbial load. Additionally the hypothesis that a microbial load greater than 10^5 cfu/ g of tissue is diagnostic for infection was challenged by measuring bacterial load in patients with colonised diabetic foot ulcers.

3.3 Ethical approval

This study was a part of a large on-going trial in which the potential use of larval therapy was compared with two different methods of non-pharmacological treatment of diabetic foot ulcers (Silver dressings and Biogun). Ethical approval for the study was obtained from the Department of Biological Sciences Research Committee at Manchester Metropolitan University. Ethical permission for the larger trial was obtained from NHS Research Ethics Committee (07/MRE08/48).

3.4 Participants and study criteria

The samples were collected from 41 adults with chronic foot ulceration of at least 3 weeks duration attending the Manchester Diabetes Centre, Manchester Foot Hospital, the in-patients clinic at the Manchester Royal Infirmary, the Diabetes Centre at Tameside General Hospital and Trafford General Hospital. Patients (above or equal to 18 years of age) with diabetes (type 1 or 2 diabetes) were included in the study. The microbiological inclusion criteria were, that the patient was colonised with MRSA but did not have clinical signs of infection. The presence of MRSA was confirmed by obtaining two MRSA positive wound surface swab samples by the hospital pathology laboratory. Eligible patients were invited to enter the study, and informed consent was obtained from them.

3.5 Patient demographics

Out of the 41 patients studied aged between 18-75 years old, 19 (46.3 %) were women and 22 (53.7%) were men. Patients were mainly Type 2 diabetics (n= 31, 75.6%) with the remaining patients being Type 1 (n=10, 24.4%). 33 patients (80.5%) had diabetes for a duration longer than 10 years. 6 patients (14.6%) had diabetes for a period of 5 to 10 years and only 2 patients (4.9%) had diabetes for less than 5 years (Table 3.1).

Table 3.1 Demographics of the patients included in the study (data provided by Dr Frank Bowling).

Sex	**No of patients (%)**
Male	22 (53.7%)
Female	19 (46.3 %)
Patients age	18-75 years
Type of diabetes	
Type 1	10 (24.4%)
Type 2	31 (80.5%)

Duration of diabetes	
<5 years	2 (4.9%)
5 to 10 years	6 (14.6%)
>10 years	33 (80.5.%)
History of recurrent ulcers	
Yes	40 (97.6%)
No	1 (2.4%)
Presence of peripheral neuropathy	34 (82.9%)
Presence of diabetic neuropathy	13 (31.7%)
Presence of PVD	17 (41.5%)
Neuropathic complications	23 (56.1%)
Neuro-ischaemic complications	18 (43.9%)
Ischaemic complications	2 (4.9%)
Presence of diabetic nephropathy	13 (31.7%)
Presence of diabetic retinopathy	19 (46.3%)
Recent hospital admission	13 (31.7%)
Recent antibiotics	38 (92.7%)
Palpable pedal pulses	28 (68.3%)
ABPI>0.9	12 (29.3%)
Diabetic medication	39 (95.1%)
Aware of complications	39 (95.1%)
Smokers	5 (8.8%)

3.6 Study design

41 patients with MRSA colonised diabetic foot ulcers were included in the study. It was a randomised controlled trial and all patients were receiving one of the three different therapies – Larvae Therapy, Biogun or Silver Dressing. Patients underwent the course of several treatments depending on the healing progress (minimum 3 and maximum 12 treatments). MRSA was used as an indicator to compare semi-quantitative and quantitative methods of bacteria isolation from wounds. After each treatment, two types of samples were obtained for identification and quantification of MRSA from each foot ulcer: a superficial swab and a deep tissue biopsy. A deep tissue biopsy was only collected if there was no possible damage to healthy granulation tissue. Treatment was continued for minimum of two applications and until the wound was fully cleared of MRSA or a maximum of 12 applications. The impact of the treatments was not assessed in this part of the study and it was reported elsewhere (Bowling, 2009). This

study concentrated only on the diagnostic validity of semi-quantitative and quantitative culture methods.

3.7 Method of sampling

The ulcer from each patient was initially cleaned with sterile saline and a superficial swab sample was then obtained using the zigzag method. The zigzag technique involves rotating the swab between the fingers as the swab is manipulated in a 10-point zigzag fashion (side to side across the wound without touching the wound edges or the peri-wound skin from one edge to the other). The size of the wound was measured to calculate the number of microorganisms per cm^2.

A deep tissue biopsy was taken using a sterile scalpel blade (biopsy cutter) and placed in a sterile specimen collection tube (BD Vacutainer 288341).

Two hundred and fifty one superficial swabs and eighty-two tissue biopsies were collected from 41 patients before and during the course of treatments.

3.8 Materials and methods of processing semi-quantitative and quantitative superficial swabs and tissue biopsies

3.8.1 Media, reagents and identification methods

3.8.1.1 Blood Agar (Oxoid, Basingstoke, UK CM0331)

Columbia Blood Agar was prepared according to the manufacturer's instructions and sterilised at 121ºC for 15min. On cooling to 50ºC, 5% (v/v) sterile defibrinated

horse blood (TCS Bioscience Ltd.) was added, mixed and the medium dispensed in 25 ml volumes in sterile Petri dishes.

3.8.1.2 MRSA Chromogenic Agar (E&O Laboratories Ltd, Bonnybridge, UK, PP3046)

Chromogenic agar (composition in g/l Agar 15.0: Peptone and Yeast extract 40.0: Salts 25.0: Chromogenic mix 2.5: ph: 6.9 +/- 0.2) was purchased ready prepared.

3.8.1.3 ISO –Sensitest (Oxoid, Basingstoke, UK , CM0471)

Sensitivity test medium (ISO-sensitest) was made according to the manufacturer's instructions and sterilised at 121°C for 15 minutes. Plates were poured to a depth of 4mm +/- 0.5 and set on a level surface to set.

3.8.1.4 Catalase Test

The catalase test was used to differentiate staphylococci from streptococci. *Staphylococcus* produces an enzyme catalase, which causes the conversion of hydrogen peroxide to water with the concomitant release of oxygen gas, seen as bubbles in the reaction tube (Gagnon *et al*., 1959).

A small amount of culture from the plate was transferred carefully into the tube containing 3% hydrogen peroxide solution. The immediate formation of oxygen bubbles in the tube indicated the activity of catalase.

3.8.1.5 DNase Test

Staphylococcus aureus produces a DNase that can diffuse from a colony and hydrolyze DNA within a plate. Test colonies were inoculated into tryptone agar medium containing DNA. Control strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* were used as positive and negative controls. Plates were incubated at 37ºC

for 18-24h. After incubation the plates were flooded with 1M HCl (Sigma-Aldrich) which precipitated DNA and turns the medium cloudy. Organisms that degrade DNA produce a clear zone around an inoculum streak.

3.8.1.6 Staph Latex Kit (Pro-Lab diagnostic)

The Prolex Staph Kit provides a rapid method to distinguish *Staphylococcus aureus* which possess coagulase (clumping factor) and/or protein A from other species of *Staphylococcus.*

The Prolex Staph Kit utilizes polystyrene latex particles which have been sensitized with fibrinogen and IgG. When staphylococcal colonies which possess clumping factor are mixed with the latex reagent, the latex particles agglutinate strongly within 20 seconds.

3.8.2 Microbiological processing of the samples

3.8.2.1 Semi-quantitative isolation of bacteria

The samples were processed for the isolation of *Staphylococcus* species by inoculating onto Blood Agar and MRSA Chromogenic agar and then streaked out using a sterile loop to obtain single colonies (streak plate method) and examined after incubation for 24h at 37°C under aerobic conditions. A semi-quantitative count was recorded according to growth in each of the four sequential streaks providing an estimation of the relative predominance of all pathogens (e.g. 1+, 2+, 3+, 4+) (Standard Operating Procedure: Aseptic Technique, 2001).

3.8.2.2 Preparing tissue biopsy for processing

A sterile plastic bijoux containing 1 cm^3 sterile saline was weighed and recorded. The tissue biopsy was then aseptically transferred to the bijoux, re-weighed and

recorded. Then the biopsy and saline was transferred to a sterile grinder and ground until the tissue was evenly homogenised. The homogenised tissue was transferred back to a sterile universal container and mixed for five minutes. This procedure was carried out in the Class 1 Safety cabinet to minimise the risk from inhalation of aerosols.

3.8.2.3 Quantitative isolation of bacteria

All wound swabs after inoculation onto the same media as described in paragraph 3.8.2.1, were cut and aseptically transferred to a sterile plastic bijoux containing 1 cm^3 saline and mixed for 5 min. The samples were then aseptically diluted to perform a quantitative count. The dilution was made by adding 10µl of the original sample to 9.99ml of sterile saline (dilution 1:1000) or 100µl of the original sample to 9.90ml of sterile saline (dilution 1:100). Using the spiral plater (Don Whitley Scientific, BS5687) a total aerobic and MRSA count was performed by plating 50µl of the diluted sample onto the MRSA Chromogenic agar (Figure 3.1). After 24 h incubation colonies were count using aCOLyte Colony Counter (Don Whitley Scientific).

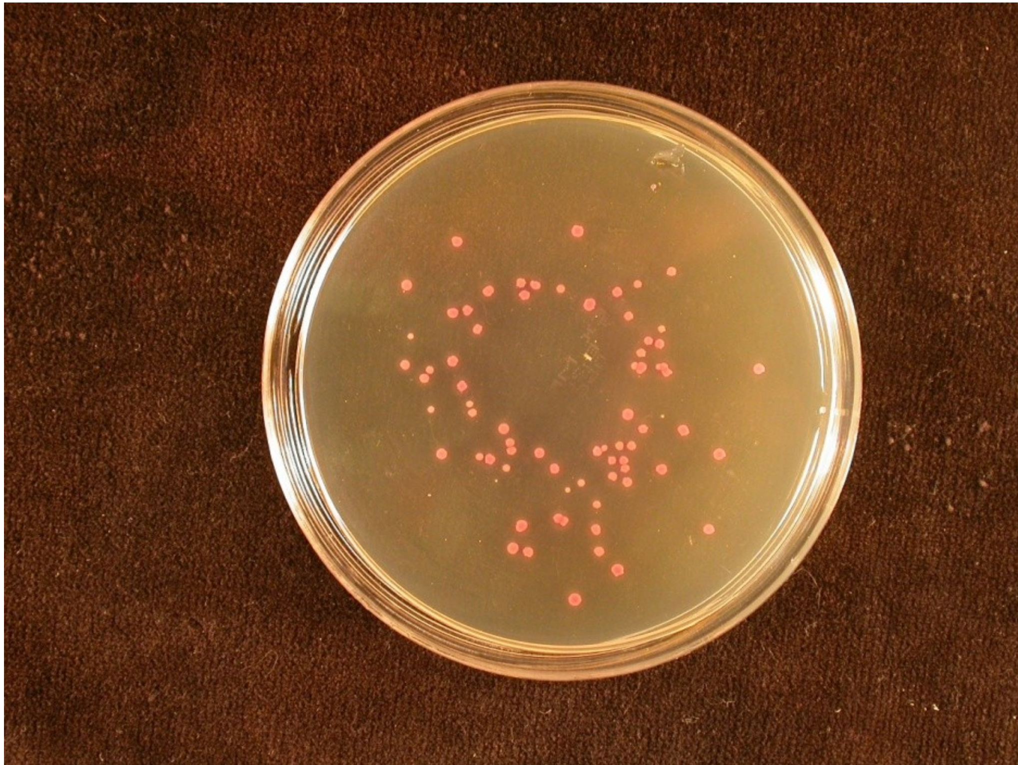


Figure 3.1 Quantitative MRSA culture on CHROMagar demonstrating single pink colonies of MRSA

3.8.3 Identification of *Staphylococcus aureus* and MRSA

MRSA Chromogenic media were examined at 24h for the presence of pink colonies (Figure 3.1) indicating a suspected MRSA isolate. Standard identification tests of catalase, tube coagulase, DNAase and latex agglutination were used to confirm the identification of *Staphylococcus aureus*. An accurate count of MRSA was performed using an image analysis system, the Acolyte counter (Don Whitley Scientific) on the respective culture media.

3.8.4 Methods for Antimicrobial Susceptibility Testing of MRSA

Antibiotic susceptibility tests were carried out by the agar disc diffusion method to confirm the presence of MRSA. The following discs (purchased from Oxoid Ltd.) were placed onto ISO Sensitest agar: Methicillin (5μg), Penicillin (1 unit),

Erythromycin (5µg), Tetracycline (25µg), Chloramphenicol (25µg), Fusidic Acid (10µg) and Streptomycin (10µg). *Staphylococcus aureus* Oxford strain (NCTC 6571) was used as a quality control strain. Plates were incubated at 30˚C for 24h. The zones of inhibition around antibiotic disks were assessed according to BSAC standards (BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 2.1.3; 2003) (Table 3.2).

Table 3.2 Zone breakpoints for MRSA (BSAC Disc Diffusion Method for Antimicrobial Susceptibility Testing, Version 2.1.3; 2003).

Antibiotic	Interpretation of zone in diameters (mm)		
	R ≤	I	S≤
Methicillin	14	-	15
Penicillin	24	-	25
Erythromycin	19	-	20
Tetracycline	19	-	20
Chloramphenicol	14	-	15
Fusidic Acid	29	-	30
Streptomycin	23	-	24

3.9 Statistical Analysis

A statistical analysis was performed using SPSS Statistics (version 19.0). The correlation between semi-quantitative and quantitative results of swabs and biopsies was performed using Spearman's correlation test, which is a non-parametric measure of statistical dependence between two variables. A p value less than 0.05 was considered significant.

3.10 Results

3.10.1 Correlation between semi-quantitative and quantitative swab culture

Two hundred and fifty one MRSA swabs were collected from 41 patients before and during the course of treatments to investigate the correlation between semi-quantitative and quantitative swab culture. Swabs were processed using semi-quantitative (Section 3.8.2.1) and quantitative methods (Section 3.8.2.3) and the results were correlated using Spearman's correlation test. A test's ability to identify a positive result is referred to as its sensitivity. One hundred and thirty three quantitative swabs contained 10^5 or more cfu/cm^2 (52.9%) of MRSA. One hundred and four out of 133 swabs containing 10^5 or more cfu/cm^2 had growth in quadrant III or quadrants III and IV for a sensitivity of 78.2% (Table 3.3).

Non-infected wounds are usually predicted by growth in quadrants less than III. One hundred and fourteen swabs contained 10^4 or less cfu/cm^2 MRSA. Seventy four swabs out of these 114 swabs had growth in quadrants less than III with a sensitivity of 64.9% (74/114).

If growth of MRSA in quadrant III and quadrants III and IV using the semi-quantitative technique is defined as 10^5 cfu/cm^2 or greater then in this study there was 15.9% (40/251) incidence of false positives where the quantitative count was actually less than 10^5 cfu/cm^2. From the opposite perspective, twenty seven quantitative counts were 10^5 cfu/cm^2 or greater and there was no growth of MRSA in quadrant III or quandrants III and IV of semi-quantitative technique. This means a false negative rate of 13.1% (33/251). Spearman's correlation analysis demonstrated a statistically significant strong correlation between the two techniques with ρ *(rho)* = 0.67 and $p < 0.001$.

Table 3.3 Comparison of semi-quantitative and quantitative MRSA swab cultures obtained from patients before and during the course of treatments.

Semi-Quantitative swabs	Quantitative Swabs (cfu / cm^2)					
	10^2	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**
Quadrant I	5	20	4	3	0	0
Quadrant I,II	2	11	32	30	0	0
Quadrant I,II,III	0	3	36	52	14	0
Quadrant I,II,III,IV	0	0	1	10	28	0
Total number of swabs = 251	**7**	**34**	**73**	**95**	**42**	**0**

3.10.2 Correlation between semi-quantitative and quantitative tissue biopsy culture before and during treatment

Sixty-two positive for MRSA deep tissue biopsies were collected from thirteen patients before and during treatment. Thirty two biopsies contained 10^5 or more cfu / g of tissue (51.6%) and they were correlated with 24 tissue biopsy cultures that had growth in quadrant III or quadrants III and IV with a sensitivity of 75% (24/32) (Table 3.4). Twenty six swabs processed semi-quantitatively correlated with thirty swabs processed using the quantitative technique with MRSA count less than 10^5 bacteria/g of tissue for a sensitivity of 86.6% (26/30). False positive results accounted for 6.45% (4/62). An incidence of false negative results was 12.9% (8/62). Spearman's correlation analysis demonstrated a statistically strong significant correlation between the two techniques with a coefficient of ρ*(rho)* = 0.70 with $p<0.001$.

Table 3.4 Comparison of semi-quantitative and quantitative tissue biopsy MRSA counts obtained from patients before first treatment and during therapy.

Semi-Quantitative Tissue biopsy	Quantitative tissue biopsy (cfu/g)					
	10^2	10^3	10^4	10^5	10^6	10^7
Quadrant I	3	3	9	1	1	0
Quadrant I,II	1	2	8	5	1	0
Quadrant I,II,III	0	2	2	9	5	2
Quadrant I,II,III,IV	0	0	0	1	6	1
Total number of swabs = 62	**4**	**7**	**19**	**16**	**13**	**3**

After the comparison of semi-quantitative and quantitative methods for all swabs and biopsies, the samples were divided into two groups: samples collected and analysed before first treatment and samples collected and analysed during the course of treatments. The reason for discriminating the samples into two groups was mainly to look at the possible impact of treatments on the diagnostic validity of the method of sample collection. We hypothesised that the treatment can have an impact on the correlation between quantitative swab and tissue biopsy results. Biopsies were not collected if the wound was too superficial or small to justify a full-thickness punch biopsy or if there was a possibility of damage to healthy tissue.

3.10.3 Correlation between semi-quantitative swab and quantitative tissue biopsy cultures obtained from patients before first treatment

Due to the small numbers of samples received (only seven deep tissue biopsies), it was not possible to compute the correlation of semi-quantitative swab and quantitative MRSA biopsy cultures obtained from patients before the first treatment. This situation could be explained by the invasiveness of the biopsy method and the reluctance of the patient to undergo the procedure.

3.10.4 Comparison of semi-quantitative swab and quantitative biopsy cultures techniques obtained from patients during therapy

Fifty three biopsy/swab pairs were collected from 17 patients out of 41 included in the study before and during the course of treatments. The median bacterial count from biopsies was 1.55 x 10^6 cfu/gram of tissue (SD= 3.38 x 10^6) and from superficial swabs was 5.5 x 10^5 cfu/ml (SD= 9.8 x 10^5).

Twenty-six tissue biopsies contained 10^5 or more cfu/g of tissue (49%) and they were correlated with 21 superficial swab cultures that had growth in quadrant III or quadrants III and IV with a sensitivity of 81% (21/26) (Table 3.5). Ten swabs processed semi-quantitatively correlated with twenty-five swabs processed using quantitative technique with MRSA count less than 10^5 bacteria/g of tissue for a sensitivity of 38% (11/29). False positive results accounted for 32% (17/53). An incidence of false negative results was 9.43% (5/53). Spearman's correlation analysis demonstrated a statistically weak correlation between the two techniques with a coefficient of $\boldsymbol{\rho}$ *(rho)* = 0.40 with $p<0.005$.

Table 3.5 Comparison of semi-quantitative swab culture and quantitative tissue biopsy MRSA counts obtained from patients during treatments.

Semi-Quantitative Swab	Quantitative tissue biopsy (cfu/g)					
	10^2	10^3	10^4	10^5	10^6	10^7
Quadrant I	1	0	2	0	0	0
Quadrant I,II	2	0	5	2	3	0
Quadrant I,I,III	1	6	9	6	2	0
Quadrant I,II,III,IV	0	0	1	4	8	1
Total number of swabs/biopsy pairs = 53	**4**	**6**	**17**	**12**	**13**	**1**

3.10.5 Quantitative microbiology of diabetic foot ulcers

One hundred and thirty seven swabs (54%) out of two hundred and fifty one collected from patients before and during the treatment had MRSA count of 10^5 cfu/cm^2 or greater (137/251). Thirty-seven biopsies (54%) out of sixty nine had MRSA count of 10^5 cfu/g of tissue or greater (37/69). The median MRSA count from biopsies was 1.06 x 10^6 cfu/g of tissue and from superficial swabs was 7.08 x 10^5 cfu/ cm^2. In this study none of the patients was showing clinical signs of infection. These findings disproved the theory based on the findings of Robson *et al.,* (1970) that bacterial count 10^5 cfu/g of tissue or greater is diagnostic for infection.

3.11 Discussion

There are very few studies correlating results of superficial swab culture and tissue biopsy culture obtained from chronic wounds and which looked at the diagnostic validity of the semi-quantitative superficial swab. In this study, superficial swabs and tissue biopsies were collected from diabetic foot ulcers colonised with MRSA, processed and compared using quantitative and semi-quantitative techniques. Wounds

usually contain a variety of different bacterial species (contaminating and colonising the wound). Comparing semi-quantitative and quantitative swab and biopsy culture by using total bacterial count does not give information on the correlation for the specific strains. This can be important especially if there is a possibility of overgrowth by other bacteria present such as *E. coli.* In this study MRSA was used as an indicator microorganism to validate microbiological culture methods.

The findings of this study demonstrated statistically strong correlations between semi-quantitative and quantitative methods for both swabs and biopsies. However there was a weak correlation between semi-quantitative swab and quantitative tissue biopsy culture observed. All patients were colonised with MRSA and underwent the course of treatments (three to twelve treatments depending on healing progress). The superficial swabs and tissue biopsies were collected before the first treatment and then during the course of therapy. Correlation between semi-quantitative swab culture and quantitative biopsy for samples obtained from patients before the first treatment was not performed due to the small number of samples received. For samples collected during the course of treatments, a quantitative tissue biopsy correlated with a semi-quantitative swab for the sensitivity of 81% for the detection of bacterial count 10^5 or greater. There was only 38% sensitivity achieved for samples with bacterial count 10^4 or less and this is why there was a weak correlation observed between semi-quantitative swab and quantitative biopsy culture. It is an important finding especially for patients on larvae therapy. The maggots secrete proteolytic enzymes that break down and liquefy dead tissue which they then ingest. They also have an ability to ingest and destroy bacteria (Huberman *et al.,* 2007) which may have an impact on correlation between superficial sampling and deep tissue biopsy and thereby lead to misleading results.

In previous studies, it was documented that quantitative superficial swab results were highly correlated with semi-quantitative results, but this was demonstrated on

infected burn wounds and the total bacterial count was taken into consideration (Herruzo-Cabera *et al*., 1992)..

The findings of this study demonstrated a statistically strong correlation between semi-quantitative swab and quantitative swab culture. Two hundred and fifty one swabs were collected from patients before and during the treatments. Of the 133 swabs containing 10^5 cfu/ ml or more bacteria, 104 swabs had bacterial growth in quadrant III resulting in a sensitivity of 78.2%. For biopsies collected before and during the course of treatments there was also a statistically strong correlation observed between semi-quantitative and quantitative biopsy culture with a sensitivity of 75% for samples with MRSA counts 10^5 cfu/g of tissue and 86.6% for samples containing 10^4 or less cfu/ gram of tissue.

Ratliff and Rodeheaheaver (2002) correlated 124 swabs processed quantitatively with swabs processed semi-quantitatively to determine the clinical acceptability of the semi-quantitative technique in identifying chronic wounds containing greater than 10^5 bacteria / cm^2. They documented that the two techniques were correlated with a coefficient of r=0.84. The sensitivity of the semi-quantitative technique to detect wounds with 10^5 or greater bacteria was 79%, which is similar to our findings.

Buchanan *et al*., (1986) compared 78 semi-quantitative and quantitative burn wound biopsy cultures and found a 96% correlation between both techniques. For prediction of wound sepsis, the semi-quantitative procedure had a positive predictive value of 100% and a negative predictive value of 93.7%. They concluded that the technique provides accurate information to the physician while saving both time and materials.

Thomson *et al*., (1990) compared tissue biopsy and semi-quantitative swabs collected from 24 burn patients. They concluded that the surface swab may be used as an indicator of numbers of organisms with reliability.

Steer *et al.,* (1996) compared qualitative results and quantitative bacterial counts of 141 surface swabs and 141 wound biopsy samples taken from 74 burn patients. Although there was significant correlation between the bacterial counts obtained by biopsy and swab, the counts obtained by one method were poorly predictive of the counts obtained by the other. In addition, parallel cultures taken on multiple occasions showed a significant correlation between bacterial counts obtained from two biopsies or two swabs taken simultaneously, but there was wide variation in bacterial densities from the same burn wound at the same time. These investigators concluded that the use of quantitative microbiology in burns is limited by the unreliability of a single surface swab or biopsy sample to represent the whole burn wound.

Sjoberg *et al*., (2003) reported that quantitative tissue biopsies gave a better prediction of sepsis than surface swabs but concluded that the amount of labour involved in collection and analysis of multiple biopsy samples limited the clinical relevance of this approach.

According to Robson *et al*., (1976) finding a bacterial count of 10^5 bacteria per gram of tissue is considered as an indicator of infection. In our study none of the patients had wounds that were clinically considered as infected but all were colonised with bacteria. The median MRSA count from tissue biopsies was 1.06×10^6 cfu/ gram of tissue and from superficial swab 7.08×10^5 cfu/ ml. Despite MRSA colonisation all wounds were colonised by other bacteria. The median total bacterial count for biopsies was 8.32×10^7 cfu/ g of tissue and for superficial swab 3.22×10^6 cfu/ cm^2. These findings show that wound assessment should be based mainly on clinical observation of

the wound and patient. Identification and quantification of the pathogens is very useful especially to manage antibiotic therapy, but quantification of bacteria should not be used as an indicator of infection especially in chronic wounds. The Robson findings may be useful on sterile skin or in diagnosing acute wound infections (burns), which are usually caused by single pathogens, but it should not be used to diagnose infection in chronic wounds.

The semi-quantitative technique offers a reproducible and cost effective method compared to quantitative serial dilution culture. Although semi-quantitative swabs are slightly less sensitive than quantitative swabs in detecting greater than 10^5 bacteria in wounds, they are beneficial for clinicians who want to adjust their wound care based on bacterial bioburden. The additional advantage of our study was the fact that we used MRSA as an indicator organism to compare two methods. Most of the previous studies correlated semi-quantitative and quantitative culture methods taking total bacterial count into consideration instead of looking at the ability of the method to detect and quantify a specific pathogen. This may be an important factor, especially in chronic wounds which are polymicrobial in nature. The ability of the method to detect the causative pathogen is crucial for an accurate diagnosis and treatment of the patient as the overgrowth with other microorganisms may mask the presence of the causative pathogen.

Currently, there are very limited studies that evaluate the semi-quantitative swab as a method for determining bacterial bioburden in chronic wounds. This method is cheap and available to anyone in healthcare regardless of the practice setting. More studies evaluating this technique in chronic wounds are needed. For example, additional research might compare different types of transport swab systems to see if the quality of swab has an impact on the correlation between superficial swab and tissue biopsy. Also, molecular methods able to look at the diversity of microflora within the sample such as

DGGE-sequencing or multiplex RT-PCR should be employed and used to compare and validate superficial swab and biopsy methods. Tissue biopsy is an invasive method and could cause pain and/or bleeding, enlarge the wound, or introduce contaminants. A full thickness punch biopsy may not be justified in the presence of a small or superficial wound. Therefore, because of the invasiveness and limited number of qualified practitioners, swab culture is the most frequently used method to determine the microbiology of chronic and acute wounds and it should be properly validated (Drinka *et al*., 2012).

3.12 Conclusions

In this study the reliability of semi-quantitative and quantitative swab and biopsy culture was assessed and compared. There was a strong correlation between semi-quantitative and quantitative swab culture and semi-quantitative and quantitative biopsy culture observed. There was also a weak correlation between semi-quantitative swab culture and quantitative tissue biopsy culture for samples collected during the course of treatments which suggests that therapy could have an impact on correlation between both methods. These results show that semi-quantitative methods can be successfully used in laboratory settings for the management of chronic wounds, however if the time is important, rapid and direct techniques such as quantitative RT-PCR should be employed. RT-PCR for direct and rapid detection of MRSA was developed in the next part of the study and compared with conventional culture results.

The semi-quantitative swab method is an efficacious and less expensive technique than serial dilution culture. Quantitative tissue biopsy is an invasive and expensive method, which requires trained professionals to perform it. Additionally our results revealed that the mean bacterial count from swabs and biopsies was more than 10^5 cfu per gram of tissue. None of our patients showed any signs of infection.

According to the findings of Robson *et al.,* (1976) the count of 10^5 cfu/ gram of tissue is an indicator of infection thus our results confirm that a diagnosis of infection should be based on clinical signs and symptoms rather than quantitative microbiology of the wound.

CHAPTER 4

Development of a RT-PCR assay for the comparison of MRSA detection and quantification with conventional microbiology of diabetic foot lesions

4.1 Introduction

Clinical microbiology laboratories still routinely use phenotypic assays for the identification, quantification and antibiotic susceptibility testing of microorganisms collected from chronic wound samples. It requires between 24 h to 72 h to identify most of the pathogens, including MRSA, by conventional methods. It is documented that culture methods detect only about 1% of all bacteria present in chronic wounds (Wolcott and Dowd, 2008). Furthermore, conventional methods are not able to isolate all bacterial species present in a specific wound (Gentili *et al*., 2012).

Recently, studies have shown the polymicrobial nature of infection in chronic lesions. Additionally, interactions among colonising bacteria are thought to play a major role in non-healing chronic wounds (Thomsen *et al*., 2010; Martin *et al*., 2010).

In a study by Rhoads *et al.,* (2012) organisms isolated by conventional cultures were compared with those detected by molecular methods. To identify a specific gene, molecular methods such as RT-PCR and the use of specific primers to identify organisms were combined with pyrosequencing. In the 168 wound samples evaluated, molecular techniques identified 338 unique genera whereas culture methods revealed only 17 different genera. Culture failed to grow the vast majority of bacteria present in the wound. The important conclusion from this study was that culture methods are not adequate in identifying bacteria in polymicrobial infections (Rhoads *et al*., 2012).

In recent years, RT-PCR has been introduced into clinical microbiology laboratories especially for the diagnosis of infectious disease. As a adjunct to culture, PCR- based methods have been evaluated for the detection of bacteria in blood (Rothman *et al.,* 2002; Wellinghausen *et al*., 2004), joint fluid (Yang *et al*., 2008), cerebrospinal fluid (Poppert *et al*., 2005), heart tissue (Breitkopf *et al*., 2005) and burn wounds (Pirnay *et al*., 2000). Several assays have been developed for the rapid detection

of a variety of commonly isolated bacteria such as *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *Listeria monocytogenes*, *Neisseria meningitidis, Haemophilus influenzae* and Shiga-Toxin producing *E. coli*. There are also assays available for slow growing, fastidious or uncultivable microorganisms such as *Mycobacterium* species, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Rickettsia*, *Coxiella*, *Bartonella*, *Mycoplasma pneumoniae*, *Helicobacter pylori* and *Clostridium difficile* (Maurin *et al.,* 2012). Multiplex RT-PCR was developed for diagnosis of syndromes and specific conditions such as bacteraemia (Josefson *et al*., 2011; Wallet *et al*., 2010), meningitis (Wang *et al*., 2012; Abdeldaim *et al*., 2010), pneumonia (Johansson *et al*., 2010, Thurman *et al*., 2011), enteritis (de Boer *et al*., 2010) and sexually transmitted infections (Murunyi *et al*., 2011; Lee *et al.,* 2012).

RT-PCR can be also used to quantify the bacteria in clinical samples. Because these tests quantify DNA from both viable and non-viable bacteria, results may be higher than those obtained by quantitative culture. A high correlation between both methods was reported for *S. pneumoniae* (Abdeldaim *et al*., 2008).

Previous molecular analyses of chronic wound microflora were based on the initial amplification of bacteria with universal primers for 16S rRNA genes followed by specific identification approaches. These approaches included sequencing of PCR products (Hill *et al*., 2003), DGGE followed by cloning and sequencing (Davies *et al*., 2004) and pyrosequencing (Dowd *et al*., 2008). However, these identification methods are costly, time consuming and not easy to implement in clinical practice. RT-PCR is faster than traditional PCR and does not require post amplification manipulation for bacterial identification (Wellinghausen *et al.,* 2004; Yang *et al.,* 2008). RT-PCR comprises both detection and quantification of a DNA target in the same step. There are two main methods for detection of amplified products (Barken *et al.,* 2007; Espy *et al*.,

2006). The first using a fluorescent dye such as SYBR® Green, which binds non-specifically to double stranded DNA and the second uses fluorescent resonance energy transfer (FRET) probes, which binds specifically to the amplified DNA. Using these probes increases sensitivity and specificity when compared with conventional PCR.

RT-PCR presents a number of advantages over conventional PCR including a lower risk of DNA cross contaminants, a faster turn-around time and the possibility to quantify target DNA in clinical specimens. RT-PCR has the same limitations as standard PCR, including false negative results due to the inhibition of DNA polymerase or variations in the target nucleic acid sequence among strains of the same bacterial species. False positive results can occur because of clinical sample contamination (Martin *et al*., 2010; Kaltenboeck and Wang, 2005).

RT-PCR assay may be a useful adjunct for the rapid and accurate identification of microorganisms in chronic wounds which can simultaneously target multiple species. There are very few commercially available multi-species PCR-based tests such as SeptiFast (Westh *et al*., 2009; Mussap *et al*., 2007), however they have not been applied to chronic wounds. Melendez *et al*., (2010) reported the development of a RT-PCR assay for rapid identification of bacteria directly from tissue samples. The assay targets fourteen common, clinically relevant, aerobic pathogens and demonstrates a high degree of sensitivity and specificity using a panel of organisms commonly associated with chronic wound infection. Thirty-nine tissue samples from twenty nine chronic wounds were evaluated and the results compared with those obtained by culture. The most common organisms identified were methicillin-resistant *Staphylococcus aureus* (MRSA) followed by *Streptococcus agalactiae* (Group B streptococcus) and *Pseudomonas aeruginosa*. The sensitivities of the PCR assays were 100% and 90% when quantitative and qualitative culture results were used as the reference standard,

respectively. The assays allowed the identification of bacterial DNA from ten additional organisms that were not revealed by quantitative or qualitative cultures.

Bacterial resistance to antibiotics is an increasing public health problem worldwide. The detection of antimicrobial resistance (especially MRSA) is important for infection control measures and for the treatment of patients. Numerous molecular techniques that reduce the time for identification of MRSA have been developed recently. However, most of these methods cannot be applied for the direct detection from clinical specimens, without previous time consuming isolation, capture or enrichment of MRSA. Methicillin-resistant and -sensitive *Staphylococcus aureus* strains are of a major concern due to their ability to cause difficult skin and underlying tissue infections. Bacteria constantly develop mechanisms of resistence to antibiotics. Intermediate vancomycin or glycopeptide resistant *Staphylococcus aureus* (VISA or GISA) were first detected in Japan in 1997 (Hiramatasu *et al*., 1997) and subsequently in other countries (Tenover *et al*., 1998; Kim *et al*., 2000). In June 2002, the first clinical isolate of *Staphylococcus aureus* resistant to vancomycin (VRSA) was isolated in the USA (Srinivasan *et al*., 2002). Despite intensive attempts at eradication during the last 20 years, MRSA continues to be the major nosocomial pathogen worldwide (National Nosocomial Infections Surveillance System, 2004).

Rapid screening and identification of MRSA has become a crucial procedure in medical diagnostics. Most of the molecular tests developed up to date are based on detection of the *mecA* gene and they have become a useful tool in diagnostic microbiology (Bignardi *et al*., 1996). PCR detection assays for MRSA screening are based on a simultaneous detection of the *mecA* gene and *S. aureus* –specific marker gene, e.g the *nuc, Sa442* or the *femB* gene (Fang and Hedin, 2003; Grisold *et al*., 2002; Jonas *et al*., 2002; Reischl *et al*., 2000; Tan *et al*., 2001). However, all these duplex-PCR assays carry the risk of co-amplifying the *S. aureus* –specific marker gene together

with the *mecA* gene from MR-CNS (Methicillin Resistant Coagulase Negative Staphylococci) leading to false-positive results when both microorganisms are present in the sample.

The rapid and accurate identification of bacteria in clinical specimens has important implications for the therapy and management of both colonised and infected patients. Based on the recent findings of the polymicrobial nature of chronic wound infection, rapid and cost-effective approaches such as multiplex RT-PCR should be developed for the detection of the most prevalent aerobic and anaerobic microorganisms in wounds.

4.2 Aims and objectives

The aim of this study was to develop a quantitative RT-PCR assay for the direct and rapid detection of MRSA in chronic wounds. If sufficient time is available, a multiplex PCR for the most common pathogens will be developed based on the results of a PCR-DGGE sequencing study in which the diversity of microflora of chronic wounds will be assessed. This chapter is presented to show the potential for future work. The RT-PCR assay was initially optimised and validated using control strains before it was used on samples collected from patients.

4.3 Materials and Methods

4.3.1 Materials

- DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) – the method was described in Chapter 2, section 2.3.3.3

- SYBR Green Master Mix – Agilent (Berkshire, UK)

- Brilliant III QPCR Master Mix – Agilent ((Berkshire, UK)

Primers and probes were supplied by Integrated DNA Technologies Ltd (Leuven, Belgium). Discriminate detection of the two targets in the duplex assay was possible by use of two different fluorophors: 6-carboxyfluorescein (FAM: emits a fluorescent signal at 525 nm) for the detection of *mecA*, and 6-carboxy-20, 40, 70, 4, 7-hexachlorofluorescein (HEX: emits a fluorescent signal at 560 nm) for the detection of *femB*. The *mecA* probe was labelled with a 5' FAM fluorophor and a 3' TAMRA quencher. The *femB* probe was labelled with a 5' HEX fluorophor and a 3' TAMRA quencher (Table 4.1)

Table 4.1 Sequences of the primers and probes used in the development of the RT-PCR assay for MRSA detection and quantification (Saeed *et al*., 2010).

Primer name	**Sequence (shown 5' to 3')**
*fem*BF1	**GACATTTGATAGTCAACGTAAACGTAATATT**
*fem*BR1	**GCTCTTCAGTTTCACGATATAAATCTAAGA**
*mec*Af	**CATTGATCGCAACGTTCAATTT**
*mec*AR	**TGGTCTTTCTGCATTCCTGGA**
Probe name	
*fem*BVICP1	**TCATCACGTTCAAGGAATCTGACTTTAACACCATAGT**
*mec*AP	**TGGAAGTTAGATTGGGATCATAGCGTCAT**

4.3.2 Methods

4.3.2.1 Ethical approval

Ethical approval for development of multiplex RT-PCR was obtained from the NHS Research Ethics Committee (11/H1011/4). This study was performed at the Manchester Royal Infimary and Chorley and South Ribble Hospitals. R&D approval was given from both hospitals.

4.3.2.2 Participants and study design

The samples were collected from adults (age 18 – 75 years old) with chronic wounds attending the Manchester Diabetes Centre, Manchester Foot Hospital or in-patients in Manchester Royal Infirmary and Chorley and South Ribble Hospital (Appendix 2 – Trial Protocol).

A total of 32 patients with chronic wounds were recruited to the study. The patients were appropriately informed and consented to the trial (Appendix 3 – Patient Information Sheet; Appendix 4 – Consent form). In the first instance the wound was cleaned with sterile saline. Two swabs were obtained using the Sigma dry swab (Medical Wire & Equipment Co. Ltd., Corsham, England). The samples were collected using a rolling zigzag method. One swab was tested using conventional microbiological culture techniques and the second swab was used for the development of a RT-PCR assay and bacterial population analysis using PCR-DGGE and sequencing (Chapter 5).

4.3.2.3 RT-PCR assay

To ensure efficient and accurate quantification of the target template, the RT-PCR assay was optimised and validated. Serial dilutions of a MRSA phage type 16 (NCTC 13143) control strain were prepared, inoculated onto the media (Section 3.8.7) to confirm colony count and then the DNA was extracted from each dilution using the DNeasy Blood and Tissue Kit. The optimisation of RT-PCR was performed using two different methods to identify which method gave more sufficient DNA extraction. The first approach involved the extaraction of DNA separately from each serial dilution prepared. In the second approach DNA was extracted from the first serial dilution and then serial dilutions were prepared from the extracted DNA sample. After extraction, the RT-PCR was carried out using the Stratagene Mx3000P System (Stratagene, USA). The

primers were initially optimised with SYBR Green I (Agilent) and then they were optimised together with probes using Brilliant III QPCR Master Mix (Agilent).

Two sets of primers were used to target two genes present in MRSA strains – *mecA* and *femB*. The *mecA* gene encodes the extra PBP2a, which is unique to methicillin-resistant staphylococci (Table 5.1). The *femB* gene codes for an enzyme important in cross-linking peptidoglycan in various different *Staphylococcus* spp. The specificity of the *femB* and *mecA* PCR primers used for DNA amplification of the species *S.aureus* has been demonstrated previously in orthopaedic infections (Saeed *et al*., 2010). Two sets of primers was used for the presence of *mecA* (this lies within the staphylococcal cassette chromosome *mec* (SCCmec) mobile cassette, encodes methicillin resistance) and *femB* (factor essential for methicillin binding, and is targeted at a *S. aureus* – specific sequence gene**.** Clinical sensitivity and specificity was evaluated by comparing the optimised extraction/PCR method with conventional CLSI culture-based methods for the identification of *S. aureus* and methicillin resistance.

The slope of the standard curve was used to determine reaction efficiency. Since the PCR reaction is based on exponential amplification, if the efficiency of PCR amplification is 100%, the amount of template will double with each cycle and the standard curve plot of the log of starting template vs. PCR cycles which generate a linear fit with a slope between approximately -3.1 and -3.6 are typically acceptable for most applications requiring accurate quantification (90%-110% reaction efficiency). If the amplification reaction is not efficient at the point being used to extrapolate back to the amount of starting material, the calculated quantities may not be accurate.

Rsq (regression coefficient) is the fit of all data to the standard curve plot and can be influenced by accuracy of the dilution series, and overall assay sensitivity. If all the data lie perfectly on the line, the Rsq value will be 1.00. As the data fall further from the line, the Rsq decreases. An Rsq value ≥0.985 is acceptable for most assays. The slope and

Rsq values of the standard curve help determine the sensitivity of a given assay. If the slope of the standard curve is lower than -3.322 the Rsq is below 0.985 and the data points indicate an upward trend in the standard curve plot at the lower starting template concentrations. This may indicate the reaction is reaching the threshold of sensitivity.

4.4 Optimisation of RT-PCR assay

4.4.1 Optimisation of RT-PCR with SYBR Green I for *mecA* and *femB* genes from DNA extracted from each serial dilution.

SYBR Green I dye is a DNA binding dye. It generates a signal from both specific and non-specific products. When free in solution, SYBR Green I displays relatively low fluorescence, but when bound to double-stranded DNA its fluorescence increases by over 1000-fold (Introduction to Quantitative PCR, Stratagene, 2005). The fluorescence increases proportionately with DNA concentration and the more double-stranded DNA is present, the more binding sites there are for the dye. This property of the dye provides the mechanism that allows it to be used to track the accumulation of PCR product. As the target is amplified, the increasing concentration of double stranded DNA in the solution can be directly measured by the increase in fluorescence (Methods Application Guide, Stratagene, 2005). Primers were used at a working concentration of 300 nM each. To determine the detection limit of the assay, serial dilutions of MRSA phage type 16 (NCTC 13143) were prepared and inoculated onto the plates using the quantitative culture method (section 3.8.2.3). Plates were incubated in aerobic conditions for 24 h and colonies counted after incubation. DNA was extracted from each dilution using the DNeasy Blood and Tissue Kit. The PCRs were performed in a total volume of 25µl using the SYBR Green Master Mix (Agilent) and Stratagene Mx3000P® QPCR System (Staratagene). The reactions comprised 300 nM (each) forward and reverse primers and 5µl of template DNA. No-template control (NTC) with

water instead of template DNA was incorporated in each run under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 min and 60°C for 1 min. The result of each PCR is indicated by a threshold cycle (*Ct*).

4.4.1.1 Optimisation of the assay for targeting the *mecA* gene

The mean difference in Ct* values between sequential dilutions (n) for the *mecA* standard curve was 3.29. This means that the amount of product in each reaction was doubled. This is well within the acceptable range of 3.1 to 3.6 (Introduction to Quantitative PCR, Stratagene, 2005). The amplification efficiency value (Rsq) of 98.9% again was within the acceptable parameters (Figures 4.1, 4.2, 4.3). If the slope of the standard curve was lower than -3.32 the Rsq was below 0.985 and the data points indicate an upward trend in the standard curve plot at the lower starting template concentrations. The reaction efficiency for *mecA* gene assay optimisation was 101.3% (a range between 90%-110% is acceptable for most applications).

A comparison of the *mecA* optimisation assay to culture methods for the detection of methicillin-resistant *S. aureus* is shown (Table 4.2). The detection limit (sensitivity) of the PCR assay for *mecA* gene was ~40 cfu of MRSA per 5µl of sample, which equates to 8 x 10^3 cfu / ml.

***Ct** (threshold cycle)** is the intersection between an amplification curve and the threshold line. It is a relative measure of the concentration of target in the PCR reaction.

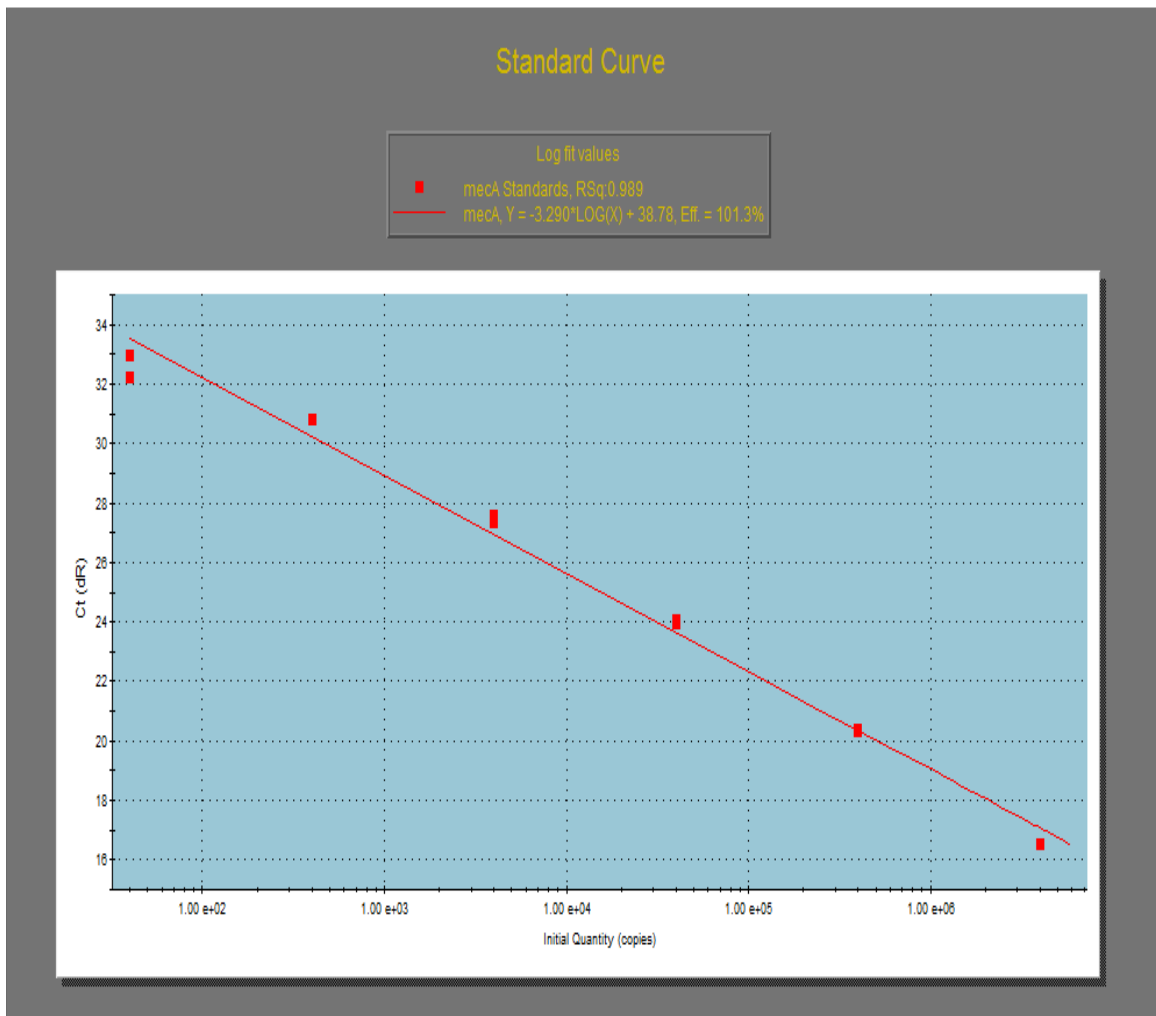


Figure 4.1 QPCR standard curve from *mecA* assay demonstrating a ten-fold dilution series of DNA extracted from each serial dilution of the MRSA phage type 16 control strain.

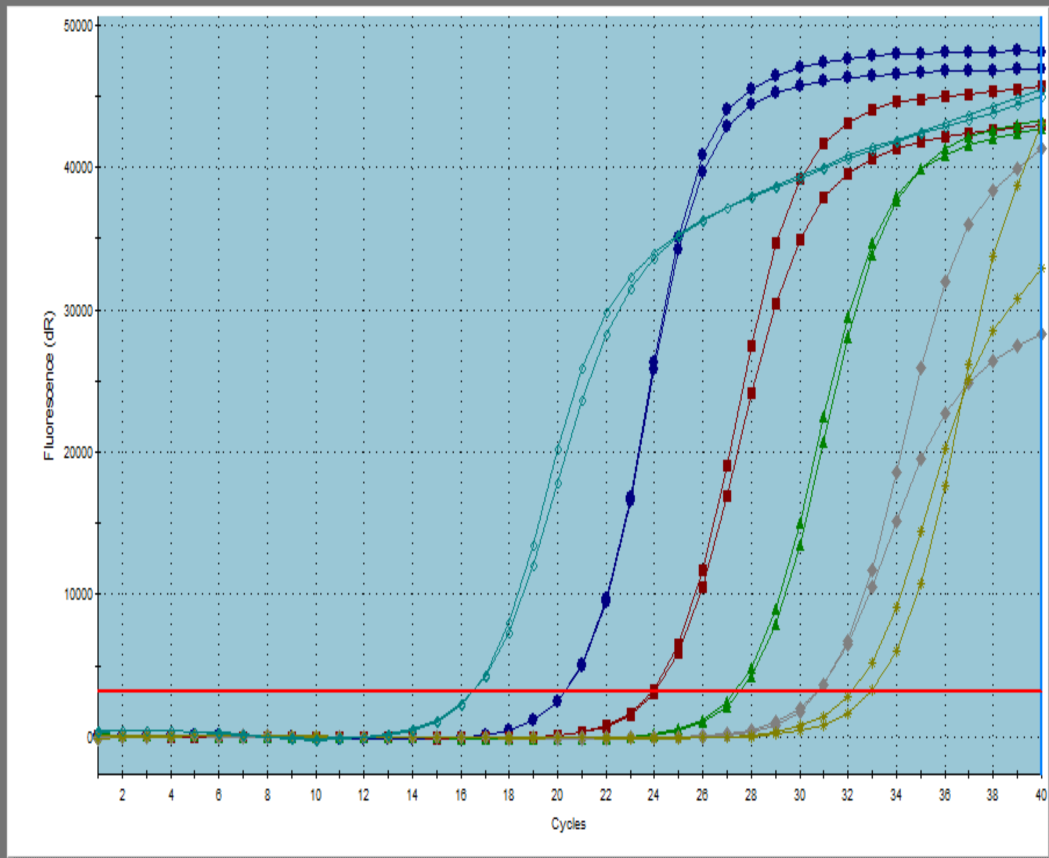


Figure 4.2 Amplification plots of *mecA* standards in a ten-fold dilution series (DNA extracted directly from each dilution)

Legend:

Amplification Line colour	Dilution of the sample
(light blue line)	Amplification plot for 1:10 dilution
(black line)	Amplification plot for 1:100 dilution
(red line)	Amplification plot for 1:1000 dilution
(green line)	Amplification plot for $1{:}10^4$ dilution
(grey line)	Amplification plot for $1{:}10^5$ dilution
(orange line)	Amplification plot for $1{:}10^6$ dilution

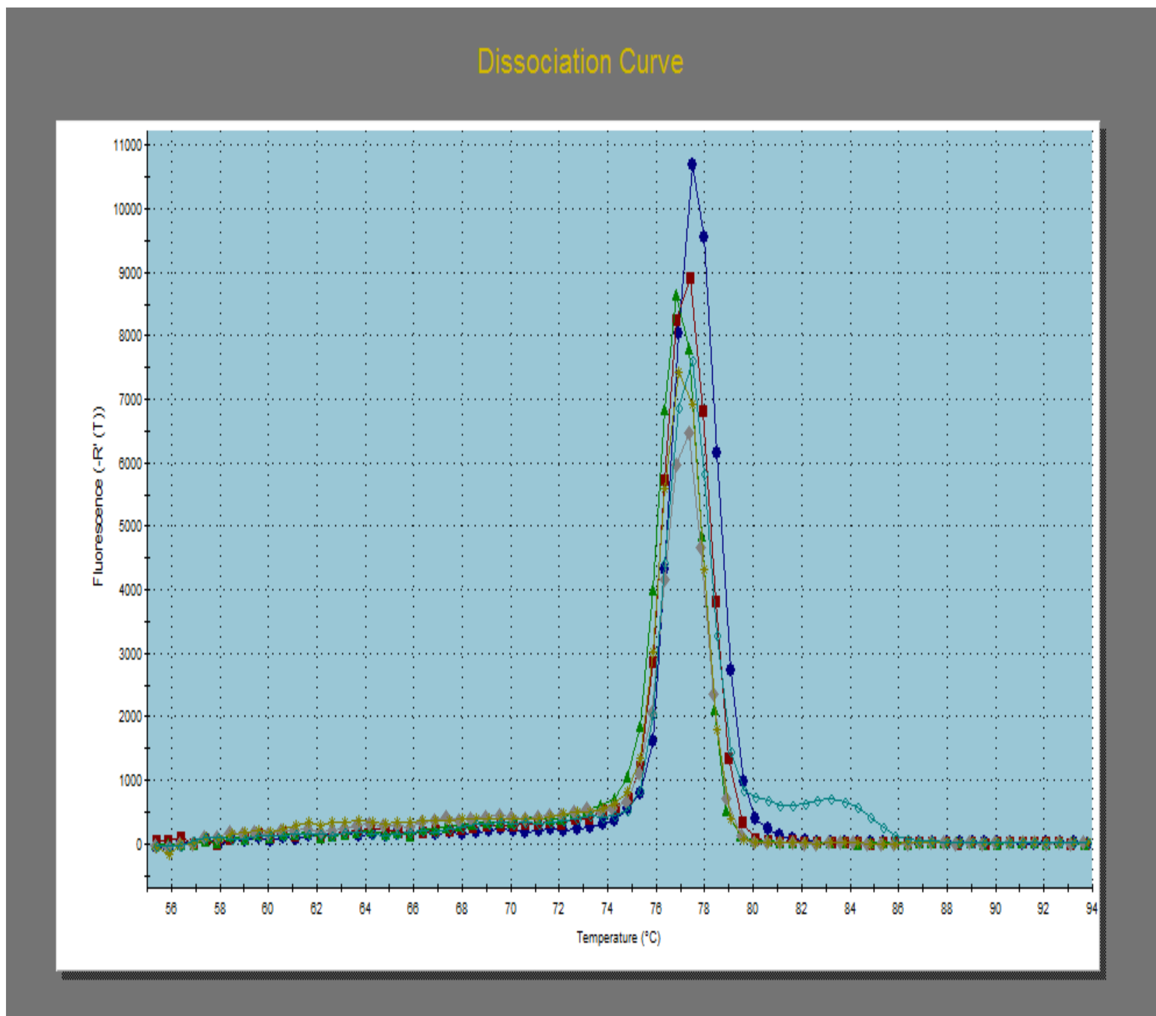


Figure 4.3 Dissociation curve of *mecA* standards in a ten-fold dilution series (DNA extracted directly from each dilution). The single melt peak indicates a single PCR product is being amplified in these samples.

Table 4.2 The *mecA* optimisation assay (DNA extracted directly from each dilution) for the detection of methicillin-resistant *S. aureus* demonstrating bacterial count of each serial dilution and its corresponding *Ct* value.

Ct	Dilution cfu/5µl of sample
16.52	4×10^6
20.35	4×10^5
24.00	4×10^4
27.46	4×10^3
30.80	4×10^2
32.55	4×10^1

4.4.1.2 Optimisation of the assay for targeting the *femB* gene

The mean difference in Ct values between sequential dilutions (n) for the *femB* standard curve was 3.32 and it was well within the acceptable range of 3.1 to 3.6. The amplification efficiency value (Rsq) was 99.9%. This means that sensitivity of assay was good (Rsq is the fit of all data to the standard curve plot and if the data lie perfectly on the line, the Rsq will be 1.00) (Figures 4.4, 4.5, 4.6). The reaction efficiency for the *femB* gene assay optimisation was 99.9% (a range of 90%-110% for reaction efficiency is acceptable for most applications).

The detection limit (sensitivity) of the PCR assay for the *femB* gene was ~40 cfu of MRSA per 5µl of sample- 8×10^3 cfu/ml (Table 4.3).

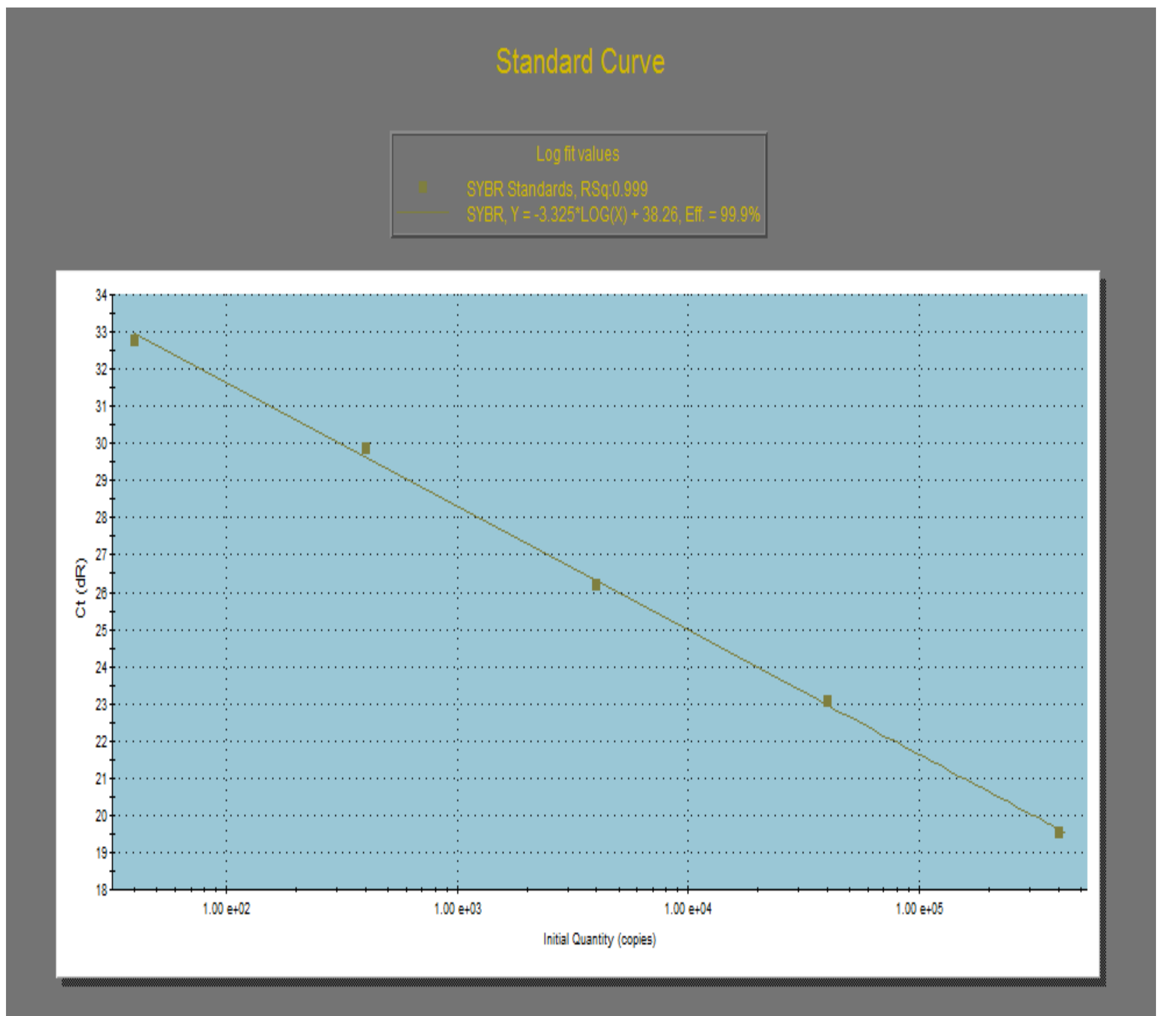


Figure 4.4 QPCR standard curve from the *femB* assay demonstrating a ten-fold dilution series of DNA extracted directly from each MRSA 16 control strain dilution.

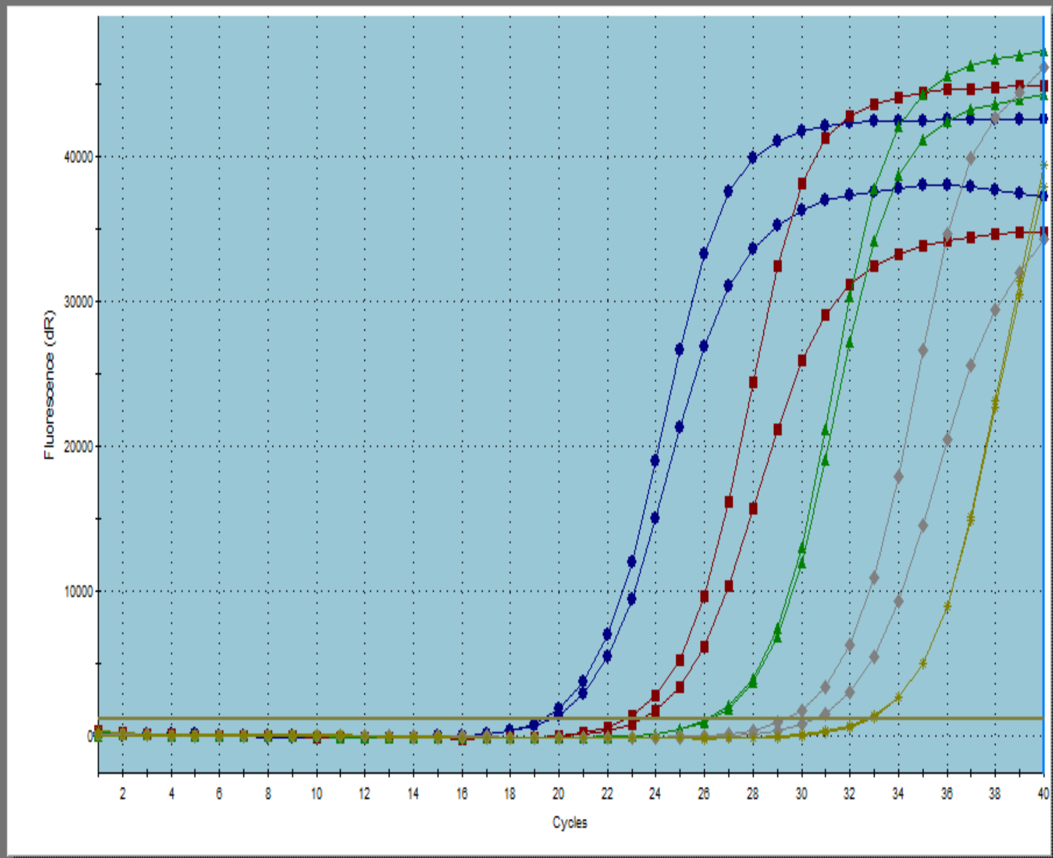


Figure 4.5 Amplification plots of *femB* standards in a ten-fold dilution series (DNA extracted directly from each dilution).

Legend:

Amplification Line colour	Dilution of the sample
(blue line)	Amplification plot for 1:10 dilution
(red line)	Amplification plot for 1:100 dilution
(green line)	Amplification plot for 1:1000 dilution
(grey line)	Amplification plot for 1:10^4 dilution
(brown line)	Amplification plot for 1:10^5 dilution

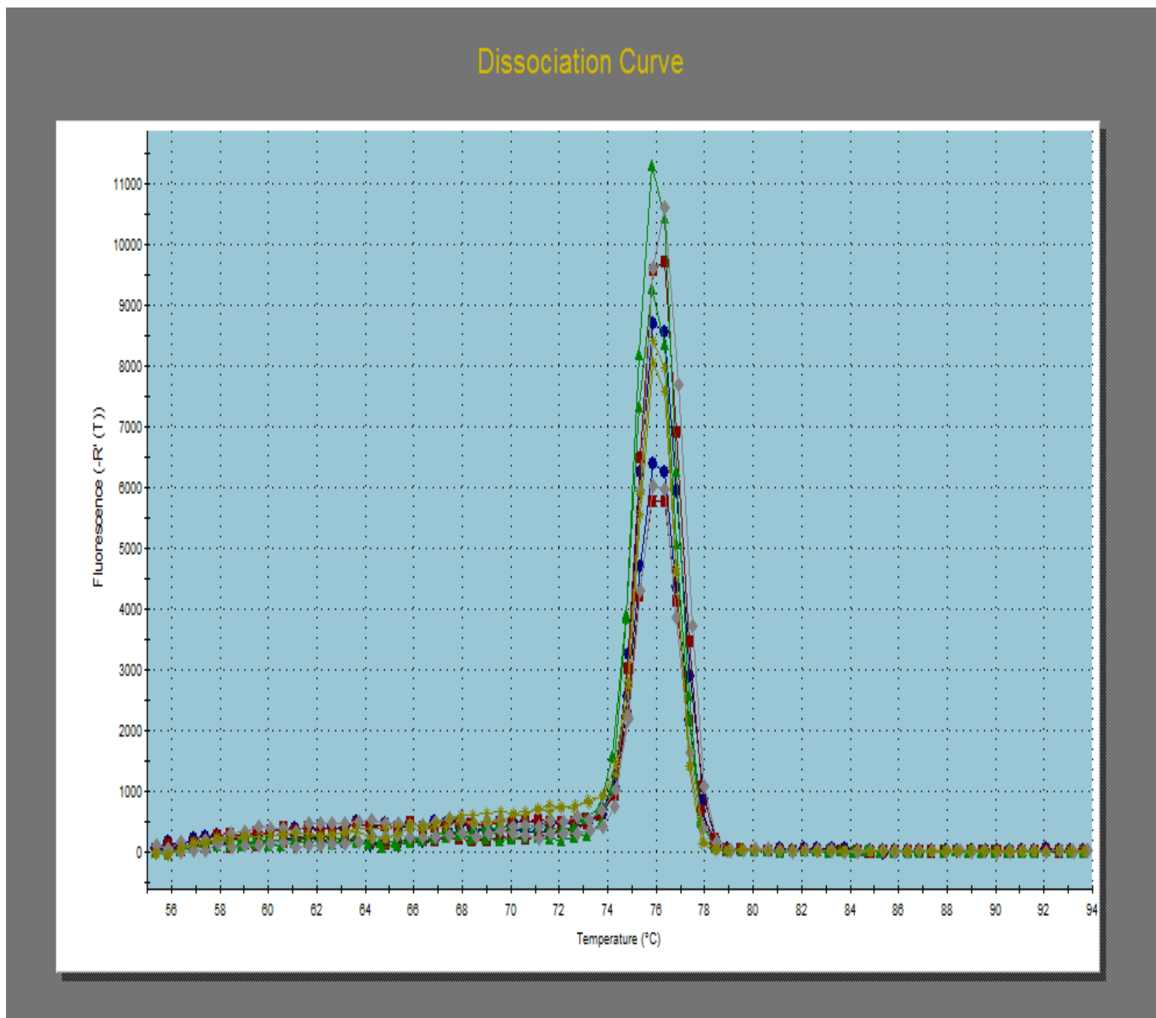


Figure 4.6 Dissociation curve of *femB* standards in a ten-fold dilution series (DNA extracted directly from each serial dilution). The single melt peak indicates a single PCR product is being amplified in these samples.

Table 4.3 The *femB* optimisation assay (DNA extracted directly from each dilution) for the detection of methicillin-resistant *S. aureus* demonstrating bacterial count of each serial dilution and its corresponding *Ct* value.

Ct	Dilution cfu/5ul of sample
19.53	4×10^5
23.09	4×10^4
26.21	4×10^3
29.85	4×10^2
32.76	4×10^1

4.4.2 RT-PCR optimisation for targeting *mecA* and *femB* with DNA dilutions prepared from DNA extracted from MRSA sample

DNA was extracted from the sample of MRSA suspension in sterile saline with approximately 10^7 cfu/ml and then dilutions of the extracted DNA were prepared. RT-PCR was performed using dilutions of DNA from the neat sample.

4.4.2.1 Optimisation of the assay for targeting *mecA* gene

The mean difference in Ct values between sequential dilutions (n) for *mecA* standard curve for DNA dilutions of the neat sample was 3.26 and it is within the acceptable range of 3.1 to 3.6. The amplification efficiency value (Rsq) of 102.4% and this is within acceptable parameters. The amplification efficiency value (Rsq) was 0.995 (see Figures 4.7, 4.8, 4.9). However, the detection limit (sensitivity) of the PCR assay for serial dilutions prepared from DNA extracted from the neat sample was ~400 cfu of MRSA in comparison to 40 cfu in the samples where DNA was extracted straight from the bacteria dilutions (Table 4.4).

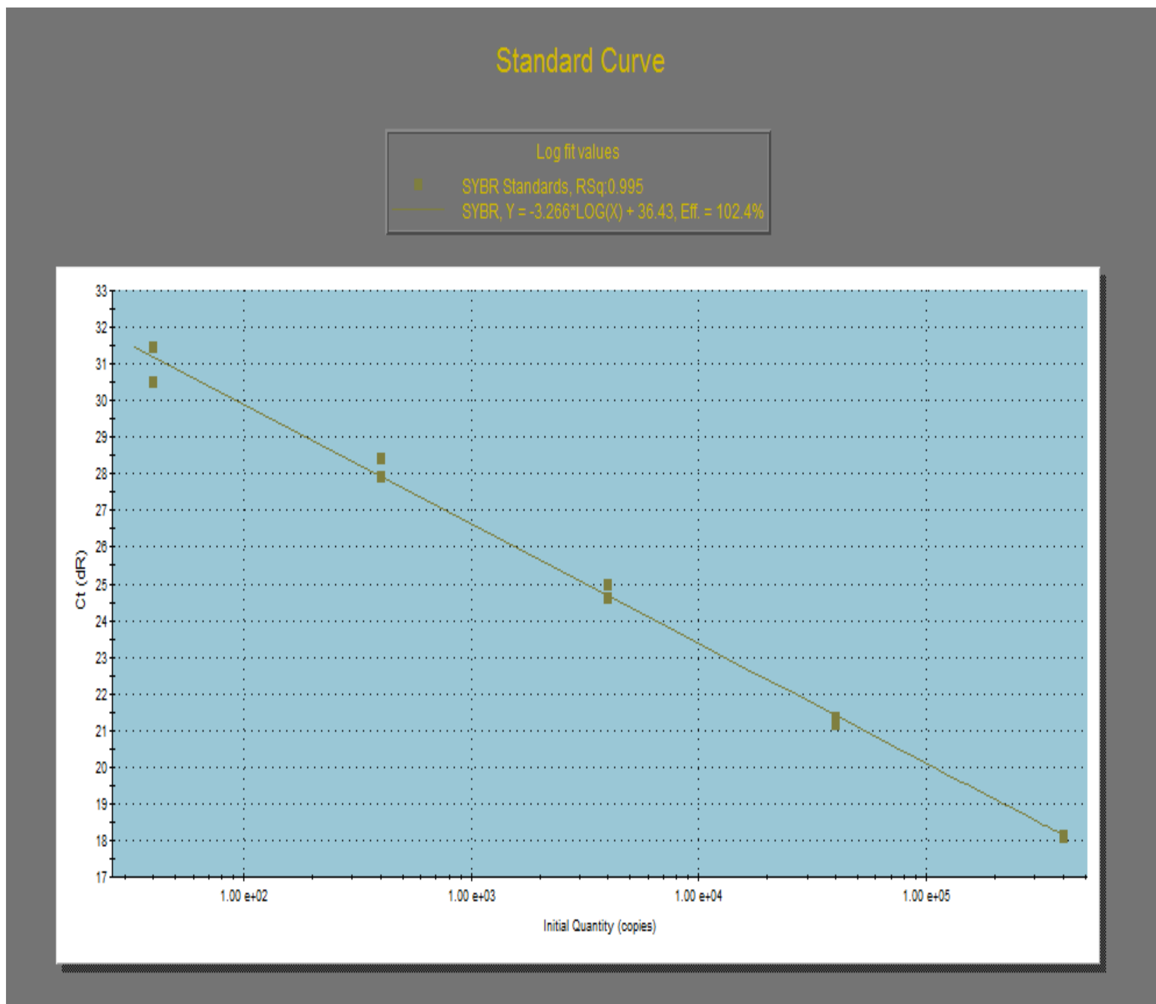


Figure 4.7 QPCR standard curve from *mecA* assay demonstrating a ten-fold dilution series of DNA extracted from MRSA 16 control strain suspension.

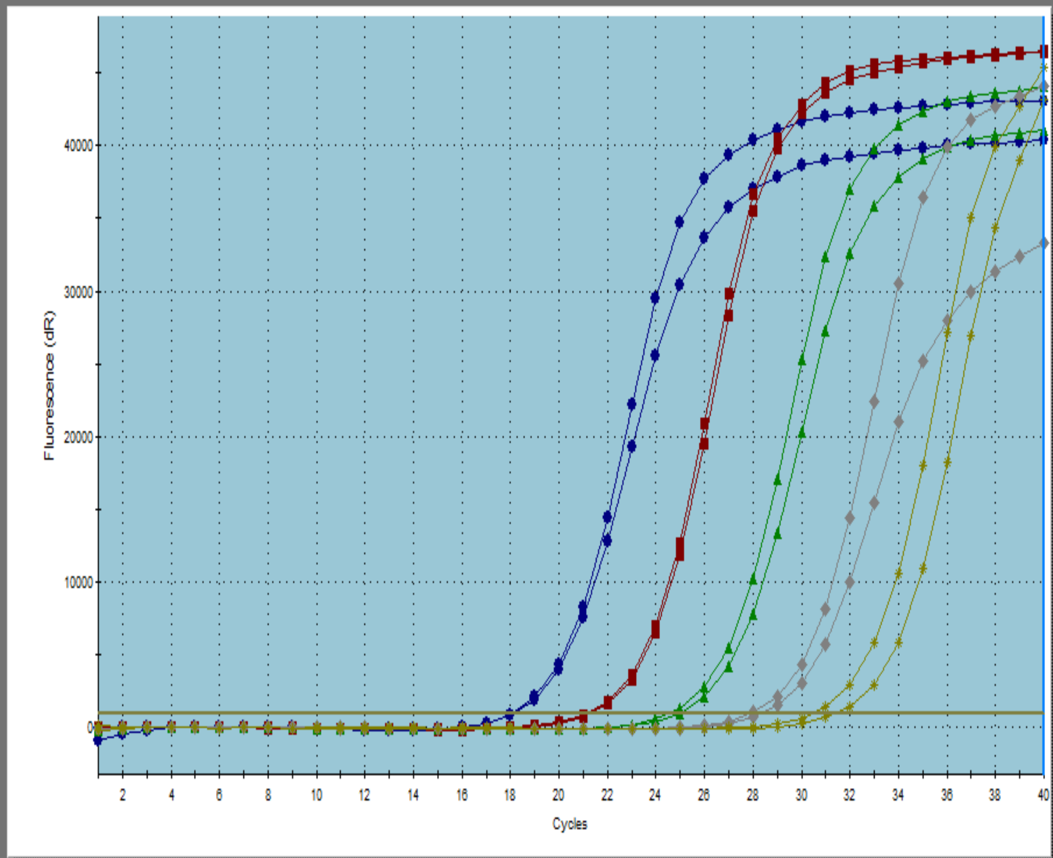


Figure 4.8 Amplification plots of *mecA* standards in a ten-fold dilution series (serial dilutions prepared from DNA extracted from the neat sample of MRSA 16 control strain).

Legend:

Amplification Line colour	Dilution of the sample
(blue line)	Amplification plot for 1:10 dilution
(red line)	Amplification plot for 1:100 dilution
(green line)	Amplification plot for 1:1000 dilution
(grey line)	Amplification plot for 1:10^4 dilution
(yellow line)	Amplification plot for 1:10^5 dilution

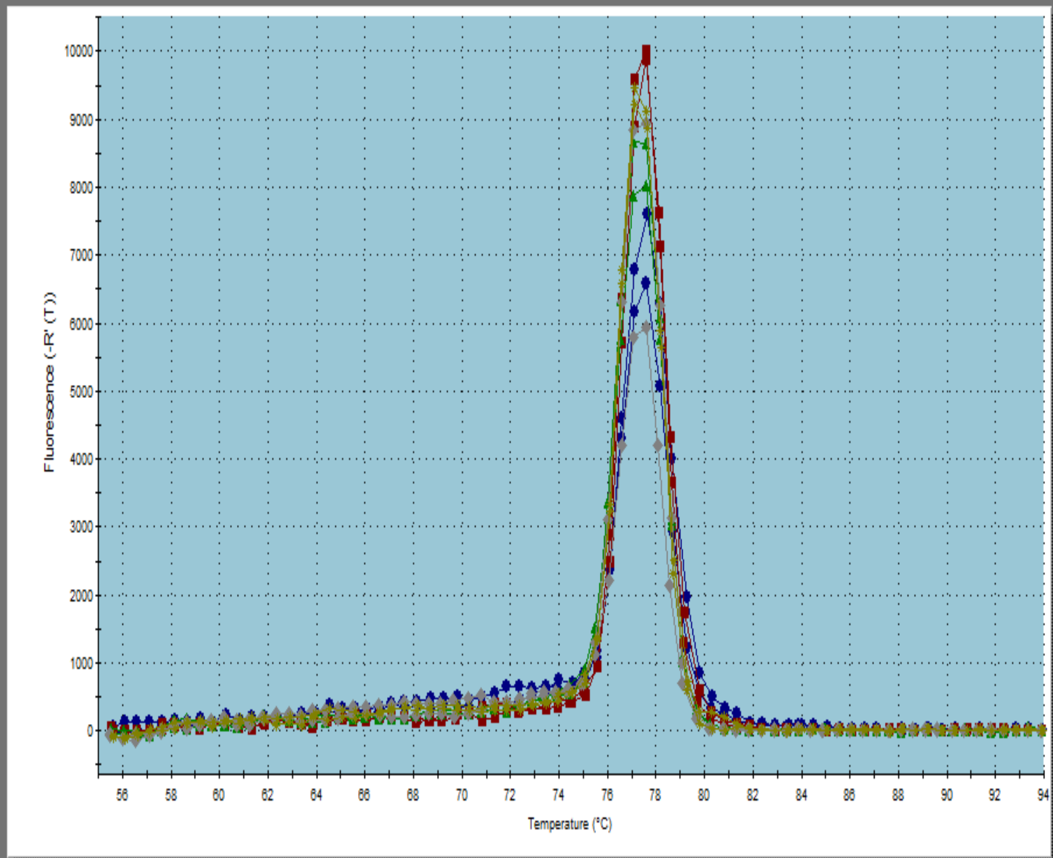


Figure 4.9 Dissociation curve of *mecA* standards in a ten-fold dilution series(serial dilutions prepared from DNA extracted from the neat sample of MRSA 16 control strain.

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Table 4.4 The *mecA* optimisation assay (serial dilutions prepared from DNA extraction from neat sample) for the detection of methicillin-resistant *S. aureus* demonstrating bacterial count of each serial dilution and its corresponding *Ct* value.

Ct	**Dilution cfu/5ul of sample**
18.12	4×10^6
21.25	4×10^5
24.81	4×10^4
28.16	4×10^3
30.89	4×10^2

4.4.2.2 Optimisation of the assay targeting *femB* Gene (serial dilutions prepared from DNA extracted from neat sample)

The mean difference in Ct values between sequential dilutions (n) for *femB* standard curve for DNA dilutions of the neat sample was 2.88 and it was not within the acceptable range of 3.1 to 3.6. The amplification efficiency value (Rsq) was 98.2% and this was also below the acceptable parameters (Figures 4.10, 4.11, 4.12). The reaction efficiency for *femB* gene assay optimisation was 118%. The data suggests that for the best assay performance and results the standard curve should be prepared by extracting DNA separately from each dilution instead of diluting the DNA from a neat sample (Table 4.5).

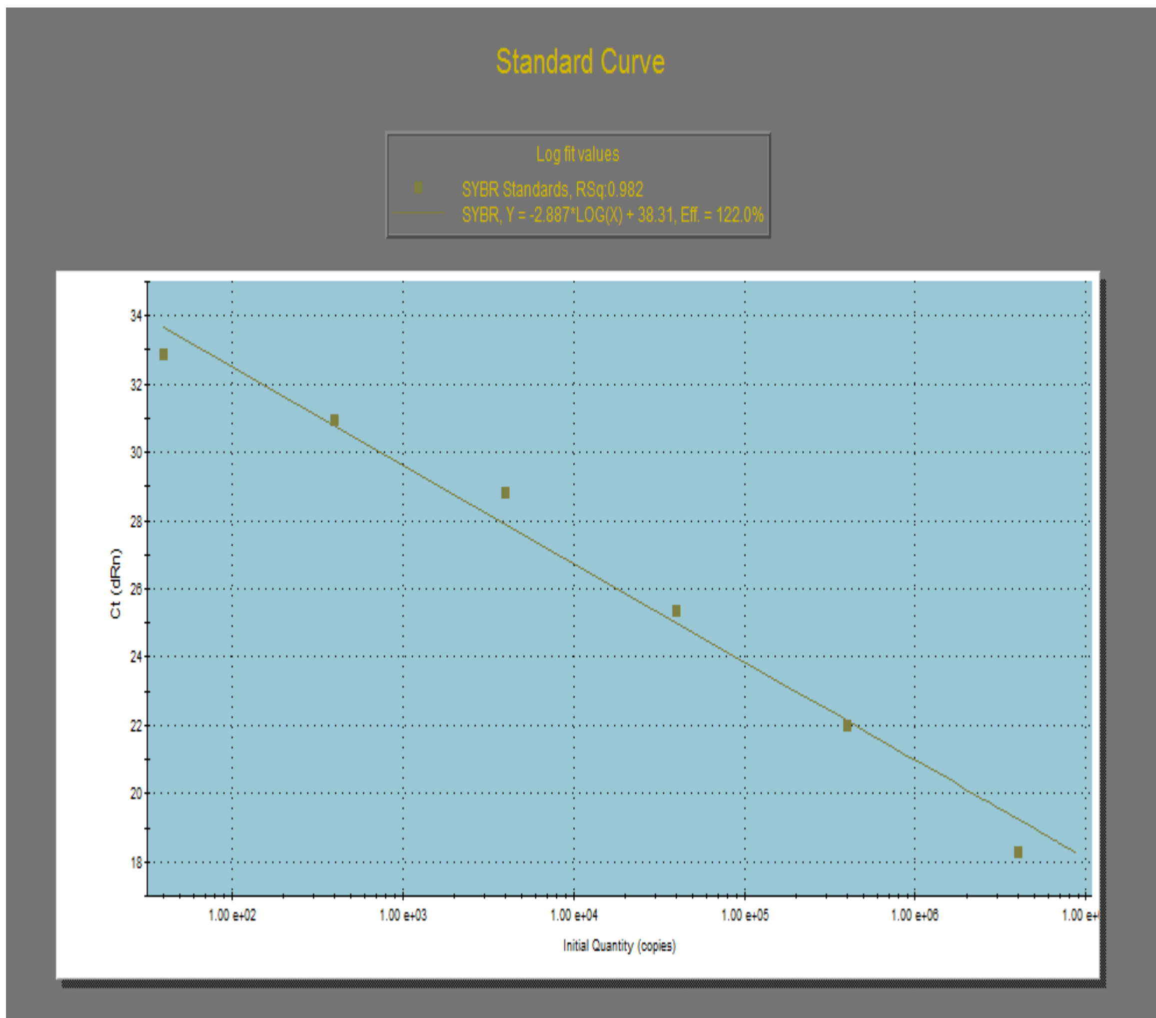

Figure 4.10 QPCR standard curve from the *femB* assay demonstrating a ten-fold dilution series of DNA extracted from MRSA 16 control strain.

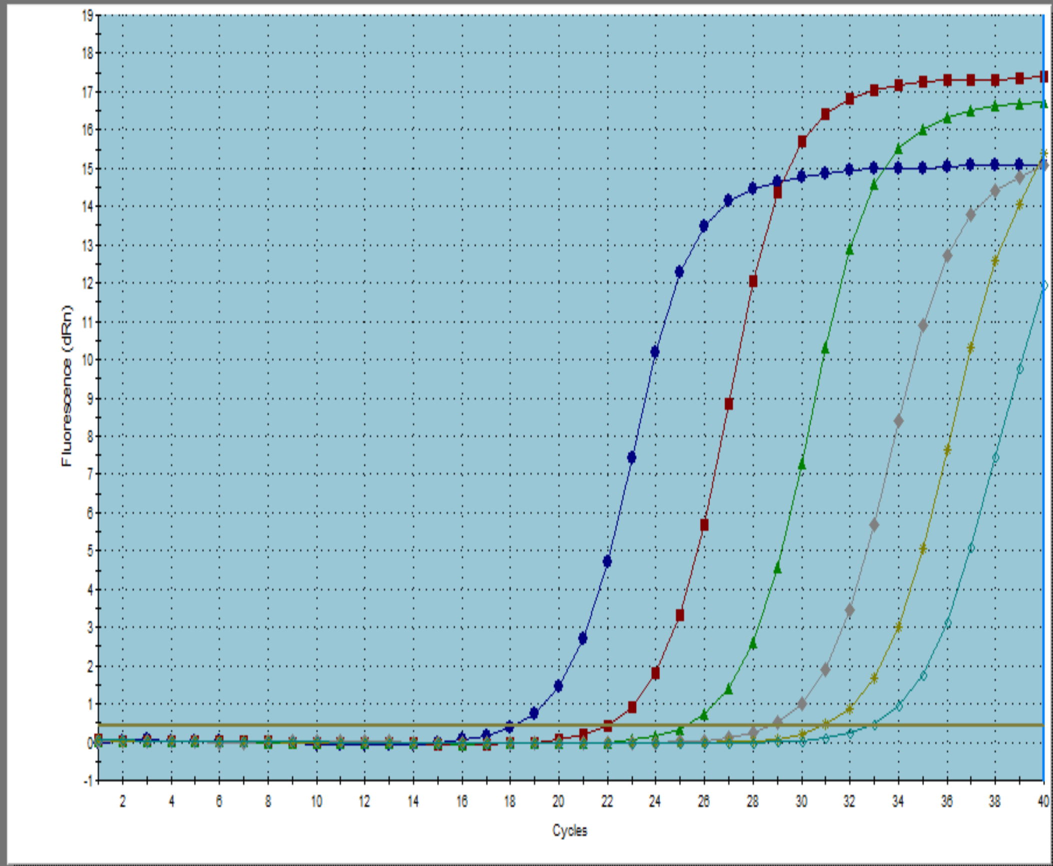

Figure 4.11 Amplification plots of *femB* standards in a ten-fold dilution series (serial dilutions prepared from DNA extracted from the neat sample of MRSA 16 control strain).

Legend:

Amplification Line colour	Dilution of the sample
(blue line)	Amplification plot for 1:10 dilution
(red line)	Amplification plot for 1:100 dilution
(green line)	Amplification plot for 1:1000 dilution
(grey line)	Amplification plot for 1:10^4 dilution
(orange line)	Amplification plot for 1:10^5 dilution
(light blue line)	Amplification plot for 1:10^6 dilution

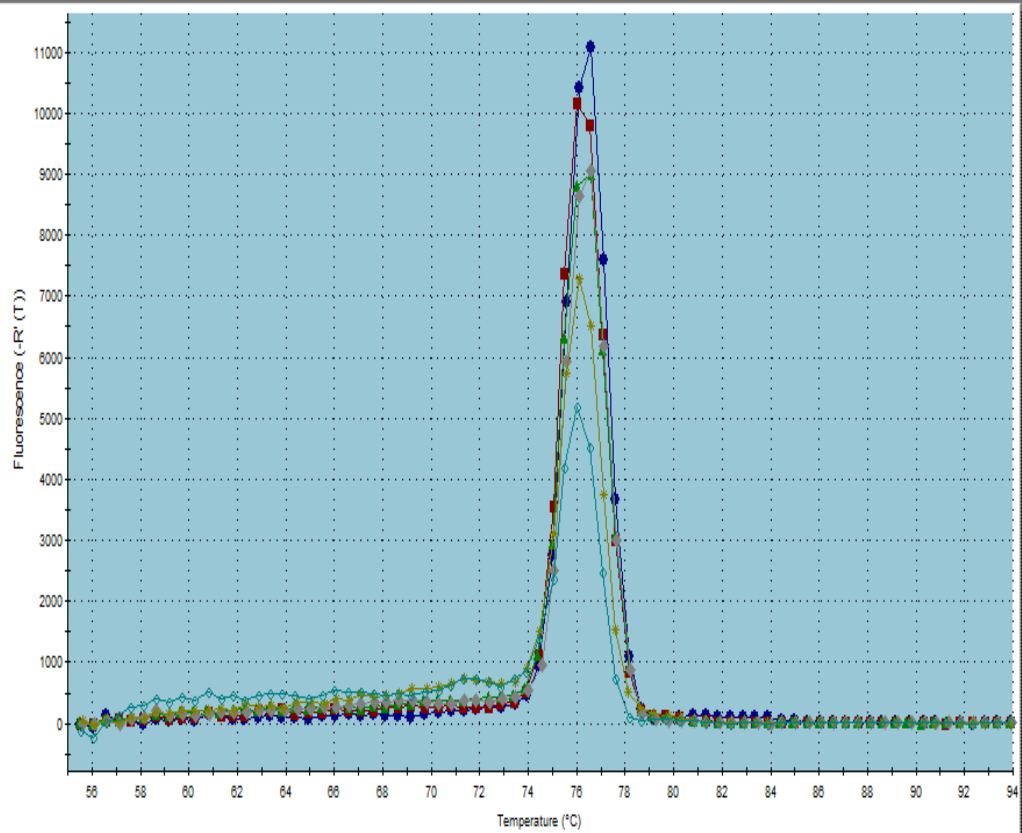

Figure 4.12 Amplification plots of *femB* standards in a ten-fold dilution series (serial dilutions prepared from DNA extracted from the neat sample of MRSA 16 control strain).

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Table 4.5 The *femB* optimisation assay (serial dilutions prepared from DNA extraction from neat sample) for the detection of methicillin-resistant *S. aureus* demonstrating bacterial count of each serial dilution and its corresponding *Ct* value.

Ct	**Dilution cfu/ml of sample**
18.27	4×10^8
21.99	4×10^7
25.35	4×10^6
28.84	4×10^5
30.95	4×10^4
32.89	4×10^3

4.4.3 RT-PCR optimisation with fluorescent probes

After assay optimisation with SYBR Green I, testing the overall performance of the RT-PCR reaction in terms of efficiency, precision and sensitivity is recommended. Data generated from serial dilutions of a positive control template (standard curve) are excellent means of determining the overall performance of the QPCR assay.

As compared to non-specific dyes such as SYBR-Green I dye, a higher level of detection specificity is provided by using an internal probe with primers to detect the QPCR product of interest. In the absence of a specific target sequence in the reaction, the fluorescent probe is not hybridized, the quencher remains and does not fluoresce. When the probe hybridizes to the target sequence of interest, the reporter dye is no longer a quencher, and fluorescence will be detected. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle. A significant advantage of using probe chemistry compared to using DNA binding dyes is that multiple probes can be labelled with different reporter dyes and combined to allow the detection of more than one target in a single reaction (multiplex QPCR).

Probes used in the study (Saeed *et al*., 2010)**:**

***fem*BVICP** TCATCACGTTCAAGGAATCTGACTTTAACACCATAGT

***mec*AP** TGGAAGTTAGATTGGGATCATAGCGTCAT

The *mecA* probe was labelled with a 5' FAM fluorophor and a 3' TAMRA quencher. The *femB* probe was labelled with a 5' HEX fluorophor and a 3' TAMRA quencher. Primers were used at working concentration of 300 nM and probes were used at the working concentration of 200 nM.

Five-fold dilutions of the known amount of template were used. Brilliant Multiplex QPCR Master Mix was used together with primers and probes in one reaction.

DNA was incorporated in each run under the following conditions: 95°C for 3 min, and 40 cycles of 95°C for 20 sec and 60°C for 20 sec. The result of each PCR is indicated by a threshold cycle (*Ct*).

The mean difference in *Ct* values between sequential dilutions for *mecA* standard curve was 3.05 and for *femB* was 2.95 (Figure 4.13). The amplification efficiency value (Rsq) for *mecA* was 99.7% (within the acceptable parameters) and 97.8% for *femB* (slightly below the acceptable parameters). The reaction efficiency for the *mecA* gene assay optimisation was 112.6% and 118% for *femB* (Figure 4.14 and 4.15). These results were the best after many attempts to improve the optimisation with probes for this assay.

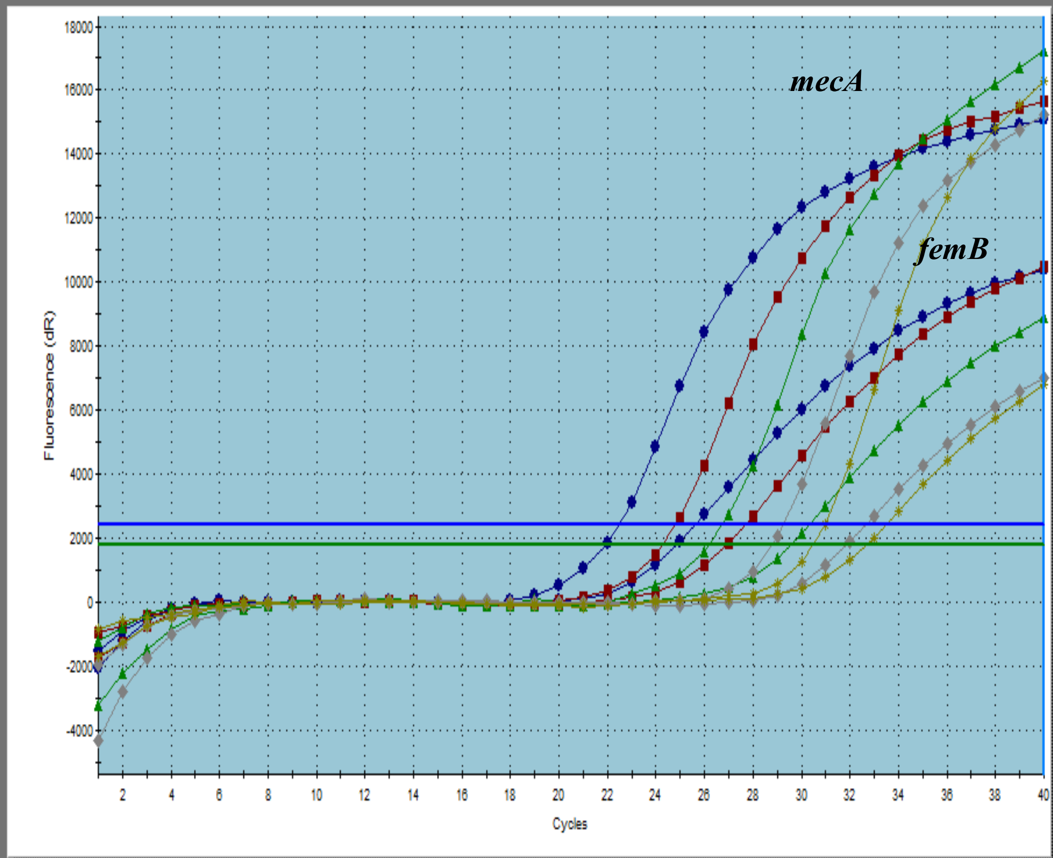

Figure 4.13 Amplification plots of the serial dilutions of the MRSA 16 control strain for the detection of *mecA* and *femB* in duplex assay.

Legend:

Amplification Line colour	Dilution of the sample
	Amplification plot for 1:10 dilution
	Amplification plot for 1:100 dilution
	Amplification plot for 1:1000 dilution
	Amplification plot for 1:10^4 dilution
	Amplification plot for 1:10^5 dilution

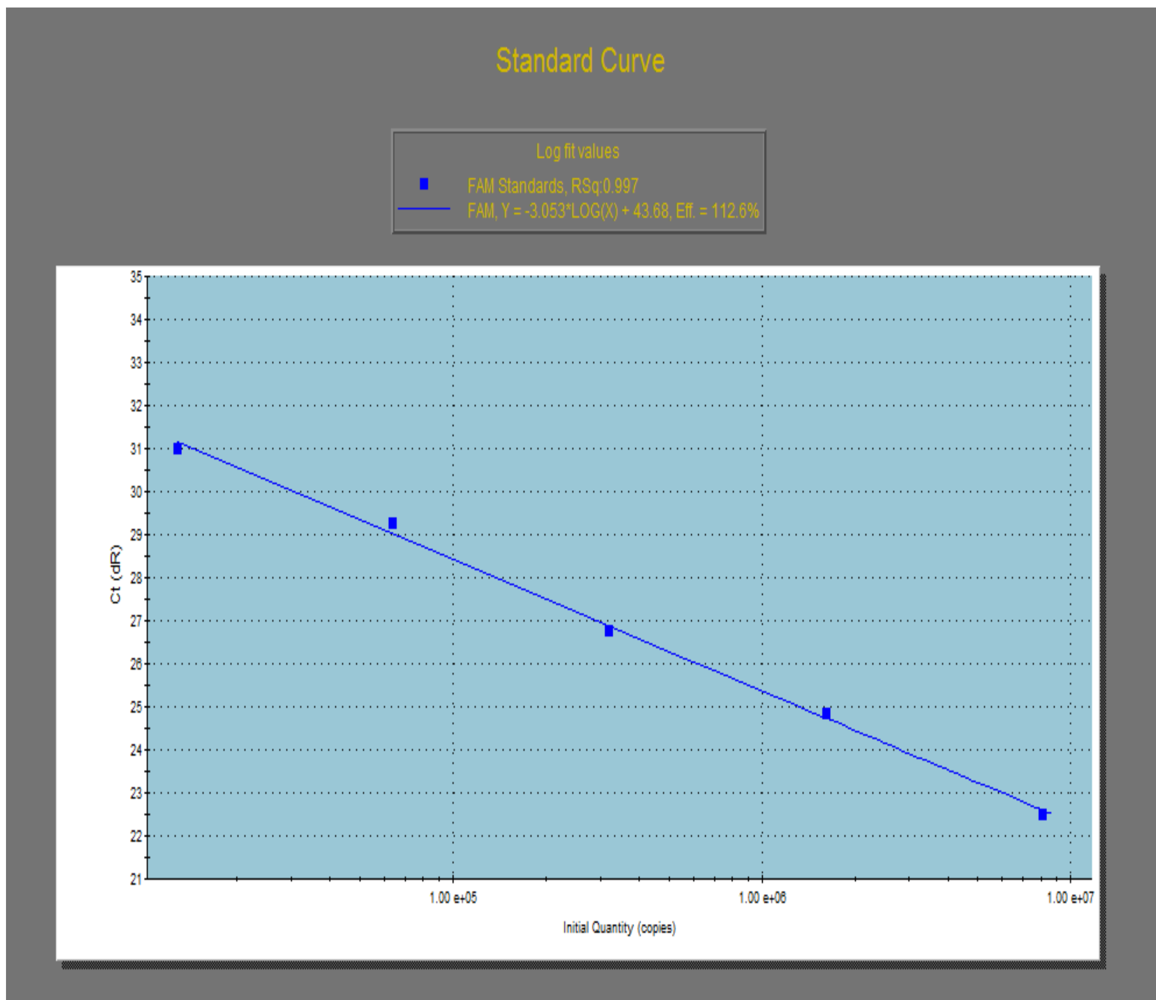

Figure 4.14 QPCR standard curve from FAM (*mecA* gene) in duplex assay. Rsq= 0.997, Efficiency= 112.6%.

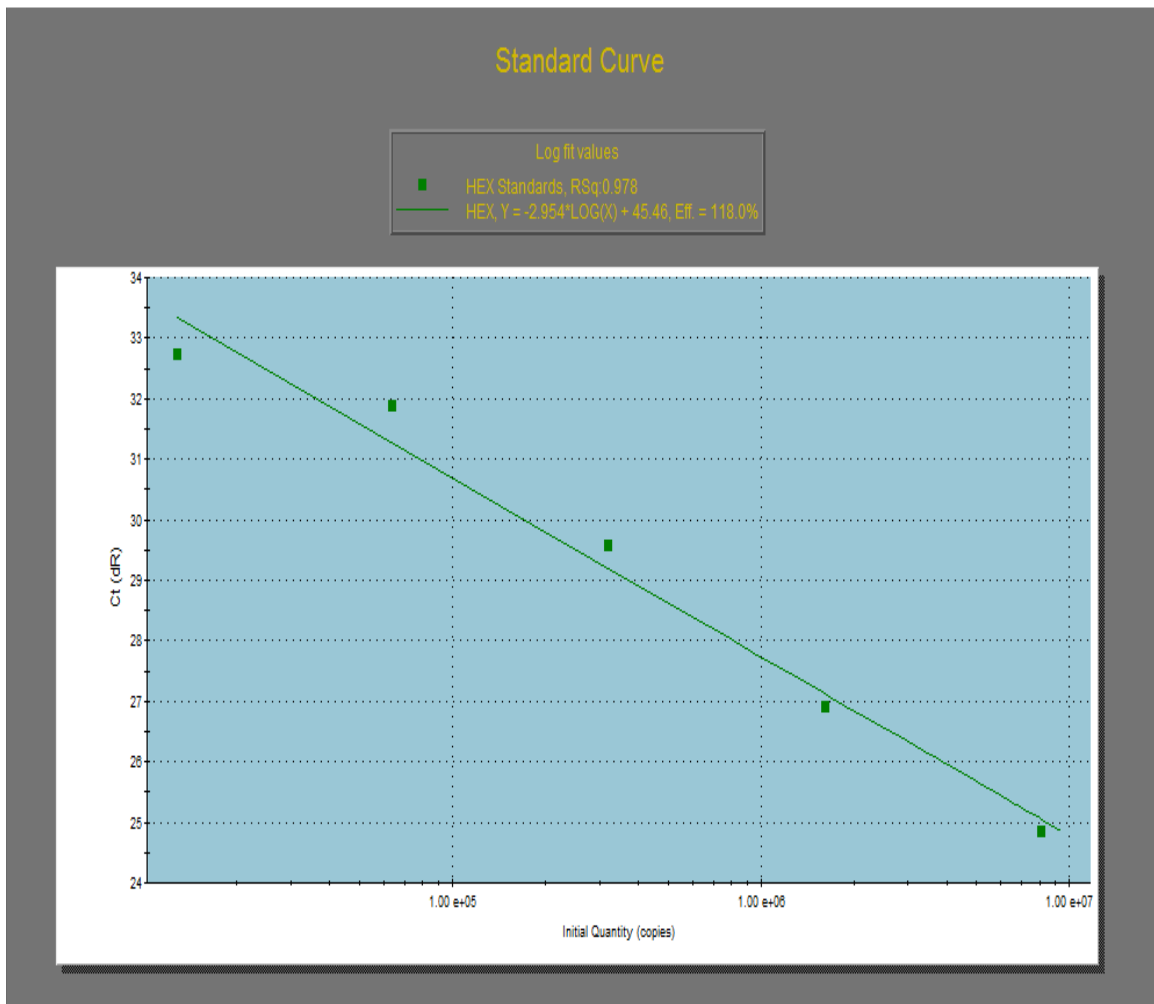

Figure 4.15 QPCR standard curve from HEX (*femB* gene) in duplex assay. Rsq= 0.978, Efficiency= 118%.

4.5 Results

4.5.1 Comparison of RT-PCR assay with conventional methods for MRSA detection and quantification

Thirty two patients with chronic wounds were included in the study. Two swabs were collected from each patient (Sigma dry swabs, Medical Wire & Equipment): one for isolation and quantification of *Staphylococcus* spp. including MRSA using conventional microbiological methods and the second one for detection and quantification of MRSA using RT-PCR assay. The performance of both methods for isolation and quantification of MRSA was assessed and compared.

4.5.2 MRSA isolation and identification using conventional culture methods

Ninety six percent of wounds were colonised with bacteria. MRSA was isolated from six wounds (18.75%) out of thirty two using conventional culture methods. Isolates were confirmed as MRSA using Gram stain, catalase, coagulase and DNAse activity (Section 3.8.1). Resistance to oxacillin was confirmed using the disc diffusion method. Three wounds (9.37%) were colonised with MSSA. Nineteen wounds (59%) were colonised with different strains of coagulase negative staphylococci (Table 4.6). *Staphylococcus* species were not isolated in six out of thirty two wounds. The identification of CNS was performed using the API Staph test.

Table 4.6 The results of *Staphylococcus* species identification using conventional culture methods in samples collected from thirty two patients with chronic wounds (NG – No Growth).

Patient number	MRSA	MSSA	CNS
1	**present**	NG	NG
2	**present**	NG	NG
3	**present**	NG	NG
4	NG	**present**	*S. epidermidis*
5	NG	**present**	NG
6	**present**	NG	NG
7	NG	NG	NG
8	NG	NG	*S. epidermidis*
9	NG	NG	*S. epidermidis*
10	NG	NG	*S. epidermidis*
11	NG	NG	*S. simulans*
12	NG	NG	NG
13	NG	NG	*S. simulans*
14	NG	NG	*S. haemolyticus*
15	NG	NG	NG
16	NG	NG	*S. warneri*
17	NG	NG	*S. xylosus. S. cohnii*
18	NG	NG	*S. haemolyticus*
19	NG	NG	NG
20	NG	NG	*S. haemolyticus*
21	NG	NG	*S. haemolyticus, S.capitis*
22	NG	**present**	*S. warneri, S. chromogenes*
23	NG	NG	*S. capitis, S. epidermidis*
24	NG	NG	NG
25	NG	NG	*S. haemolyticus*
26	NG	NG	*S. cohnii*
27	NG	NG	*S. simulans*
28	NG	NG	*S. warneri*
29	**present**	NG	NG
30	NG	NG	*S. capitis*
31	NG	NG	NG
32	**present**	NG	NG

4.5.3 MRSA identification using RT-PCR assay.

The previously optimised RT-PCR assay to identify the presence of two genes (*mecA* and *femB* genes) was tested for its performance on patient samples.

Interpretation of RT-PCR results was as follows:

Positive *femB* and *mecA* – MRSA present

Positive *femB* and negative *mecA* – MSSA present

Negative *femB* and positive *mecA* – MRCNS present

Negative *femB* and *mecA* - staphylococcal DNA not detected.

Both genes *mecA* and *femB* were detected in eight samples out of thirty two tested, which can confirm the presence of MRSA, but also the presence of MSSA and coagulase negative staphylococci in the same specimen (Figure 4.17). Out of these eight samples, five samples were confirmed with MRSA presence using conventional culture methods. The two remaining samples contained both MSSA and CNS which lead to false positive results. One sample was detected with *mecA* and *femB* genes, but conventional microbiology confirmed only the presence of CNS (Table 4.7).

One sample out of thirty two was identified as a MSSA (only the *femB* gene was detected). The presence of *mecA* with the absence of *femB* (Figure 4.18) was detected in fifteen samples out of thirty two which suggests the presence of coagulase negative staphylococci. Eight samples showed the absence of both *mecA* and *femB* genes in comparison to six samples free from staphylococci confirmed by conventional methods. Two samples (11, 32) contained very low number of microorganisms below the detection limit $\leq 10^2$ cfu/ml.

Table 4.7 The results of MRSA detection using RT-PCR and culture methods. The presence of *mecA* and *femB* genes was confirmed in eight samples using RT-PCR, however, five samples out of eight were confirmed with MRSA using culture methods. False positive results in two samples were due to the presence of MSSA and Coagulase Negative Staphylococci (CNS) in the same specimen.

Patient number	*mec*A and *fem*B presence confirmed by **RT-PCR assay**	MRSA presence confirmed by **culture**	CNS presence confirmed by **culture**
1	(*mec*A / *fem*B) MRSA	MRSA	-
2	(*mec*A / *fem*B) MRSA	MRSA	-
3	(*mec*A / *fem*B) MRSA	MRSA	-
4	(*mec*A / *fem*B)	MSSA	CNS
5	(*fem*B)	MSSA	-
6	(*mec*A / *fem*B) MRSA	MRSA	-
7	-	-	-
8	*mec*A	-	CNS
9	*mec*A	-	CNS
10	*mec*A	-	CNS
11	-	-	CNS
12	-	-	-
13	*mec*A	-	CNS
14	*mec*A	-	CNS
15	-	-	-
16	*mecA*	-	CNS
17	*mec*A	-	CNS
18	*mec*A	-	CNS
19	-	-	-
20	*mec*A	-	CNS
21	*(mecA / femB)*	-	CNS
22	(*mec*A / *fem*B)	MSSA	CNS
23	*mec*A	-	CNS
24	-	-	-
25	*mec*A	-	CNS
26	*mec*A	-	CNS
27	*mec*A	-	CNS
28	*mec*A	-	CNS
29	(*mec*A / *fem*B) MRSA	MRSA	-
30	*mec*A	-	CNS
31	-	-	-
32	-	MRSA	-

The number of patients correctly identified with MRSA (true positives) using RT-PCR assay was five. The number of patients correctly identified as negative for MRSA (true negatives) was 23. Three patients were incorrectly identified as MRSA carriers due to the presence of MSSA and CNS in the same sample. In one patient MRSA was not detected using the RT-PCR assay (Table 4.8). The diagnostic values of the RT-PCR assay for detection of *mecA* and *femB* genes were as follows: sensitivity 83.3%; specificity, 88.5%; positive predictive value, 62.5%; and negative predictive value, 95.8%. The performance of the RT-PCR assay was compared with the results obtained using conventional culture methods.

Table 4.8 The RT-PCR assay performance demonstrating the number of true positive and true negative results and false positive and negative results.

True Positive	5
True Negative	23
False Positive	3
False Negative	1

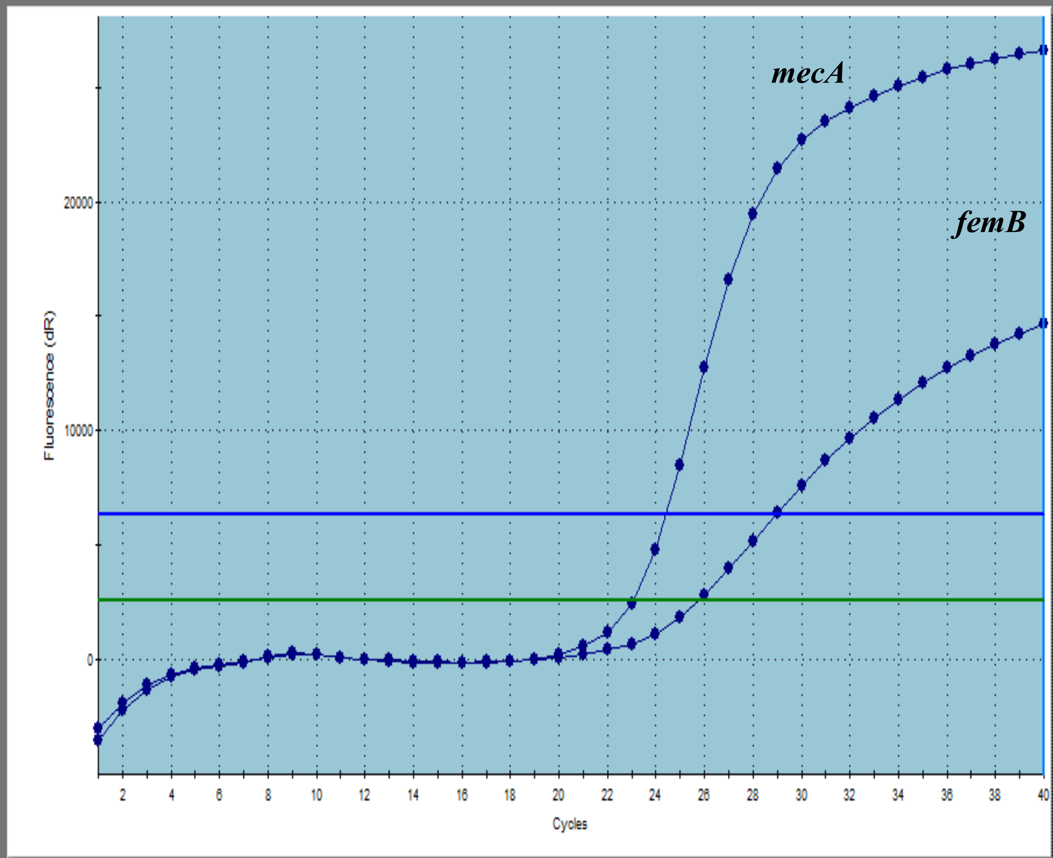

Figure 4.17 Amplification plots showing the expression of *mecA* and *femB* gene – MRSA or both MSSA and CNS present.

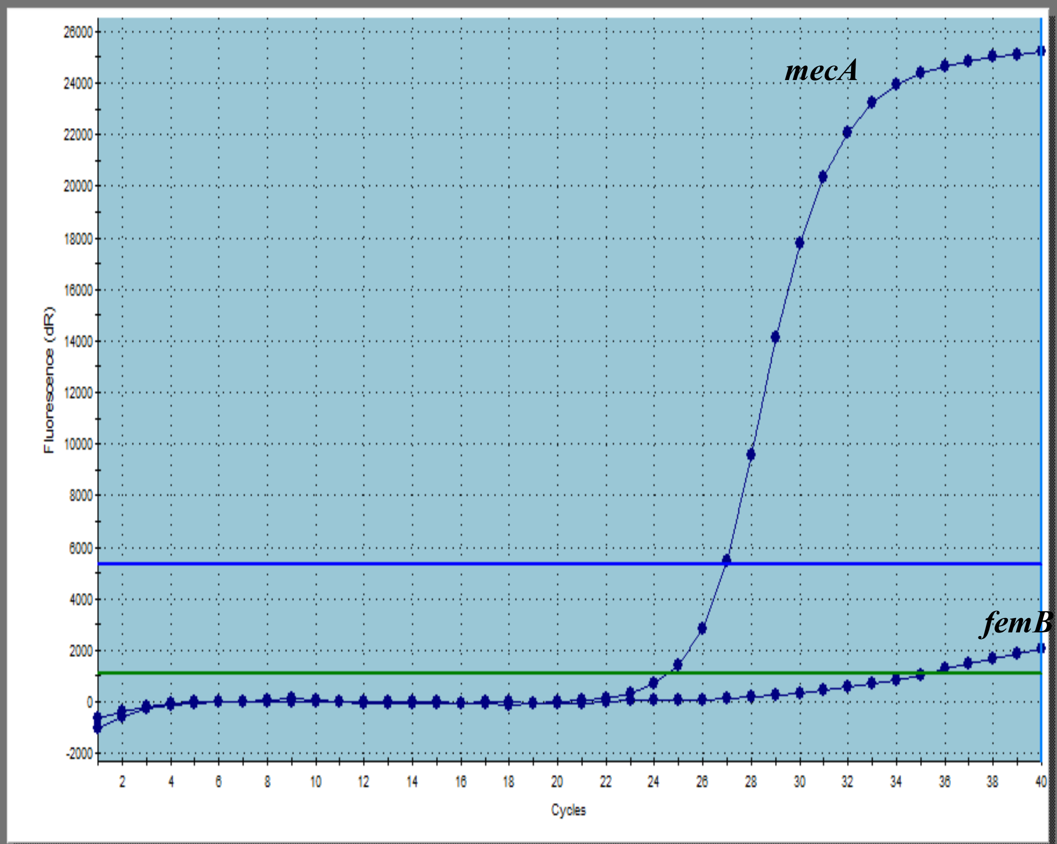

Figure 4.18 Amplification plots showing the expression of *mecA* only – CNS present.

4.5.4 MRSA quantification using RT-PCR assay

Six samples out of thirty two were identified with MRSA using conventional culture methods, however only five samples out of these six were positive for MRSA using the RT-PCR assay. The MRSA phage type 16 control strain was used for determination of MRSA count and as a positive control in RT-PCR set up. This was used to generate a standard curve of MRSA count / CT value. The number of cells in the patient samples was determined according to the standard curve. Means and standard deviations (SDs) of Ct values were calculated using the statistical software package Minitab16. It was observed that the average difference between MRSA count obtained using real-time PCR assay and conventional culture results was 0.61 log (Table 4.9). Unfortunately, the number of MRSA positive samples was too small to perform Pearson's coefficient and Chi-square test to examine whether the results of 2 methods (culture and real-time PCR) were independent or not.

Table 4.9 Comparison of the number of cells detected by RT-PCR with number of cells detected by conventional microbiology methods from clinical sample

R* The difference between the number of cells detected by RT-PCR assay and the number of cells detected by conventional methods.

Patient	**Ct - value**	**Number of cells detected by RT-PCR cfu/ml**	**Number of cells detected by conventional methods cfu/ml**	**R***
1	25.15 ± 0.92	1.81×10^6	1.06×10^5	1.70 log
2	24.36 ± 2.10	3.31×10^6	5.77×10^6	0.24 log
3	20.50 ± 1.94	3.65×10^7	7.44×10^6	0.38 log
4	26.66 ± 0.33	7.78×10^5	2.12×10^5	0.57 log
5	25.14 ± 1.16	1.79×10^6	3.50×10^6	0.17 log
6	-	No detection	1.30×10^2	-

4.6 Discussion

Early detection of MRSA helps to implement preventive infection control strategies and reduce costs (Cox *et al*., 1995). Conventional culture of screening

samples requires two to three days before definitive MRSA identification can be achieved. RT-PCR has the potential to provide faster results than conventional methods for the identification of MRSA.

In this study a RT-PCR for rapid and direct detection and quantification of MRSA from wound swabs was developed and compared with conventional tests. It was a preliminary development stage of a multiplex RT-PCR assay for the rapid and direct detection of common microorganisms found in wounds. In Chapter 5, the diversity of microflora in chronic wounds was investigated using PCR-DGGE sequencing. Based on the results of PCR-DGGE sequencing, multiplex RT-PCR for chronic wounds could be developed and compared with culture methods as part of the future work.

The specificity of the *mec*A and *fem*B primers used for identification of *S. aureus* has been demonstrated previously (Jonas *et al*., 2002; Thong et al., 2011). The major problem of this method is the high probability of co-amplification of *fem*B and *mec*A from mixed cultures consisting of MR-CNS and MSSA as it would mimic a false-positive MRSA result. Most up-to-date studies have been performed on pure cell cultures or using MRSA selective enrichment overnight broth, which leads to extending the time of detection, but improves specificity of the assay. In this study, six samples out of thirty two were shown to contain MRSA using conventional methods. In the RT-PCR assay eight samples were identified with both *mec*A and *fem*B genes, but two out of these eight contained both MSSA and coagulase negative staphylococci confirmed by culture. This means that for direct detection of MRSA from clinical samples the presence of both MSSA and CNS could give false positive results. One sample was identified with coagulase negative staphylococci only, which means that the conventional method failed to identify MRSA or MSSA in the sample. It is especially important in samples collected from chronic wounds as they usually contain mixed microflora. One sample identified with MRSA using conventional culture was not

detected with RT-PCR due to low numbers of cells. The limit of detection for this duplex assay was $\geq 10^3$ cfu/ml and the swab contained 1.3×10^2 cfu/ml of MRSA. One sample out of 32 was identified with MSSA using the RT-PCR assay, whereas conventional methods confirmed MSSA presence in three of the samples. The presence of CNS resistant to methicillin was confirmed using culture methods in 19 of the chronic wounds. Fifteen of the wound swabs were identified with only the *mec*A gene which confirmed the presence of MR-CNS. Eight samples showed the absence of both *mec*A and *fem*B genes in comparison to six samples free from staphylococci confirmed by conventional methods. Two samples contained low number of CNS species ($\leq 10^2$ cfu/ml) and this is possibly the reason for the unsuccessful detection of CNS using RT-PCR.

The diagnostic values of the RT-PCR assay for the detection of *mec*A and *fem*B genes were as follows: sensitivity, 83.3% (5 of 6); specificity, 88.5% (23 of 26); positive predictive value, 62.5% (5 of 8); and negative predictive value, 95.8% (23 of 24). The performance of the RT-PCR assay was compared with the results obtained using conventional culture methods. Higher specificity and sensitivity could be achieved by incubating samples overnight in a selective enrichment broth containing oxacillin. Jonas *et al.,* (2002) tested 439 swabs for the detection of *mec*A and *fem*B gene with prior enrichment in selective broth and achieved 100% sensitivity and 100% specificity for this assay.

Saeed *et al*., (2010) examined 19 samples, which were negative on culture using conventional methods by using duplex RT-PCR assay for the presence of *mec*A and *fem*B. The samples were collected from patients with bone and joint infections or prosthetic joint infections. Ten samples (57.8%) gave positive results RT-PCR- four for MSSA, two for MRSA and four for MR-CNS. In seven patients identification of a causative organism was of great reassurance for the surgical team. These individuals

were on effective and adequate antibiotic therapy. In three patients the results of the RT-PCR led to a complete change of antibiotics as two of them were identified with MSSA (change of therapy from vancomycin to flucloxacillin) and one with MRSA (change of therapy from flucloxacillin to vancomycin). In the remaining nine samples (42.2%) the RT-PCR results were negative and in concordance with conventional cultures. This may be attributed to true negatives, prolonged antibiotic therapy before sampling or infection caused by microorganism other than *S. aureus*. Specific duplex staphylococcal PCR has been previously reported to be a rapid method of detection of *S.aureus*, including MRSA, with a sensitivity and specificity of 100% from screening swabs (Jonas *et al*., 2002). Saeed *et al*., (2010) reported that duplex RT-PCR assay had similar sensitivity and specificity from direct colonies of *Staphylococcus* spp. They found that by using a specific duplex staphylococcal RT-PCR of *mec*A and *fem*B genes, it was possible to identify *Staphylococcus* spp. including MRSA, directly from orthopaedic samples. Unfortunately, they did not report the detection limit for this assay. The findings of the present study show that the presence of MSSA and MR-CNS in the sample can lead to false positive results especially if the bacteria exist in high numbers. In the study by Saeed *et al*., (2010) samples were collected from patients with presumed bone, joint, or prosthetic joint infection. These infections are usually caused by a single pathogenic strain and this may be the reason for such a good sensitivity of the method. In the study presented here the specimens were collected from chronic wounds which are polymicrobial in nature and usually contain mixtures of different staphylococcal species.

Rajan *et al*., (2007) compared culture methods with PCR for MRSA detection from 170 swabs collected from 63 patients between September and December 2005 attending the intensive care unit of Beaumont Hospital, Dublin, Ireland. Three patients out of five were initially identified with MRSA. The remaining two patients were

positive for MRSA on testing of repeat specimens. The quickest time to detection using conventional culture was with CHROMagar MRSA (48 h), which detected 80% of MRSA. Real-Time PCR was rapid (2.25 h) and facilitated the optimisation of antibiotic therapy in two of three positive PCR patients, but PCR was less specific and more expensive than CHROMagar MRSA. They concluded that PCR facilitates the rapid detection of MRSA and has a potential to contribute to preventing spread, but should continue to be used in conjunction with culture.

Stratidis *et al*., (2007) evaluated the real-time PCR for detection MRSA directly from positive blood culture bottles. One hundred and forty-two blood cultures of Gram-positive *cocci* in clusters were detected using the Gram staining method. Each blood sample was tested for the presence of MRSA by PCR analysis (SmartCycler) based on detection of the *mec*A and *orf*X genes. In parallel, they were subcultured onto standard media for identification and characterisation. Of the 57 MRSA blood culture bottles tested using the SmartCycler system, all isolates were correctly identified when compared with both culture and susceptibility testing using conventional methods. The sensitivity and negative predictive value of the SmartCycler methodology both were 100% with a negative likelihood ratio approaching zero. Seventy-nine (95%) of the 85 MRSA-negative blood culture samples tested negative for MRSA because of the absence of detectable *mec*A and *orf*X genes, yielding a specificity of 95% and a positive predictive value of 93%. The false-positive rate was < 5% with 4 of 83 MRSA-negative samples being identified as MRSA. They concluded that RT-PCR assay for the detection of MRSA in blood samples was found to be both sensitive and specific. The rapid detection time of 2 h makes this an important addition to patient care and infection control practice with potential reductions in health-care-related costs. Blood stream infections are usually caused by one pathogen and the sensitivity and specificity of the

assay is much higher for MRSA detection from blood samples in comparison to mixed culture samples.

Huletsky *et al.,* (2004) developed a Real-Time PCR assay for the rapid detection of MRSA directly from clinical specimens containing a mixture of staphylococci. The staphylococcal clinical isolates were part of the SENTRY program collection and several supplier's collections and originated from many countries around the world. The set of primers specific to the various types of SCCmec right extremity and a primer specific to the *S. aureus orf*X sequence were used. These primers were used in a multiplex assay along with a molecular beacon probe specific to the *orf*X sequence. The assay allowed the detection of 2 to 10 genome copies of MRSA from 15 clinical isolates obtained from different areas in North America, Europe and Asia. With the PCR assay, two of 205 (1%) MRSA strains tested were not detected while 13 of 252 (5.2%) MSSA strains were misidentified as MRSA. The higher number of primers used by Huletsky *et al*., (2004) increases probability to hybridize to similar structures like SCC*mec*-like elements devoid of *mec*A present in MSSA (Luong *et al*., 2002). Overall there was a 96.7% correlation between the PCR results and the identification of MRSA. None of the 10 methicillin-sensitive or 14 methicillin-resistant CNS species tested were detected using the real-time PCR assay. Using five different forward primers in combination with three different probes and one reverse primer they were able to show that the majority of a world-wide collections of MRSA isolates was detected by their PCR assay.

Our quantitative analysis revealed that the average difference between MRSA count obtained using real-time PCR assay and conventional culture results was 0.61 log. This can be due to the fact that the RT-PCR assay counts both viable and non-viable cells whereas conventional method quantify only viable microorganisms. Unfortunately, due to the small amount of patients identified with MRSA, we could not a perform statistical analysis. There are not many studies looking at a quantitative comparison of

RT-PCR results with results obtained using conventional methods. Most of the current studies concentrate on rapid detection of MRSA especially when the infection is present and time is important for treatment and survival of the patient.

The optimisation of RT-PCR was performed using the MRSA phage type 16 control strain with the DNA extracted directly from each serial dilution and with the dilutions of DNA prepared from the original sample. The higher sensitivity and specificity of the assay for both *mec*A and *fem*B genes were achieved with the DNA extracted directly from each serial dilution of MRSA control strain rather than serial dilutions of the DNA prepared from the original sample. This is the first report comparing the specificity and sensitivity of the RT-PCR assay optimisation using two different methods. It is an important finding as most of the laboratories dilute DNA from the original sample to prepare standard curves instead of preparing the serial dilutions of MRSA and extracting DNA directly from each dilution.

Modern medicine challenges us to develop new and rapid techniques to improve the care of patients. RT-PCR can be very useful diagnostic tool in the identification of different *staphylococci* species especially when conventional culture fails to grow. These techniques could have the potential to support clinicians in quickly choosing the most appropriate antibiotics which should lead to reduction in morbidity and total cost of care. However, these techniques should be used in conjunction with culture methods.

4.7 Conclusion

RT-PCR assay for the detection of *mec*A and *fem*B genes directly from samples collected from chronic wounds has a potential for the rapid identification of MRSA. However, the samples should be incubated in enrichment broth prior to analysis or supported by culture analysis due to the high probability of co-amplification of *fem*B and *mecA* from mixed cultures consisting of MR-CNS and MSSA as this would mimic a

false-positive MRSA result. The DNA for standard curve preparation should be extracted directly from each serial dilution of the control strain instead of extracting the DNA from the original sample and preparing serial dilutions of the DNA.

CHAPTER 5

A Molecular and Culture Based Assessment of the Microbial Diversity of Chronic Wounds and Healthy feet

5.1 Introduction

There are many factors affecting the diversity of microbial flora of human skin such as age (Noble *et al*., 1974; Sultana *et al*., 2003; Percival *et al*., 2012), sex (Wilburg *et al*., 1984; Staudinger *et al*., 2011), skin site, climate, race, occlusion, level of hygiene and type of cleansers used, occupation and whether the patient is hospitalised (Larson *et al*., 2000).

In order to understand the microbiology of chronic wounds it is important to have an understanding of the microflora of normal skin. Firstly, a newborn's skin is covered by a white waxy coating called *Vernix caseosa,* which is primarily composed of sebum, cells that have sloughed off the foetus's skin and shed lanugo hair. *Vernix caseosa* provides the skin with a neutral pH (Hoath *et al*., 2006). A short time after birth the vernix spreads resulting in a lowering of the skin's pH with ranges from 3 to 5.9 have been reported (Aly *et al*., 1978). Vernix has protective properties before and after birth (Rissmann *et al*., 2009) such as multiple AMPs (adenosine monophosphates) which together with barrier properties and the ability to suppress bacterial adhesion creates an effective defence mechanism against infection (Akinbi *et al*., 2004; Marchini *et al*., 2002; Yoshio *et al*., 2003). The most frequently isolated bacteria from neonates are CNS (Keyworth *et al*., 1990; Keyworth *et al.,* 1992) and studies have found that development of skin's microflora of babies born by surgical and natural births is the same as bacteria found on the skin within 6h postnatal (Keyworth *et al*., 1992). In a study by Keyworth *et al*., (1992) CNS were found in 92% of cases, with a rapid increase in bacterial counts over the first 7 days. Of the CNS 82% were identified as a *S. epidermidis* and were isolated from all sites. Other less commonly isolated microorganisms were *Propionibacterium* spp, α-haemolytic streptococci, aerobic spore bearing bacilli, aerobic coryneforms, *C. albicans*, *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *E. coli*.

Further studies showed that as children get older, microorganisms such as *Propionibacterium* and yeasts are found in abundance especially during and after puberty (Ashbee *et al*., 2002; Juncosa *et al*., 2002).

The numbers of microorganisms on adult skin have been estimated between 6 x 10^2 and 2 x 10^6 cfu / cm^2 depending upon the body site. Skin can be divided into three distinct regions, each differing in their microbiology (Aly *et al*., 1977; Bojar and Holland, 2002). The regions include moist areas (groin, toe web areas and the armpits); oily areas (forehead, nose) and dry areas (Leyden *et al*., 1987). Moist areas provide suitable conditions for bacteria to grow and multiply. They are heavily colonised by Coryneforms and bacteria belonging to the Micrococcaceae. On the perineum there is a high number of Gram positive and Gram negative rods of faecal origin found (Bieber and Kahlmeter, 2010). At oily areas of the skin relatively low levels of Micrococcacaeae and Coryneforms are found compared with high levels of *Propionibacterium* species (Evans and Crook, 1984). *S. hominis*, *S. epidermidis*, *Malassezia* spp are also found in these areas (Webster, 2007). In dry areas there is a mixture of species and the most commonly identified microorganisms are *Staphylococcus* especially CNS with 50% identified as *S. epidermidis*, *Micrococcus* sp, *Corynebacterium sp*, *Propionibacterium sp*, *Brevibacterium sp*, *Acinetobacter sp*, *Dermabacter sp* (Harmory and Parisi, 1987; Vuong and Otto, 2002). *S. epidermidis* plays a role in maintaining the balance of skin microbiota, however it is also a source of antibiotic resistance genes and the cause of nosocomial infections (Otto, 2009). Other CNS isolated from adult skin are *S. saprophiticus*, *S. hominis*, *S. warneri*, *S. haemolyticus* and *S. capitis.*

High numbers of bacteria and yeast are recovered from elderly individuals mainly because of decreased sweat production and the development of dry skin (Somerville, 1980). *S. aureus* colonisation increases with age and is highest in those

over 70 years. Many hospitals report high MRSA colonisation rates among elderly patients (Hoefnagels-Schuermans *et al*., 1997; O'Sullivan and Keane, 2000).

Damage to the skin, such as cuts, surgical or traumatic wounds or leg ulcers enable access of microorganisms to normally sterile tissue and provide a much different environment for microbial growth than intact skin (Dryden, 2009; James *et al*., 2008). In acute wounds, the healing process, including the immune response, is capable of controlling invasion by microbes and repairing the wound in a relatively short time frame (days to weeks). However, in some cases wounds become chronic and fail to heal within a reasonable time frame (months to years) and often remain in an inflammatory state (James *et al*., 2008).

The bacteriology of acute and chronic wounds is different, with the latter tending to harbour more diverse microbial communities that include anaerobic species. The bacterial flora in wounds appears to change over time. In the early acute wound natural skin microflora predominate (Gram positive cocci are the most common). Facultative aerobic Gram negative rods and anaerobic bacteria enter the wound after about four weeks post injury (Kaftandzieva *et al*., 2012). Acute wounds are less diverse in microflora than chronic wounds and infections of acute wounds are mostly caused by single pathogens. Chronic wounds are polymicrobial in nature and recent studies using molecular techniques have emphasised the complex ecology of these wounds (Davies, 2003; Davies *et al*., 2004).

The most predominant aerobic species isolated from clinical wound samples included *S. aureus*, CNS, *P. aeruginosa*, *E. coli*, *Enterobacter cloacae*, *Klebsiella* species, *Streptococcu*s species, *Enterococcus* species and *Proteus* sp (Howell-Jones *et al*., 2005; Bowler *et al*., 2001). *S. aureus* is the most commonly isolated species from wounds (Bowler, 1998) and is a recognised pathogen containing a variety of virulence

factors. It is isolated from both acute and chronic wounds (infected and non-infected). The most commonly isolated anaerobes from wound samples include *Peptostreptococcus* sp, *Clostridium* sp, *Bacteroides* sp, *Propionobacterium* sp, *Prevotella* sp, *Fusobacterium* sp and *Veillonella* sp (Howell-Jones *et al*., 2005; Wall *et al*., 2002; Bowler *et al*., 2001). *Peptostreptococcus* in particular has been recognised as a barrier to healing in chronic wounds due to production of short chain fatty acids and hydrolytic enzymes as potential factors in delaying wound healing (Wall *et al*., 2002). In polymicrobial communities, the growth of specific bacterial species may be aided by synergistic effects from growing in the presence of other bacterial species. For example in a study of leg ulcers, *S. aureus* appeared to increase growth rates of Gram negative anaerobes (Bowler and Davies, 1999).

The impact of bacteria on the process of wound healing is complex and the subject of much debate in the literature. It is well documented and accepted that all open, chronic wounds are polymicrobial in nature and colonised with bacteria. Most physicians believe that the bacteria do not interfere with the healing process if the wound does not show clinical signs of infection. However, some physicians are starting to believe that high levels of bacteria may disturb healing even in the absence of clinical signs of infection (Edwards and Harding, 2004).

In 2001, White and Cutting introduced the term "critical colonisation", which applies to the wounds that do not display the standard clinical signs of infection, but contain high levels of bacteria that inhibit wound healing (White and Cutting, 2001). To properly diagnose infection in this situation, additional criteria are required (Cutting and Harding, 1996). Gardner *et al*., (2001) assessed the validity of those additional criteria, which include serous exudate, foul odour, discoloured or friable granulation tissue, and delayed healing or wound deterioration. They also performed quantitative biopsies and

used Robson's (Robson, 1997) definition of greater than 100,000 (> 10^5) organisms/g of tissue as being infected. Eleven of the 36 (31%) wounds in their study were infected; 91% of those wounds contained necrotic tissue. For these 11 infected wounds not displaying traditional signs of infection, 80% demonstrated delayed healing and friable granulation tissue.

There is a close correlation between the microorganisms present in wounds and those found in the normal flora of the gut or oral cavity (Brook, 1987/1989; Brook and Frazier, 1997). Minor, healing wounds may allow sufficient time for only a relatively small numbers of skin contaminants to colonise the wound. The continued exposure of devitalised tissue together with the delayed healing process is likely to promote the colonisation with a wide variety of endogenous microorganisms (Bowler *et al*., 2001). Dental plaque, the gingival crevice, and the contents of the colon contain approximately 10^{11} to 10^{12} microorganisms / g of tissue, of which, up to 90% of the oral microflora (Bartlett and Gorbach, 1976) and up to 99.9% of the colonic microflora (Hentges, 1989) are anaerobes. Wounds with disturbed oxygen supply and reduced environment are susceptible to colonisation by a range variety of anaerobic endogenous bacteria (Bjarnsholt *et al*., 2011).

For many years, medical microbiologists have relied on culture techniques to investigate the complexity of infections including chronic wound pathogenic biofilms (McGuckin *et al*., 2000). Whilst culture methods are useful and are a well-established approach for the detection of many common pathogenic bacteria associated with wound infections, they may underestimate microbial diversity (Wilson *et al*., 1997; Oates *et al*., 2012). These culture methods can be used to identify the "culturable" bacteria associated with such biofilms. However, laboratory culture techniques are only able to detect organisms which grow relatively quickly and easily on laboratory media. This

presents an important problem because many of the bacteria in wound biofilms do not grow on culture media (Davies *et al*., 2004). Thus, there is a lack of information about the diversity of populations that occur in association with chronic wounds biofilms. Various culture-independent methods have been investigated in a limited number of studies as potential adjuncts or replacements of culture for the microbial characterization of chronic wounds (Dowd *et al*., 2008, Gontcharova *et al.,* 2010 , Hill *et al*., 2003). Culture-independent investigations of the bacterial diversity utilizing PCR-denaturing gradient gel electrophoresis (DGGE) (Davies *et al*., 2004), pyrosequencing (Dowd *et al.,* 2008), other DNA fingerprinting techniques (Singh *et al*., 2009) and quantitative PCR (Melendez *et al*, 2010) have generally identified a greater range of bacteria than traditional culture techniques, and taxa not previously detected in wounds have been reported (Oates *et al.,* 2012). Whilst it is well documented that culture-independent methods may provide more information on microbial diversity, the role that taxa thus identified play in infection remains poorly understood. This contrasts with isolation methods where the pathogenicity of prominent culturable organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* has been well established (Bourke *et al*., 1994; Davies *et al.,* 2001; Gorbert *et al*., 2005; Lyczak *et al.,* 2000).

In environmental microbiology, denaturing gradient gel electrophoresis (DGGE) has been used as a tool for profiling complex microbial populations for many years and the technique has now been applied to the study of a limited number of human microbial populations (Muyzer and Smalla, 1998; Possemiers *et al*., 2004; Liu *et al*., 2010; Dowd *et al*., 2008). PCR-DGGE is classified as part of the new discipline of molecular microbial ecology, which investigates the interactions among microorganisms and between microorganisms and their environment (Muyzer and Smalla, 1998). This involves long-term study, which includes various and numerous environmental sample analysis (Muyzer and Smalla, 1998). Conventional cloning, hybridisation and culture

methods are not always practical for such investigations as these methods do not provide any information on the dynamics of the microorganism populations in complex ecosystems and potential effects of environmental changes on such populations (Muyzer *et al*., 1993; Sheffield *et al*., 1989). They also require an extended knowledge of the microorganisms to develop adapted probes that target particular individuals among diverse populations (Muyzer and Smalla, 1998). PCR-DGGE has the advantage of not requiring previous knowledge of microbial populations. It is a fingerprinting approach that can generate a pattern of genetic diversity in complex microbial ecosystems including biofilms of humans (Muyzer *et al*., 1993). This method has the potential to visually profile and monitor changes occurring in various microbial communities that are undergoing different treatments or modifications. It is a rapid and efficient separation technique of same length DNA sequences (amplified by PCR), which may vary as little as a single base pair (Sheffield *et al*., 1989). PCR-DGGE is a flexible method that allows a unique combination of different approaches for a more accurate identification of, for example, functional genes present in particular bacterial populations or specific bacterial species by using hybridization or species-specific probes (Walter *et al*., 2000). It can be utilized in diverse subject areas such as clinical and environmental microbiology.

There are very limited studies which have utilized DGGE to evaluate the diversity of microbial populations that occur within the pathogenic biofilms associated with chronic wounds (Dowd *et al*., 2008; Rhoads *et al*., 2012). A better understanding of the wound's microbiota will allow us to better manage the wound. Considering the bacterial populations within pathogenic biofilms is very important for many reasons. These reasons typically relate to the fact that the higher bacterial population diversity within a pathogenic biofilm provides the bacterial community as a whole with an

increased ability to persist and thrive in a variety of antagonistic situations, even in spite of combined host and medicinal attack (Boles *et al*., 2004).

5.2 Aims and objectives

The aim of this study was to apply DGGE and sequencing to the analysis of the chronic wound (diabetic wound) microflora and healthy skin and compare the data with conventional culture techniques. DGGE-sequencing was initially developed on samples collected from healthy participants before applying it to swabs obtained from chronic wounds. DGGE sequencing allowed an analysis of the bacterial population of individual wounds and healthy skin by using a single PCR. These samples were run alongside one another on the same gel for direct comparison. Based on the results of this study and the literature this was to inform a future development of a multiplex PCR assays for chronic wounds.

5.3 Materials and methods

5.3.1 Ethical approval

Ethical approval for the molecular detection of bacteria from healthy feet was obtained from the School of Biology, Chemistry and Health Sciences Research Committee at Manchester Metropolitan University.

Ethical permission for the molecular detection of microorganisms from chronic wounds was obtained from the NHS Research Ethics Committee (11/H1011/4). The samples, collected from the same patients as described in Chapter 4 section 4.3.2.1, were used for development of the PCR-DGGE sequencing method.

5.3.2 Participants and study design

5.3.2.1 Healthy feet study

This was a pilot study to develop and optimise DGGE methods for a larger study on chronic wounds (Appendix 5 – Study Protocol). A total of 20 healthy volunteers (10 female and 10 male) aged 18-75 years were included in the study (Appendix 6 – Participant Information Sheet; Appendix 7 – Consent form). Four samples were taken from each volunteer – two swabs from the sole of each foot. The soles of the foot (central part of the plantar) were initially cleaned using sterile saline and then an area of approximately 2 cm^2 swabbed using zigzag method (description of zigzag method in Chapter 3, section 3.6). Samples were collected from participants using Sigma dry swab (Medical Wire & Equipment).

One swab from each foot was processed using conventional microbiological methods. Bacterial DNA was extracted from the second swab and analysed by PCR-DGGE sequencing.

Qualitative microbiology was performed by inoculating the samples onto a variety of selective media and identifying bacteria after incubation using standard microbiological methods described in Chapter 3 section 3.5.2.

The cultural methods were compared with molecular methods (DGGE, sequencing) population studies to study bacterial diversity in normal, intact skin.

5.3.2.2 Chronic wounds

Thirty two patients with diabetic foot ulcers were recruited to the study and the study was carried on as described in section 4.3.2.2. DNA was extracted from each wound sample using a Qiagen kit and PCR-DGGE sequencing was performed. PCR-DGGE sequencing results were compared with conventional culture methods.

5.3.3 Materials, media, reagents and identification methods – conventional culture method

Identification of *Staphylococcus aureus* including MRSA was based on the same methods as in Chapter 3, section 3.7. In order to identify other bacteria species present in healthy skin and chronic wounds, additional media, reagents and methods were employed.

5.3.3.1 MacConkey Agar (Oxoid, Basingstoke, UK, CM0115)

The medium was made up according to the manufacturer's instructions, sterilised and dispensed in 25 ml volumes in sterile Petri dishes.

5.3.3.2 Fastidious Anaerobic Agar (LabM, Heywood, UK, BO90-A)

Ready prepared Fastidious Anaerobe Agar with 7% (v/v) Horse Blood and 75ml/l Neomycin was purchased.

5.3.3.3 Sabouraud Agar (Oxoid, Basingstoke, UK CM 0041)

The medium was made up according to the manufacturer's instructions, sterilised and dispensed in 25 ml volumes in sterile Petri dishes.

5.3.3.4 Prolex Streptococcal Grouping Latex Kit (Pro-Lab Diagnostic, Wirral, UK)

Streptococcus species were grouped according to Lancefield's system (Lancefield, 1933) using commercially prepared antisera A, B, C, D, F and G (Prolex Streptococcal Grouping Latex Kit, Pro-Lab Diagnostic). The Prolex Streptococcal Grouping Latex Kit is based on liberation of specific antigen from bacteria cell walls by modified nitrous acid extraction.

5.3.4 Microbiological processing of the samples

The samples were processed using the semi-quantitative and quantitative culture methods described in Chapter 3, section 3.7.2.1 and 3.7.2.3.

5.3.5 Identification of isolates

Most isolates, including MRSA, were identified to the species level using standard methods such as typical colonial morphology on culture media, catalase test, coagulase test, DNase test, oxidase test (Pro-Lab Diagnostics), and the Analytical Profile Index (API) system (bioMerieux UK Ltd.) - API 20E, API 20NE, APIStaph used as per the manufacturer's instructions. An accurate count of the appropriate bacteria was performed using an image analysis system, the Acolyte counter (Don Whitley Scientific) on the respective culture media.

5.3.5.1 Isolation of Gram-negative bacteria

MacConkey agar no. 3 was used to isolate Gram-negative bacteria. This agar is a more selective modification of MacConkey medium which is suitable for the detection and enumeration of coliform and *Pseudomonas* spp. Due to the inclusion of a specially prepared fraction of bile salts in addition to crystal violet, the medium gives improved differentiation between coliforms and non-lactose fermenting organisms whilst Gram-positive cocci are completely inhibited. The presence of *Pseudomonas* spp. was confirmed using oxidase test.

5.3.5.2 Anaerobic bacteria

A zone of inhibition around the metronidazole disc indicated the presence of anaerobic bacteria. Clearing around the disc indicated obligate anaerobes. In addition, if there was an increased growth on this plate compared to the aerobic plates this was investigated for the presence of anaerobes.

5.3.6 Materials, media, reagents and identification methods – PCR-DGGE sequencing

5.3.6.1 DNA extraction

DNA extraction methods were used as described in Chapter 2, Section 2.3.3.3.

5.3.6.2 PCR Reagents

PCR reagents, methods and thermocycling conditions were the same as those decribed in Chapter 2, Sections 2.3.3.4 and 2.4, respetively.

5.3.6.3 Horizontal Gel Electrophoresis reagents

Horizontal Gel Electrophoresis reagents and methods were used as described in Chapter 2, Section 2.3.3.5.

5.3.6.4 DGGE

The composition of acrylamide/bisacrylamide solutions were as follows:

100% denaturant polyacrylamide solution

- 17.5 ml acryl/bisacrylamide ratio 19:1 (Sigma-Aldrich, Dorset, UK)

- 42g urea (Sigma-Aldrich, Dorset, UK)

- 40ml formamide (Sigma-Aldrich, Dorset, UK)

- 9.5ml sterile dH_2O

- 1 ml 50x TAE buffer

0% denaturant polyacrylamide solution

- 17.5ml acryl/bisacrylamide

- 81.5ml sterile dH_2O

- 1ml 50x TAE buffer

Silver Gel staining:

- Fixing solution: 0.5 % (v/v) of Glacial Acetic Acid was added to 50ml of 10% (v/v) ethanol and sterile water added to adjust to 500ml.

- Silver staining solution: 0.1g of silver nitrate was added to previously prepared 200ml of fixing solution.

- Developing solution: 3g of NaOH and 2ml of formaldehyde were added to 200ml of sterile water.

5.3.6.5 Steps in the process to obtain individual 16S rDNA sequences from a mixed population (Figure 5.1)

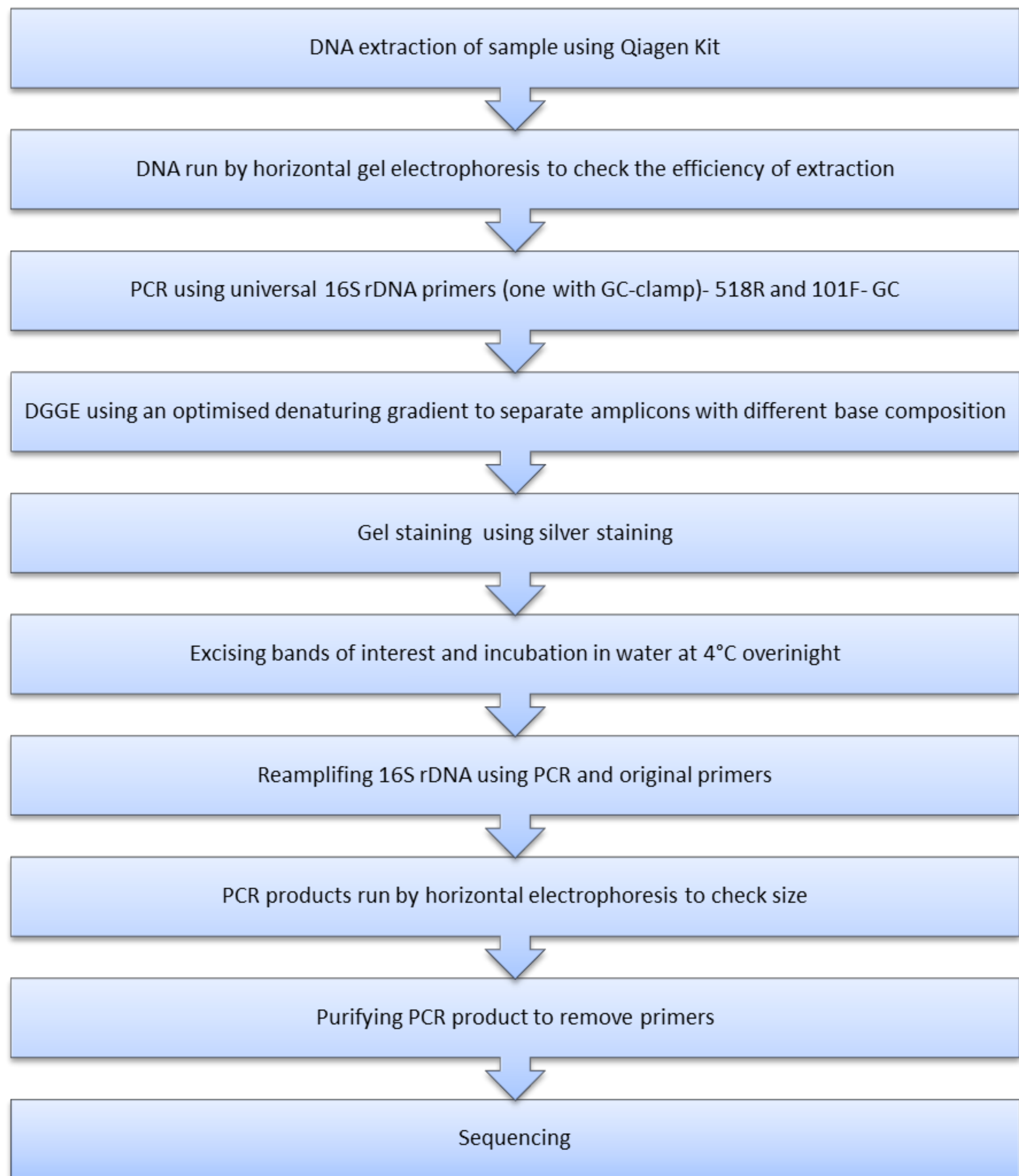

Figure 5.1 Steps in the process to obtain individual 16s rDNA sequences from mixed populations of bacteria (adapted from Dr P. Linton, personal communication).

5.3.6.6 DNA extraction from samples

Swabs collected from patients were placed into a vial containing 180µl of molecular grade water, vortexed and the DNA was extracted from the suspension using the DNeasy Blood and Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

5.3.6.7 Horizontal Gel Electrophoresis

Horizontal Gel Electrophoresis was performed as described in Chapter 2, Section 2.3.3.5.

5.3.6.8 PCR set up

PCR reactions were carried out with reaction volumes of 25 µl and PCR conditions for universal 16S primers were as described in Chapter 2, Section 2.4, Table 2.2.

5.3.6.9 Denaturing Gradient Gel Electrophoresis (DGGE)

The DGGE was performed using the INGENYphorU system (Ingeny, The Netherlands). The method was adapted from Dr. P. Linton (personal communication).

5.3.6.9.1 Set-up of DGGE glass plate cassette

The glass plates were washed with detergent; rinsed with ddH_2O and left to dry. Both plates were cleaned with 95% (v/v) ethanol and gel bond was placed on the top of one glass with the large notches. The U-shaper spacer was covered with Vaseline especially on the edges and placed on the top of the glass plate with the large notches and then the other glass was placed on the top of it. The glass plate sandwich was transferred in the cassette and the U-shaper spacer pulled up and secured by tightening the screws at the bottom and sides of the cassette.

5.3.6.9.2 Gel casting

A 200 µl pipette tip was attached to the end of the tube coming from the left-hand chamber of the gradient former and placed in the top of the glass cassette.

Two separate Universal bottles each containing 24 ml of the high and low denaturation acrylamide/bisacrylamide solution (60% and 30%) made up from 0% and 100% solutions were prepared and stored on ice. 60% (v/v) denaturation solution was prepared by adding 14.4 ml of 100% of denaturation solution to 9.6ml of 0% denaturation solution (section 5.3.6.4). 30% (v/v) denaturation solution was made by adding 7.2 ml of 100% denaturation solution to 16.8 ml of 0% denaturation solution (section 5.3.6.4).

An aliquot of 20% APS (ammonium persulfate) was defrosted and 100 µl added into each universal and mixed. Then 10 µl TEMED (tetramethylethylenediamine) was added into each tube and inverted to mix and the gel was cast immediately. The high denaturing solution was poured into the left-hand chamber and the valve was opened into the other chamber briefly and closed again quickly to remove any air bubbles. The solution which migrated into the other chamber was transferred back into the left-hand chamber. The magnetic stirrer was started gently. The low denaturing solution was poured into the right-hand chamber and the peristaltic pump (flow-rate of 5ml/min) turned on and the valve quickly opened so that the chambers were connected.

The gel was polymerised for 1h and any liquid that formed on the top of the gel was poured off. In the study on diversity of microflora in chronic wounds the gel was left to polymerise overnight. A Bijou bottle with 6ml of 0% denaturant acryl/bisacryl (section 5.3.6.4) was prepared and stored on ice. 60 µl of 20% APS and 6 µl TEMED were added and mixed thoroughly. The solution was pipetted onto the top of the resolving gel and the comb was pushed down into it without forming any bubbles, and

left to polymerise for 10-15 min. The warm buffer from the tank was flooded around the comb and the comb finally removed. The wells were rinsed with buffer and any excess of gel cleaned using clean tissue. The screws were loosed and the U-shaper spacer pushed down and secured by tightening the side screws until they just touched the cassette.

5.3.6.9.3 Sample loading

The samples were loaded in the sample buffer (10 µl concentrated PCR product with 2µl) and a low voltage power supply connected (12V). The water and sample buffer were loaded in any empty lanes. The gel was run for 5 min at high voltage without buffer re-circulation. Then the buffer re-circulation was turned on and run for the full running time of 16h at 75V.

5.3.6.9.4 Preparation of running tank

The buffer tank was prepared by filling with 0.5x TAE and heating to 60˚C. The gel cassette was added, making sure there were no bubbles under the gel, the buffer re-circulation tube was then connected and the pump on the side of the tank was turned on. The positive and negative terminals were connected to the gel cassette and the external cables connected to the power pack. The gel was run for 5 min at 250 volts.

5.3.6.9.5 Gel staining

The gel was removed from the plate and placed in a tray containing fixing solution (section 5.3.6.4). The gel was incubated with rocking for 30 minutes and then the fixing solution was decanted and stain solution (section 5.3.6.4) added to the gel tray and incubated with rocking for 20 minutes. After incubation the gel was rinsed three times in distilled water and the developing solution added (section 5.3.6.4). The gel was developed until the bands were clearly visible. The developing solution was poured off

and a fixing solution added for another 30 minutes with rocking. After staining the gel was preserved in a preserving solution (10% (v/v) glycerol, 25% (v/v) ethanol). The bands of interests were excised and added to 50µl of molecular grade water, mashed using a pipette tip and left overnight in the fridge. After incubation PCR was performed to amplify the DNA in the bands (Section 5.3.6.8). The amplified products were cleaned with Sure clean (Bioline) according to the manufacturer's instructions before sequencing.

5.3.6.10 Sequencing

PCR products were sequenced using the non GC-clamp (reverse) 518R primer at the DNA Sequencing Department at The University of Manchester (Manchester, UK) using a Perkin-Elmer ABI 377 sequencer.

5.4 Results

5.4.1 Conventional microbiology of healthy feet

Twenty healthy participants were included in the study. Two samples were collected from the clean sole of each foot (one for culture and one for molecular analysis). Samples collected for culture analysis were processed and bacteria isolated and identified using conventional microbiology methods. Bacterial isolates cultured from swabs were identified to genus or species level when possible. The mean number of bacterial species recovered using culture methods was 2.4 (1-5 species per sample). One participant was identified with five bacterial species, two participants were identified with four bacterial isolates, five participants with three species, eight with two microorganisms and four participants with only one isolate. There was a significant correlation between the number of organisms recovered from the right and left feet

($p<0.026$). The most predominant microflora found on healthy skin were CNS (Coagulase Negative Staphylococci*)* – *S. epidermidis* (12 participants), *S. warneri* (9 participants*), Micrococcus* sp (4 participants), *S. cohnii* (4 participants), *S. hominis* (3 participants). Three participants (2 female and 1 male) were colonised with *Candida cifferi*, two females with *Candida pelliculosa* and one female with *Corynebacterium* spp. *S. aureus* was found only in one female participant. All participants were colonised only with Gram positive staphylococci species and *Candida* spp (Table 5.1).

Table 5.1 Bacterial species isolated from the healthy feet samples collected from female and male.

Bacterial strain	**Gender**	
	Female	**Male**
Staphylococcus aureus	1	0
Staphylococcus epidermidis	5	7
Staphylococcus cohni	2	2
Staphylococcus warneri	4	5
Staphylococcus caprae	0	1
Staphylococcus hominis	1	2
Staphylococcus capitis	1	1
Staphylococcus sciuri	1	0
Candida pelliculosa	2	0
Candida cifferrii	2	1
Candida fermata	0	1
Cryptococcus terreus	1	0
Micrococcus	2	2
Kocuria varians	0	1

Twenty five (62.5%) out of 40 samples (20 swabs from the right and 20 swabs from the left foot) collected from healthy participants had total bacterial count 10^5 cfu/cm^2, fourteen samples (35%) 10^6 cfu/cm^2 and 2.5% of specimens contained 10^7 cfu/cm^2.

All samples collected from the healthy feet contained 10^5 or more cfu/cm^2 of bacteria on the skin. The average total bacterial count was 1.88 x 10^6 cfu/cm^2. None of the samples contained Gram negative rods (Appendix 1, Table 1 and 2).

5.4.2 Comparison of the conventional methods with DGGE-sequencing for bacteria identification in samples collected from healthy participants

Two samples from each participant (left and right foot superficial swabs) were processed using PCR-DGGE and sequencing for the diversity of microflora and the comparison with conventional methods. Only PCR positive samples were run on the gel. The study was also limited to the analysis of single participant samples, due to the lack of bands present in many samples, high costs and labour – intensive nature of sequencing. However, the most discernible bands were sequenced and microorganisms identified. Nine samples out of twenty three were carefully analysed, visible bands cut, DNA amplified and sequencing performed. Figure 5.2 shows the 16S rDNA fragments from the samples collected from soles of the left and right feet of healthy participants. The bands were labelled and sequenced. The discernible bands were assumed to represent the dominant members of the mixed bacterial community present in each sample; i.e. those that are present in highest numbers (Linton *et al*., 2007).

All 16 bands excised from 9 samples were sequenced. The closest relative of the clones were identified by performing a BLAST search of the sequences at http://www.ncbi.nlm.nih.gov/blast. The percentage identity of all clones to known database sequences was > 95%, with only one sequence having a lower identity < 93%, namely, band 4 *(Prochlorococcus marinus*). The individual profiles proved to be complex. Although participants had some bands in common, only bands 2, 10 and 11 were identified with the same bacterial isolate and results correlated to those obtained

by culture analysis. In three samples, the DGGE profiles had bands present that were not represented by the bands in the profiles obtained from the cultured isolates from these samples (Figure 5.2).

For most sequences affiliated with *Staphylococcus* the sequence similarities obtained from the EMBL database were the same for different species within the same genus, which prevented identification to the species level.

Twelve (75%) DGGE bands were identified with the same species as bacteria identified using cultural methods (Table 5.2). One band (6.25%) was found to not show significant similarity to any bacteria from the nucleotide sequence database. Three (18.75%) bands were identified with bacteria that were not detected using culture methods (*Propionobacterium acne*, *Prochlorococcus marinus* and *Corynebacterium tuberculostearicum*).

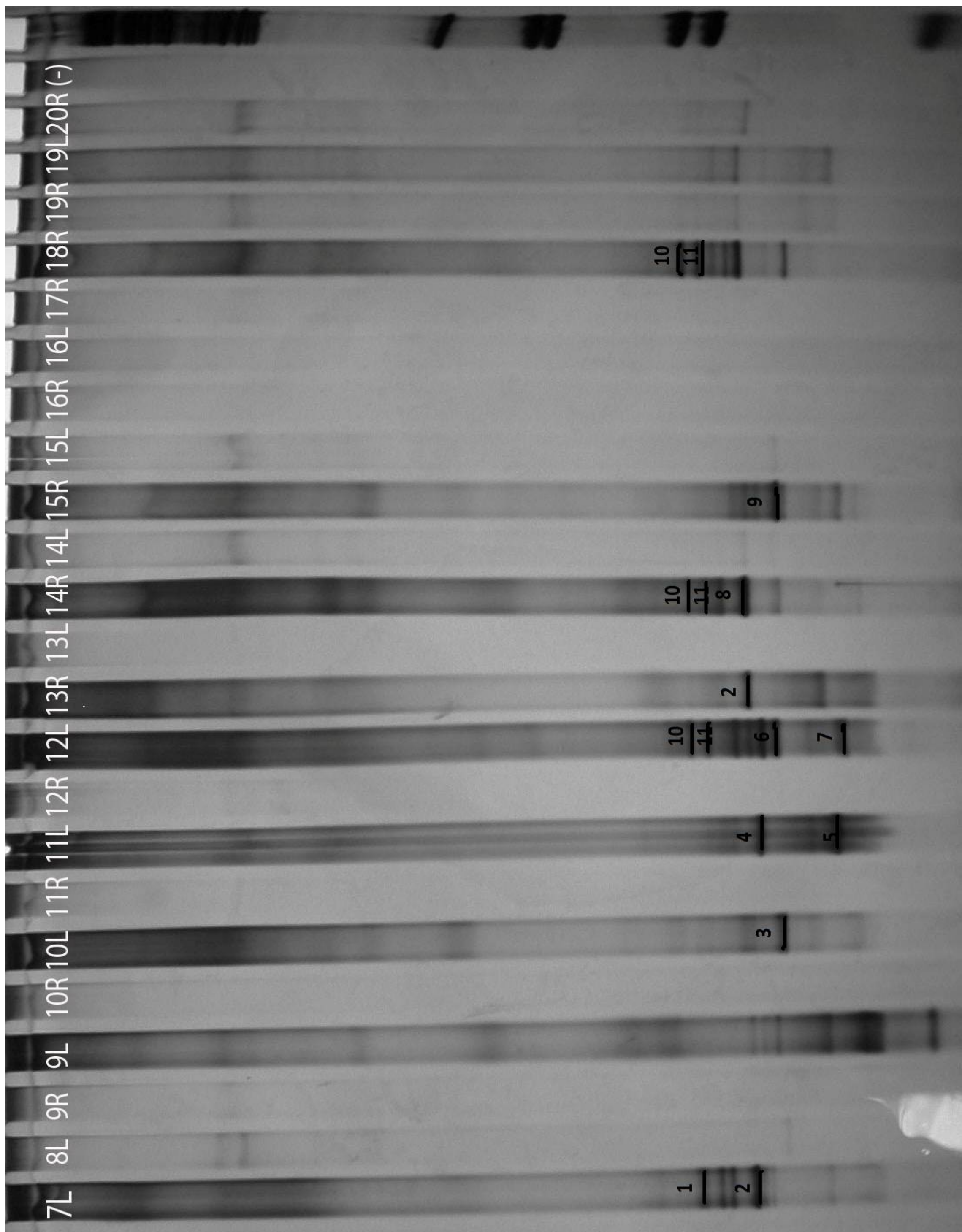

Figure 5.2 DGGE analysis of 16S rRNA gene fragments of the samples collected from healthy participants (7-20 patients numbers; L- left foot, R-right foot); 1-11 – band number corresponding to particular bacterial strain (see Table 5.3). The bands were additionally marked for better visibility. #

Table 5.2 Comparison of cultured bacterial isolates with DGGE analysis from swabs obtained from individual participants (L- left foot; R- right foot).

*Percent similarity of partial 16S rDNA coding sequence to sequence of their closest bacterial relatives available in the EMBL nucleotide sequence database.

Patient	Corresponding band	Identification using culture methods	Identification using DGGE-sequencing	%*
7L	1	*Staphylococcus cohnii*	*Staphylococcus cohnii spp. urealyticus*	100%
7L **13R**	2/ two bands	*Staphylococcus cohnii*	*Staphylococcus cohnii spp. urealyticus*	99%
10L	3	Only CNS identified	*Propionobacterium acne*	99%
11L	4		*Prochlorococcus marinus*	90%
	5	*Staphylococcus warneri*	*Staphylococcus warneri*	100%
12L	6	*Staphylococcus epidermidis* and *S.hominis*	No significant similarity found	
	7		*Corynebacterium tuberculostearicum*	98%
14R	8	*Staphylococcus epidermidis* and *S.warneri*	*CNS*	99%
15R	9	*Staphylococcus hominis*	*CNS*	99%
12R **14R** **18L**	10 / 3 bands	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	99%
	11 / 3 bands	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	99%

5.4.3 Conventional microbiology of chronic wounds

Two samples (superficial swabs) were collected from each wound – one for culture analysis and one for molecular testing (PCR-DGGE and sequencing). Wound swabs were cultured and microorganisms identified to the genus or species level when possible. The mean number of bacteria species recovered from chronic wounds using culture methods was 1.83 (1-4 isolates per sample). Fifteen patients were identified with Coliforms, six patients were identified with MRSA, three patients with MSSA and five patients with *Streptococcus* spp (Group A, B and D). Anaerobes, *Proteus mirabilis* and *Pseudomonas* spp were detected in single patients. Coagulase negative staphylococci were the most predominant microflora. Five patients were identified with *S. epidermidis* and fifteen patients were identified with other CNS (*S. haemolyticus*, *S. simulans*, *S. capitis*, *S. warneri*, *S. cohnii, S. chromogenes*, *S. xylosus*) (Appendix 1, Table 3).

5.4.4 Comparison of the culture methods with DGGE sequencing analysis in chronic wounds

Denaturing gradient gel electrophoresis (DGGE) was performed using 30 samples. However, only 21 samples out of 30 showed visible and good quality PCR bands and these patients' data were analysed and included in this study.

After completion of electrophoresis, gels were stained using silver staining and the visible bands were excised. Sequence identification was carried out only with bands showing a PCR product after a re-amplification process. The obtained sequences were compared with sequences of known bacteria listed in the EMBL nucleotide sequence database. In total 21 of 30 investigated patients samples showed reproductible PCR products after amplification. 40 samples (bands) were cut from 21 investigated samples.

The results of comparative sequence analyses obtained from EMBL database are shown (Table 5.3). Sequence homologies to sequences of known bacteria in the EMBL

database ranged between 92% and 100%, with only one sample producing a result of 85%. In total, 3 samples out of 21 investigated had a single band and were identified with only one isolate (patient numbers 29, 19, 6), and this was confirmed by culture methods. All 18 samples showed multiple bands. 9 samples were identified with species that were not isolated using standard culture methods.

A comparison of DGGE positions of sequenced bands was performed. Although some patients had the same bands in common, each patient produced a unique banding pattern. The DGGE patterns of bands of all samples was shown (Figure 5.3). In 6 samples (patient numbers 9, 11, 12, 16, 24 and 27) two to three different positions were found among the same species. It was also found that bands located on the same level within the gel belonged to different species, whereas bands identified with the same species were located on different levels within the gel. However bands identified with *Staphylococcus* spp were located on very similar position to each other with minimal deviation (Figure 5.3 – bands 1, 2, 3, 12, 13, 21, 22, 26, 28, 29, 36, 39, 40). Many bands in the same position within a gel were represented by different bacteria. *S. epidermidis*, *Enterobacter hormaechei* and *E. faecalis* were found to produce bands in 2 different positions. *Morganella morganii*, *Proteus mirabilis* and *Duganella zoogloeoides* produced bands in 3 different positions within the gel, whereas *Acinetobacter baumannii* gave 5 different bands.

The results of both DGGE sequencing and culture methods correlated in 12 (57%) patients out of 21 investigated for the bands sequenced.

Nine (43%) patients were identified with bacteria which were not cultivated using standard microbiological methods (*Duganella zoogloeoides*, *Pseudomonas meridiana*, *Enterobacter hormaechei*, *Enterococcus faecalis*, *Acinetobacter baumannii*). The most frequently detected genera were *Staphylococcus* and coliforms, which is analogous to

culture results. For most sequences affiliated with *Staphylococcus* the sequence similarities obtained from the EMBL database were the same for different species within the same genus, which prevented identification to the species level. For one sequence no identification to the genus level could be obtained because no significant similarity was found.

It was hoped that bands on the same level corresponded to the same species however the results showed that the bands located on the same level within the gel may represent different species and bands identified with the same species may be located on different levels within the gel. Since not all bands were sequenced, a direct comparison between DGGE sequencing and traditional culture methods was not performed.

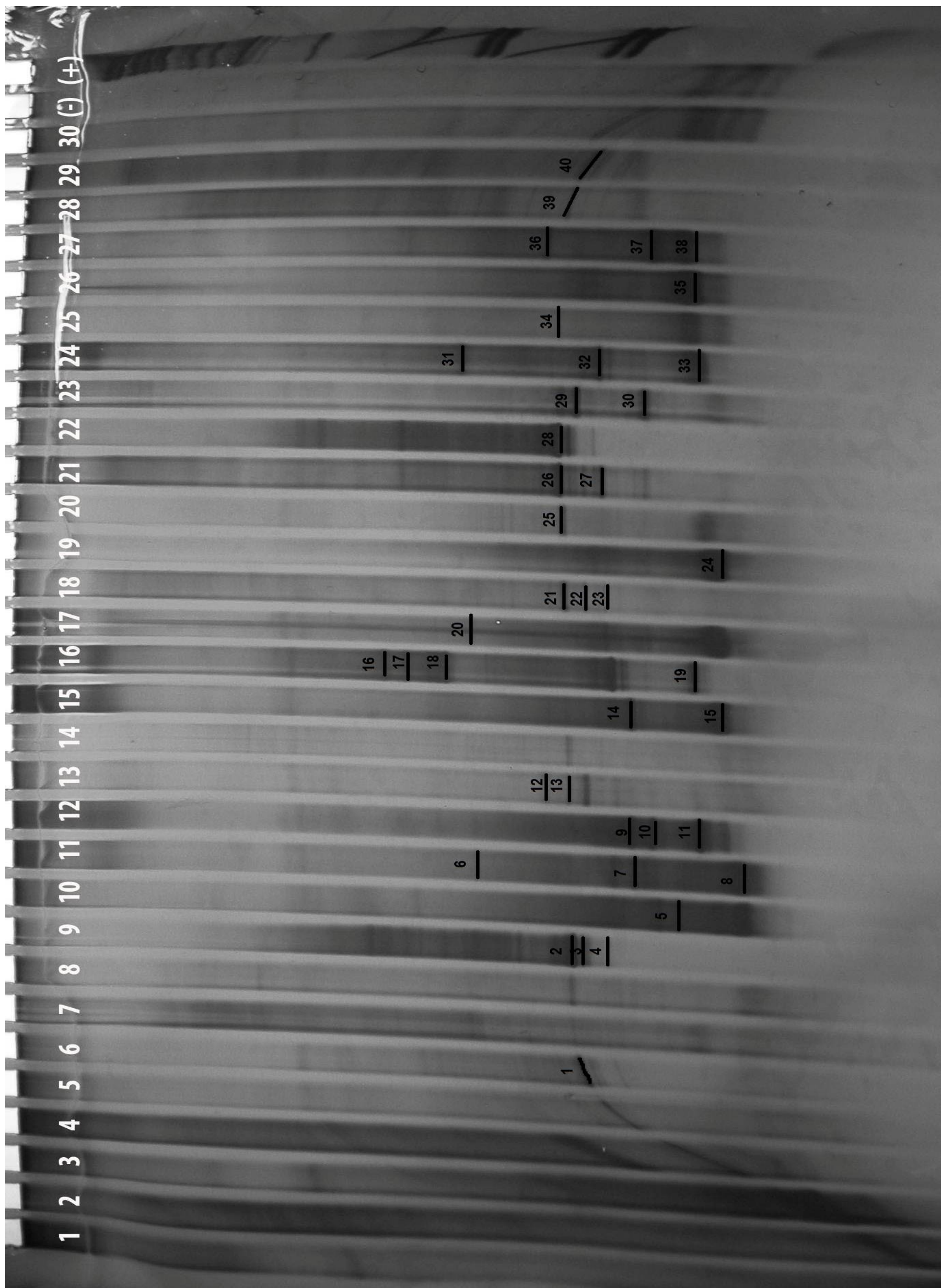

Figure 5.3 DGGE analysis of 16S rRNA gene fragments from samples collected from patients with chronic wound ulceration. (1- 30 at the top of the gel – patient's number; bands labelled 1 to 40; (-) – negative control; (+) – positive control). The bands were additionally marked for better visibility.

Table 5.3 Comparison of cultured bacterial isolates with DGGE analysis from swabs obtained from individual patients with chronic wounds.

*Percent similarity of partial 16S rDNA coding sequence to sequence of their closest bacterial relatives available in the EMBL nucleotide sequence database.

Patient	Band	Identification using culture methods	Identification using DGGE	%*
6	1	*S. aureus*	*S. aureus*	99%
9	2	*S. epidermidis*	*S. epidermidis*	99%
	3		CNS	98%
	4		*S. epidermidis*	99%
10	5	coliforms *S. epidermidis*	*Escherichia fergusonii*	99%
11	6	coliforms anaerobes *S. simulans*	*Morganella morganii*	93%
	7		*Morganella morganii*	93%
	8		*Morganella morganii*	98%
12	9	*Proteus mirabilis*	*Proteus mirabilis*	99%
	10		*Proteus mirabilis*	99%
	11		*Proteus mirabilis*	99%
13	12	*S. simulans*	CNS	95%
	13		CNS	96%
15	14	*Pseudomonas* spp	*Pseudomonas aeruginosa*	96%
	15		*Enterobacter hormaechei*	99%

Table 5.3 (Continuation) Comparison of cultured bacterial isolates with DGGE analysis from swabs obtained from individual patients with chronic wounds.

*Percent similarity of partial 16S rDNA coding sequence to sequence of their closest bacterial relatives available in the EMBL nucleotide sequence database.

Patient	Band	Identification using culture method	Identification using DGGE	%*
16	16	coliforms *S. warneri*	*Duganella zoogloeoides*	92%
	17		*Duganella zoogloeoides*	93%
	18		*Duganella zoogloeoides*	93%
	19		*Pseudomonas meridiana*	95%
17	20	coliforms *S. xylosus* *S. cohnii*	*Enterobacter hormaechei*	85%
18	21	*S. hominis*	*S. epidermidis*	99%
	22		CNS	96%
	23		*Enterococcus faecalis*	97%
19	24	coliforms	*Acinetobacter baumannii*	97%
20	25	*S.hominis*	*Acinetobacter baumannii*	95%
21	26	coliforms	*S. aureus*	99%
	27	*Streptococcus* spp *S. capitis*	*Acinetobacter baumannii*	93%
22	28	MSSA *S. warneri*	*S. epidermidis*	97%

Table 5.3 (Continuation) Comparison of cultured bacterial isolates with DGGE analysis from swabs obtained from individual patients with chronic wounds.

*Percent similarity of partial 16S rDNA coding sequence to sequence of their closest bacterial relatives available in the EMBL nucleotide sequence database.

<table>
<tr><td rowspan="2">23</td><td>29</td><td rowspan="2">S.epidermidis
S.capitis</td><td>CNS</td><td>96%</td></tr>
<tr><td>30</td><td>E,faecalis</td><td>96%</td></tr>
<tr><td rowspan="3">24</td><td>31</td><td rowspan="3">Coliforms
Streptococcus spp</td><td>Acinetobacter baumannii</td><td>97%</td></tr>
<tr><td>32</td><td>Acinetobacter baumannii</td><td>97%</td></tr>
<tr><td>33</td><td>Enterobacter hormachaei</td><td>98%</td></tr>
<tr><td>25</td><td>34</td><td>Coliforms
S.hominis</td><td></td><td>No results</td></tr>
<tr><td>26</td><td>35</td><td>Coliforms
S.cohnii</td><td>Enterobacter hormaechei</td><td>98%</td></tr>
<tr><td rowspan="3">27</td><td>36</td><td rowspan="3">Coliforms
S.cohnii</td><td rowspan="2">CNS</td><td>97%</td></tr>
<tr><td>37</td><td>97%</td></tr>
<tr><td>38</td><td>Enterobacter hormaechei</td><td>96%</td></tr>
<tr><td>28</td><td>39</td><td>Coliforms
S.hominis</td><td>S.aureus</td><td>96%</td></tr>
<tr><td>29</td><td>40</td><td>MRSA</td><td>S.aureus</td><td>96%</td></tr>
</table>

5.5 Discussion

In the present study, a 16S rDNA PCR-DGGE sequencing method was applied to the analysis of the diversity of microflora in wounds and healthy skin. This allowed analysis of the bacterial population of individual wounds by using a single PCR and also for samples to be run alongside one another on the same gel for direct comparison. In this way it was possible to obtain considerable information about the species composition of wounds and healthy skin. Bands of specific interest present in the total wound DNA were then excised and sequenced. Based on the results of the study the most common bacteria in chronic wounds would be selected for further development of multiplex RT-PCR.

The 16S rRNA gene has always been present in all microorganisms and is the main target of genetic sequencing in bacteria. Several unique characteristics make this gene the ideal candidate for mutations analysis (Tortoli, 2011). The 16S rRNA gene is characterized by an evolutionary rate high enough to produce interspecies variability but, at the same time, by a degree of conservation sufficient to minimise the intraspecies variability (Peix *et al*., 2009). The 16S rRNA gene sequence is about 1,550 bp long, contains both variable and conserved regions and is large enough to provide distinguishing and statistically valid measurements. The comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences.

The PCR products that can be used to perform DGGE analysis and allow efficient resolution must be < 500bp (Myers *et al*., 1985). This short fragment limits the amount of sequence information that can be obtained. However, the first 500bp of 16S rDNA fragment is the hypervariable region and is the best region to make phylogenetic

compositions. The primers used in the present study yielded a product of 417 bp long. These primers gave good discrimination and separation of the predominant species present in the community. For retrieving clean sequences from individual bands co-migration was a problem. It is clear from other studies with DGGE that 16S rRNA gene sequences affiliated to specific bacterial species can be found in more than one position in DGGE gels. This may be due to the presence of multiple operons or a high similarity between the base compositions. *Paenibacillus polymyxa* produces more than one DGGE band due to slight sequence heterogeneity between operons (Nübel *et al*., 1996) and as many as nine different positions were found among 10 *Corynebacterium* affiliated sequences (Schabereiter-Gurtner *et al*., 2001).

In the present study, bands on the same position within a gel were represented by different species. In the healthy feet study, only a few bands (bands 2, 10, 11) were at the same position and were identified as the same microorganism. However, bands identified as *Staphylococcus* spp were located in a very similar position to each other with minimal deviation. Additionally, *S. epidermidis* was found to produce bands in two different positions within a gel in both the healthy feet and chronic wounds study. In the chronic wound study *Morganella morganii*, *Proteus mirabilis*, *Enterobacter hormaechei*, *Duganella zoologoeoides*, *Enterobacter faecalis* and *Acinetobacter baumannii* all produced between 2 to 5 bands in different positions within a gel, which may be due to the presence of multiple operons or different subspecies within the sample.

Schabereiter-Gurtner *et al.,* (2001) identified bacteria from conjunctival swabs by PCR-DGGE and sequencing. They found that *Corynebacterium* species can give 9 different positions in DGGE. Bands at five different positions in DGGE were found among 9 *Staphylococcus*-affiliated sequences. Three different band positions in DGGE were found among 6 *Streptococcus*-affiliated sequences. All sequences affiliated with

Propionobacterium had the same position in DGGE. In the recent study, *S. epidermidis*, *Enterobacter hormaechei* and *E. faecalis* were found to produce bands in 2 different positions. *Morganella morganii*, *Proteus mirabilis* and *Duganella zoogloeoides* produced bands in 3 different positions within the gel, whereas *Acinetobacter baumannii* gave 5 different bands.

The mean number of bacterial species recovered from healthy feet and chronic wounds samples using culture methods was 2.4 and 1.83 respectively. However, the microflora of healthy feet seem to be more diverse but this diversity was mainly between *Staphylococcus* species. In chronic wounds, the diversity was observed within different genera. None of the samples collected from healthy participants was shown to contain Gram negative bacteria. The most predominant microflora of healthy skin was Coagulase negative staphylococci, *Candida* spp, *Corynebacterium* spp. Healthy skin contains mainly Gram positive cocci, whereas in chronic wounds the predominance of Gram positive bacteria in the early phase switches to Gram-negative species mostly coming from the gut (*E. coli*) 4-10 days after injury (Bowler *et al*., 2001).

PCR is known to be more sensitive than culture for the detection of bacteria in clinical samples (Rantakokko-Jalava *et al*., 2000; Schabereiter-Gurtner *et al*., 2001). In the present study, DGGE allowed the identification of a number of strains not detected by culture technique with 43% of the DGGE fragments sequence representing organisms not cultured from the wounds from which they had been amplified. This highlights the fact that a significant proportion of the resident microflora was not able to be analysed by culture methods alone. The reason for this maybe due to the fact that bacteria were isolated from samples containing large numbers of other bacteria and they may not have been detected during the initial culture screening due to competition with other more numerous species and overgrowth on the selective/nonselective media by related microflora. In the healthy foot study, three (18.75%) samples were identified

with bacteria, which were not detected using culture methods (*Propionobacterium acnes*, *Prochlorococcus marinus* and *Corynebacterium tuberculostearicum*). *Prochlorococcus marinus* is the dominate photosynthetic organism in the ocean. It accounts for up to 60% of the ocean's chlorophyll in many regions such as the subtropical Pacific and it is not associated with human microflora. *Corynebacterium* and *Propionobacterium* species are normal skin flora and they are largely commensals and part of the skin flora present on most healthy adult human skin. 43% of the chronic wound microflora not detected by culture, but identified by DGGE are intestinal bacteria. The following were found to be associated with chronic wounds: *Morganella morganii*, *Escherichia fergussonii*, *Enterobacter faecalis*, *Proteus mirabilis*. *Acinetobacter baumannii* is also associated with chronic wounds, however it is a pathogenic bacterium resistant to most antibiotics. As a result of its resistance to drug treatment, some estimates state the disease is killing tens of thousands of U.S. hospital patients each year (Pollack, 2010). The illness can cause severe pneumonia and infections of the urinary tract, bloodstream and other parts of the body. *Acinetobacter* enters into the body through open wounds, catheters, and breathing tubes. It usually infects those with compromised immune systems, such as the wounded, the elderly, children, or those with immune diseases so the proper identification and treatment of infection is very important, especially for patients with diabetes. *A. baumannii* has also been implicated in severe life threatening infections such as necrotizing fasciitis (Charnot-Katsikas *et al*., 2009; Sullivan *et al*., 2010). *Enterobacter hormaechei* was identified as a unique species in 1989 (O'Hara *et al*., 1989). It has been shown to be of clinical significance by causing nosocomial infections, including sepsis. It spreads via horizontal transfer and is often associated with extended spectrum beta-lactamase production, which increases the challenges associated with treatment by limiting therapeutic options. It can be isolated from blood, wounds and sputum. One patient was

identified with *Duganella zooloeoides,* which is not associated with wounds and skin microflora. It is an environmental microorganism and human colonisation with *Duganella* remains unclear.

The results of the present study together with previous studies have demonstrated the greater diversity of the wound microflora assessed by molecular methods in comparison to cultural techniques. Rhoads *et al.,* (2012) investigated 168 chronic wounds using both conventional culture methods and PCR-DGGE. Seventeen different bacterial taxa were identified with culture, and 338 different bacterial taxa were identified with molecular testing. The majority of bacteria identified with culture were also identified with molecular testing, but the majority of bacteria identified with the molecular testing were not identified with culture methods. The Rhoads study demonstrates the increased sensitivity that molecular microbial identification can have over culture methodologies.

Davies *et al.,* (2004), investigated the microflora of healing and nonhealing chronic venous leg ulcers using 16S PCR-DGGE and found that more that 40% of the sequences represented organisms not cultured from the wounds from which they were amplified. They also confirmed that the bacterial DNA from patient's samples had some bands in common, however each patient sample produced a unique banding pattern. Similar results have been found in this study with 43% of microorganisms identified with PCR-DGGE sequencing methods but not identified using the culture approach.

Sequencing the 16S rRNA gene is more beneficial than traditional biochemical identification methods (Clarridge, 2004). The DGGE method is a valuable approach in screening complex ecosysytems on a large scale, however it is not free of biases. The performance of the method from sample collection to identification of the bacteria is time consuming and laborious. Additionally, there are many issues arising

from the number of steps necessary to perform the whole technique (sample collection, DNA extraction, PCR amplification, DGGE, bands excision, PCR of the bands and sequencing). If anything goes wrong in any stage during the process it will have an impact on the results and require the process to be started again. DGGE and sequencing is a good research technique to investigate complex bacterial populations when the time to obtain diagnostic results for patients does not matter. In clinical microbiology there is always a pressure to quickly identifying causative microorganisms, especially in life-treatening conditions.

There are also some limitations related to the sensitivity and specificity of the method. DGGE is able to detect only microbial populations making up at least 1% of the total community (Muyzer and Smalla, 1998). Also, fragments amplified from different species might migrate to the same location or multiple bands are observed from a single species in the gel. The co-migration of different sequences to the same position shows that the assumption that one band equals one genome is not always valid. The presence of bands at similar positions in DGGE gels does not confirm the presence of the same sequence or bacterial species in each sample. These issues limit the conclusions that can be drawn from this technique alone (Kisand and Wikner, 2003). Also, PCR-DGGE sequencing methods give the results of viable and non-viable microorganisms present within the sample, in contrast to conventional culture, which identifies only viable microorganisms.

There are various molecular biological methods used to reveal the species composition of bacterial populations without cultivation. Partial ribosomal amplification and pyrosequencing (PRAPS) is a recent and powerful method, which can be used to investigate microbial diversity in depth in any type of sample (Dowd *et al*., 2008). The bacterial tag encoded FLX amplicon pyrosequencing method (bTEFAP) (Dowd *et al*., 2008) improves upon this PRAPS approach, increasing the cost benefit of diversity

pyrosequencing through the use of sample-specific sequence tags incorporated onto secondary amplification primers. Individual sample amplicons can then be pooled prior to sequencing and using bioinformatics approaches they can be identified post-sequencing and analysed separately. The use of 454FLX pyrosequencing is revolutionary because it can provide a sufficient number of sequences of adequate length to enable extrapolations that estimate bacterial diversity based on the total number of microorganisms present and the distribution of individuals among those species. The bTEFAP technique allows the characterisation of bacterial diversity with less labour compared with traditional techniques such as DGGE. Pyrosequencing technology is an easy to use method and is relatively inexpensive, however it has also limitations such as the detection of long homopolymers (repeated nucleotides) which can result in sequencing errors (Dowd *et al*., 2008).

It is important to identify bacterial populations within pathogenic biofilms for many reasons. These reasons typically relate to the fact the higher bacterial population diversity within a pathogenic biofilm provides the bacterial community as a whole with an enhanced ability to persist and thrive in a variety of antagonistic situations, even in spite of combined host and medicinal attack (Boles *et al.,* 2004). Moreover, to establish the role of microorganisms in wound healing, it is necessary to define the full diversity of bacteria within a wound. Molecular methods such as nucleic acid amplification, pyrosequencing, and development of 16S ribosomal clone libraries have revolutionized our ability to understand the microbiology of chronic wounds and may revolutionize our approach to the use of antimicrobial agents in the therapy of chronic wounds. These new approaches are based on a full investigation of the microflora within a wound and they identify the large number of organisms that are fastidious or noncultivable (Martin *et al.,* 2010).

In summary, the DGGE sequencing technique is a useful research method for

investigation of microorganism populations. In clinical settings it can be valuable to investigate infections caused by bacteria with unusual growth requirements, for patients who have been unsuccessfully treated with antibiotics or who suffer from clinical bacterial infections that cannot be cultured. The time necessary to perform the technique and also some limitations mentioned above make this technique difficult to place within rapid identification methods and within clinical laboratory settings.

5.6 Conclusions

In this study, the bacterial diversity of wounds and healthy feet was assessed using conventional culture and PCR-DGGE sequencing technique. It allowed a comparison of isolation methods to a culture independent DNA profiling technique. In some cases, bacteria indicated on DGGE gels was not detectable by culture. The assessment of bacterial diversity is important, especially in chronic wounds containing biofilms. Based on the results of this study and recent studies found in the literature the panel of clinically relevant microorganisms would be selected for future development of a multiplex RT-PCR assay: *S. aureus*, *E. coli*, *S. epidermidis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Streptococcus* sp and *Enterobacter hormaechei.*

Both DGGE and culture techniques have distinct characteristics: culture methods are easy to implement, are cost-effective and can identify dominant culturable pathogens. However they are limited by the culturability of target bacteria. PCR-DGGE sequencing can be used to analyse complex bacterial communities and can identify unculturable organisms which may only represent a little as 1% of the total bacterial population. DGGE is a time-consuming technique and is expensive due to the fact that bands at the same position within a gel may represent different species and to fully assess the microflora present within the sample all distinct bands should be analysed

and sequenced, thus rapid and a less expensive method to analyse the diversity of microflora such as multiplex RT-PCR should be developed and employed.

CHAPTER 6

General discussion and future work

6.1 General Discussion

Diagnostic results to a great extent depend on the quality of the sample received in the laboratory and the methods used to identify microorganisms. Poorly collected and transported samples fail to isolate causative microorganisms and may recover contaminants and normal flora. In this study, the aspect of sample collection and transport was investigated as well as the conventional and molecular approaches for bacterial identification and quantification. The objective of the first part of the study was to investigate the most appropriate swab transport system in terms of the ability of the swab to maintain viability of the most common wound bacteria during transport. Additionally, swabs were tested for their performance with molecular methods in order to choose the most appropriate swab for further *in vivo* studies on patients and healthy

volunteers. Four different swab systems with different media formulations were evaluated (dry Sigma Swab, Amies Healthlink Transporter, Amies Sigma Transwab and eSwab). The recovery studies revealed that all swabs met acceptance criteria and were able to maintain the viability of *S. aureus, E. coli* and *Bacteroides fragilis* over 48h. However, molecular analysis of the swabs confirmed that the Sigma dry swab performed best with DNA extraction and PCR reaction, providing sufficient DNA extraction for both *S. aureus* and *E. coli*. This is an important finding, especially for samples with low concentrations of microorganisms as insufficient extraction of DNA may lead to lack of an identification of the causative microorganism. Also, it is important when the quantitative diagnostic results are required or for research studies involving quantitative molecular techniques. Based on the results of this study, the Sigma dry swab remained the best choice for further *in vivo* studies involving both conventional and molecular techniques for identification and quantification of bacteria.

In the next part of the study, the diagnostic validity of the semi-quantitative and quantitative swab and biopsy cultures was investigated to establish the best method of culturing using MRSA as an indicator organism and known pathogen. This was an additional advantage of the study as most of the previous studies in the literature correlated semi-quantitative and quantitative methods using total bacterial counts to look at the ability of the method to detect and quantify bacteria. The quantitative MRSA count analysis disproved the hypothesis that wound infection exists when bacterial load excees 10^5 cfu/cm^2. None of the patients developed infection despite the fact that 54% of chronic wounds had a MRSA count of 10^5 or more cfu/ cm^2, which suggests that bacterial load is not an important factor in diagnosing chronic wound infection. A diagnosis of chronic wound infection should be based on the assessment of the clinical signs of infection.

My findings demonstrate a statistically strong correlation between semi-quantitative and quantitative swab and semi-quantitative and quantitative biopsy culture. There was a weak correlation observed between semi-quantitative swab and quantitative biopsy culture. Semi-quantitative methods can be successfully used and employed in the laboratory settings for the management of chronic wounds. However, when time is important, rapid methods should be used. Thus a RT- PCR assay for the direct and rapid detection of MRSA from wound swabs was developed and compared with conventional methods in the next part of the study. The sensitivity of the developed assay was 83.3% and the specificity was 88.5%. Our quantitative analysis revealed that the average difference between the MRSA count obtained using RT-PCR and conventional culture results was 0.61 log. These findings show the potential of RT-PCR assay in rapid detection and quantification of MRSA, however the assay should be further optimised to achieve higher sensitivity and specificity. The RT-PCR assay for MRSA in this study is the first step in developing multiplex RT-PCR for the most common wound pathogens. In order to achieve it in the future, the diversity of microflora in chronic wounds was investigated using PCR-DGGE sequencing and compared with conventional culture results. DGGE allowed the identification of a number of strains not detected by culture techniques with 43% of the DGGE fragment sequences representing organisms not cultured from the wounds from which they had been amplified. This highlights the fact that a significant number of bacteria were not detected by culture methods alone. The most common bacteria found in chronic wounds identified using PCR-DGGE sequencing were *S. aureus*, *E. coli*, *S. epidermidis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp, *Streptococcus* sp and *Enterobacter* sp. Based on these results, multiplex RT-PCR will be developed for chronic wound pathogens future work (Section 6.2). Previous studies have compared conventional culture to molecular methods (Dowd *et al*., 2008; Martin *et al*., 2010; Rhoads *et al.,*

2012) and agreed that molecular tests are more sensitive than culture testing and hold promise for improving patient care. This present study adds to this body of work.

In conclusion, it is important to further our understanding of chronic wound healing from a biofilm concept approach as we begin to appreciate that many disease aetiologies are now more complex than once thought in medicine, that is the 'Koch's postulates' approach. A better understanding of the impact of certain bacterial communities in chronic wounds and delayed wound healing is necessary. Currently, wound management is based on principles of reducing bacterial load and preventing infection (Fonder *et al.*, 2008) however, the complexity of the wound environment makes it likely that antimicrobial therapy could result in unintended consequences. To date there is little data on the microbiological response to antimicrobial wound therapies. The application of proper and rapid diagnostic methods to analyse wound microbial communities with respect to clinical outcomes and therapeutic interventions (particularly antibiotic treatments) will provide critical insights into the roles of microorganisms in wound healing and the impacts of wound therapies.

6.2 Future work

The first step in obtaining diagnostic results is a proper collection and transportation of the sample to the laboratory. There is now a wide range of swab transport systems available, thus it is important to choose an appropriate transport device, which is able to maintain the viability of bacteria during transport and does not interfere with the diagnostic method. Future studies will concentrate on further assessment of the swabs performance with DNA extraction methods and PCR. More swabs with different media formulations will be tested with a range of aerobic, facultative and anaerobic bacteria commonly found in chronic wounds for their

performance with DNA extraction and PCR. The possible interference from the transport media and their detection sensitivity will be investigated.

Future studies will also investigate the impact of the treatments on the correlation between semi-quantitative and quantitative swab and biopsy culture using not only conventional methods but also molecular approaches such as multiplex RT-PCR. This will allow us to look at the diversity of the microfora in wounds and the impact of treatment on the correlation between the surface microflora and deep tissue structures of the wound. In relation to the novel wound healing theory suggesting microbial imbalance and synergistic relationships between bacteria to be the main cause of non-healing wounds, future studies should concentrate on deeper investigation of molecular microbiological approaches for bacterial population analysis. Rapid techniques such as multiplex real-time PCR identification methods for MRSA and other most common wound bacteria selected based on the results of this study will be developed and established.

There are no statistically significant studies able to conclusively and consistently show a relationship between clinical outcome and the microbial composition of a chronic wound. Thus, the importance of certain bacterial strains in wounds should be investigated to the greater extent. The literature in this area is very limited and this work will contribute to a wider understanding of the microbial colonisation of wounds, the impact of microorganisms on wound healing. The development of rapid diagnostic methods to investigate the diversity of microflora in wounds will contribute to the effectiveness of the treatment and will reduce the time to obtain correct diagnosis, therefore it will contribute to better wound management. Appropriate management of the wound is important to reduce the likelihood of wound infection which may lead to bone infection (osteomyelitis) and bacteraemia.

Appendices

Appendix 1

Table 1 *Staphylococcus* species identified in samples collected from the sole of the right and left foot from healthy participants and processed using conventional culture methods (Chapter 5). ng: no growth; TBC: Total Bacterial Count; Bacterial count – cfu/cm^2.

Patient number	Gender	Right/Left foot	TBC	*S. aureus*	*S.epidermidis*	*S. cohnii*	*S. warneri*	*S. caprae*	*S. hominis*	*S. capitis*	*S. sciuri*	Micrococcus
1	**F**	right	3.30×10^6	**ng**	1.40×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
		left	2.56×10^6	**ng**	8.00×10^3	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
2	**F**	right	3.76×10^6	**ng**	**ng**	1.60×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	6.80×10^4
		left	7.30×10^5	**ng**	**ng**	2.00×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
3	**F**	right	2.04×10^7	6.00×10^4	**ng**	**ng**	4.20×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
		left	2.64×10^7	6.00×10^4	**ng**	**ng**	2.38×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
4	**M**	right	6.50×10^6	**ng**	8.80×10^5	6.20×10^5	2.34×10^6	**ng**	**ng**	**ng**	**ng**	**ng**
		left	1.34×10^6	**ng**	3.40×10^5	2.40×10^5	4.60×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
5	**M**	right	3.42×10^6	**ng**	5.00×10^5	**ng**	1.40×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
		left	1.38×10^6	**ng**	2.80×10^5	**ng**	5.60×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
6	**M**	right	2.52×10^6	**ng**	1.60×10^4	**ng**	**ng**	3.80×10^3	**ng**	**ng**	**ng**	**ng**
		left	8.40×10^5	**ng**	1.00×10^4	**ng**	**ng**	1.00×10^4	**ng**	**ng**	**ng**	**ng**

Table 1 (Continuation) *Staphylococcus* species identified in samples collected from the sole of the right and left foot from healthy participants and processed using conventional culture methods (Chapter 5). TBC: Total Bacterial Count; Bacterial count – cfu/cm^2.

Patient number	Gender	Right/Left foot	TBC	*S. aureus*	*S. epidermidis*	*S. cohnii*	*S. warneri*	*S. caprae*	*S. hominis*	*S. capitis*	*S. scuri*	Micrococcus
7	**F**	right	4.72×10^5	**ng**	**ng**	1.10×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	3.80×10^4
		left	3.47×10^5	**ng**	**ng**	4.20×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	1.40×10^4
8	**F**	right	1.42×10^6	**ng**	1.20×10^6	**ng**	8.80×10^4	**ng**	**ng**	**ng**	**ng**	**ng**
		left	6.80×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	6.00×10^3
9	**F**	right	3.30×10^5	**ng**	**ng**	**ng**	4.00×10^3	**ng**	2.00×10^4	**ng**	**ng**	**ng**
		left	2.00×10^5	**ng**	**ng**	**ng**	8.00×10^3	**ng**	**ng**	**ng**	**ng**	**ng**
10	**F**	right	1.07×10^6	**ng**	9.90×10^5	**ng**	7.20×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
		left	3.80×10^5	**ng**	1.96×10^5	**ng**	1.84×10^5	**ng**	**ng**	**ng**	**ng**	**ng**
11	**M**	right	1.16×10^5	**ng**	1.12×10^5	**ng**	4.00×10^3	**ng**	**ng**	**ng**	**ng**	**ng**
		left	1.82×10^5	**ng**	1.56×10^5	**ng**	2.60×10^4	**ng**	**ng**	**ng**	**ng**	**ng**
12	**M**	right	6.30×10^5	**ng**	1.40×10^4	**ng**	**ng**	**ng**	4.00×10^3	**ng**	**ng**	**ng**
		left	3.06×10^5	**ng**	4.40×10^4	**ng**	**ng**	**ng**	6.00×10^5	**ng**	**ng**	**ng**
13	**M**	right	2.00×10^5	**ng**	2.20×10^4	2.80×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	1.20×10^4
		left	1.36×10^5	**ng**	8.20×10^4	2.20×10^4	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**

Table 1 (Continuation) *Staphylococcus* species identified in samples collected from the sole of the right and left foot from healthy participants and processed using conventional culture methods (Chapter 5). TBC: Total Bacterial Count; Bacterial count – cfu/cm^2.

Patient number	Gender	Right/Left foot	TBC	*S. aureus*	*S. epidermidis*	*S. cohnii*	*S. warneri*	*S. caprae*	*S. hominis*	*S. capitis*	*S. scuri*	Micrococcus
14	**F**	right	7.46×10^5	**ng**	1.10×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
		left	5.48×10^5	**ng**	1.42×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
15	**F**	right	4.20×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	3.30×10^5	**ng**	**ng**	**ng**
		left	3.14×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	3.00×10^5	**ng**	**ng**	**ng**
16	**F**	right	1.66×10^5	**ng**	**ng**	**ng**	3.40×10^4	**ng**	**ng**	5.20×10^4	**ng**	**ng**
		left	2.62×10^5	**ng**	**ng**	**ng**	5.80×10^4	**ng**	**ng**	6.40×10^4	**ng**	**ng**
17	**F**	right	9.80×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	9.80×10^5	**ng**	**ng**
		left	1.06×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	1.06×10^6	**ng**	**ng**
18	**M**	right	2.42×10^5	**ng**	1.30×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	1.14×10^5	**ng**
		left	2.90×10^5	**ng**	2.24×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	1.80×10^4	**ng**
19	**M**	right	5.03×10^6	**ng**	5.03×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
		left	2.75×10^6	**ng**	2.75×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
20	**M**	right	1.28×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
		left	3.42×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**

Table 2 Bacterial strains identified in the samples collected from the sole of the right and left foot from healthy participants and processed using conventional culture methods (Chapter 5). Bacterial count – cfu/cm^2.

Patient number	**Right/Left foot**	**Gender**	***C. pelliculosa***	***Cryptococcus terreus***	***Candida cifferrii***	***Candida farmata***	***Kocuria varians***
1	**right**	**F**	8.00×10^4	3.80×10^5	**ng**	**ng**	**ng**
	left		2.20×10^5	3.40×10^4	**ng**	**ng**	**ng**
2	**right**	**F**	**ng**	**ng**	6.00×10^3	**ng**	**ng**
	left		**ng**	**ng**	8.00×10^3	**ng**	**ng**
4	**right**	**M**	**ng**	**ng**	**ng**	6.40×10^4	**ng**
	left		**ng**	**ng**	**ng**	**ng**	**ng**
7	**right**	**F**	**ng**	**ng**	4.00×10^4	**ng**	**ng**
	left		**ng**	**ng**	1.00×10^4	**ng**	**ng**
8	**right**	**M**	**ng**	**ng**	5.00×10^5	**ng**	**ng**
	left		**ng**	**ng**	5.00×10^4	**ng**	**ng**
9	**right**	**F**	8.60×10^4	**ng**	**ng**	**ng**	**ng**
	left		6.40×10^4	**ng**	**ng**	**ng**	**ng**
20	**right**	**M**	**ng**	**ng**	**ng**	**ng**	1.28×10^5
	left		**ng**	**ng**	**ng**	**ng**	3.42×10^5

Table 3 Bacterial strains identified in samples collected from chronic wounds and processed using conventional culture methods (Chapter 5).

Patient number	**Bacterial count cfu/cm^2**								
	TBC	**Coliforms**	**MRSA**	**MSSA**	***S. epidermidis***	**CNS**	***Streptococcus* sp**	**Anaerobes**	***Pseudomonas* sp**	***Proteus mirabilis***
1	7.72×10^6	1.14×10^6	1.06×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
2	5.77×10^6	**ng**	5.77×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
3	1.28×10^7	5.26×10^6	7.44×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
4	4.79×10^6	**ng**	**ng**	4.40×10^4	3.02×10^5	**ng**	8.61×10^6 Strep. Gr D	**ng**	**ng**	**ng**
5	7.40×10^4	**ng**	**ng**	4.00×10^3	**ng**	**ng**	2.00×10^4 Strep. Gr A	**ng**	**ng**	**ng**
6	2.12×10^5	**ng**	2.12×10^5	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
7	ng	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
8	1.80×10^3	**ng**	**ng**	**ng**	1.80×10^3	**ng**	**ng**	**ng**	**ng**	**ng**
9	4.29×10^6	**ng**	**ng**	**ng**	4.29×10^6	**ng**	**ng**	**ng**	**ng**	**ng**
10	1.98×10^6	5.40×10^5	**ng**	**ng**	6.00×10^4	**ng**	**ng**	**ng**	**ng**	**ng**

Table 3 (Continuation) Bacterial strains identified in samples collected from chronic wounds and processed using conventional culture methods (Chapter 5).

Patient number	**Bacterial count cfu/cm^2**									
	TBC	**Coliforms**	**MRSA**	**MSSA**	***S. epidermidis***	**CNS**	***Streptococcus* sp**	**Anaerobes**	***Pseudomonas* sp**	***Proteus mirabilis***
11	8.00 x 10^4	2.50 x 10^3	**ng**	**ng**	**ng**	1.20 x 10^2	**ng**	1.80 x 10^3	**ng**	**ng**
12	5.80 x 10^7	5.40 x 10^5	**ng**	**ng**	**ng**	ng	**ng**	**ng**	**ng**	5.80 x 10^7
13	2.40 x 10^5	**ng**	**ng**	**ng**	**ng**	2.40 x 10^5	**ng**	**ng**	**ng**	**ng**
14	2.80 x 10^4	**ng**	**ng**	**ng**	**ng**	2.80 x 10^4	8.61 x 10^6 Strep. Gr D	**ng**	**ng**	**ng**
15	3.52 x 10^7	**ng**	**ng**	**ng**	**ng**	ng	2.00 x 10^4 Strep. Gr A	**ng**	3.53 x 10^7	**ng**
16	2.64 x 10^6	2.60 x 10^4	**ng**	**ng**	**ng**	1.20 x 10^2	**ng**	**ng**	**ng**	**ng**
17	7.20 x 10^6	2.00 x 10^3	**ng**	**ng**	**ng**	2.60 x 10^5	**ng**	**ng**	**ng**	**ng**
18	4.40 x 10^4	**ng**	**ng**	**ng**	**ng**	4.40 x 10^4	**ng**	**ng**	**ng**	**ng**
19	2.00 x 10^3	1.30 x 10^6	**ng**	**ng**	**ng**	ng	**ng**	**ng**	**ng**	**ng**
20	2.80 x 10^4	**ng**	**ng**	**ng**	**ng**	2.80 x 10^4	**ng**	**ng**	**ng**	**ng**
21	2.38 x 10^5	6.00 x 10^3	**ng**	**ng**	**ng**	1.60 x 10^4	2.48 x 10^5	**ng**	**ng**	**ng**

Table 3 (Continuation) Bacterial strains identified in samples collected from chronic wounds and processed using conventional culture methods (Chapter 5).

Patient number	**Bacterial count cfu/cm^2**									
	TBC	**Coliforms**	**MRSA**	**MSSA**	***S. epidermidis***	**CNS**	***Streptococcus* sp**	**Anaerobes**	***Pseudomonas* sp**	***Proteus mirabilis***
22	5.80×10^4	**ng**	**ng**	6.00×10^3	**ng**	3.80×10^4	**ng**	**ng**	**ng**	**ng**
23	6.35×10^5	**ng**	**ng**	**ng**	2.40×10^5	3.80×10^5	**ng**	**ng**	**ng**	**ng**
24	5.48×10^5	1.72×10^5	**ng**	**ng**	**ng**	**ng**	3.77×10^5	**ng**	**ng**	**ng**
25	9.40×10^4	3.40×10^4	**ng**	**ng**	**ng**	5.20×10^4	**ng**	**ng**	**ng**	**ng**
26	9.00×10^5	6.00×10^4	**ng**	**ng**	**ng**	1.50×10^5	**ng**	**ng**	**ng**	**ng**
27	1.18×10^5	1.60×10^4	**ng**	**ng**	**ng**	1.20×10^4	5.60×10^4 Strep. Gr B	**ng**	**ng**	**ng**
28	4.00×10^3	1.90×10^2	**ng**	**ng**	**ng**	9.80×10^3	**ng**	**ng**	**ng**	**ng**
29	3.89×10^6	**ng**	3.50×10^6	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
30	9.20×10^3	2.90×10^2	**ng**	**ng**	**ng**	8.90×10^3	**ng**	**ng**	**ng**	**ng**
31	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**	**ng**
32	1.30×10^2	**ng**	1.30×10^2	**ng**	**ng**	**ng**	2.48×10^5	**ng**	**ng**	**ng**

Appendix 2

Bacteriology of chronic wounds – Protocol – (Chapter 4 and 5)

PROTOCOL

VERSION NUMBER:	Issue 2
ISSUE DATE:	27 November 2010
TITLE:	Bacteriology of chronic wounds
SPONSOR:	
CHIEF INVESTIGATOR:	Dr Frank Lee Bowling
PROJECT START DATE:	03 January 2011
PROJECT FINISH DATE:	29 April 2011

Bacteriology of wounds

1. Background:

Chronic wounds are a repository of complex polymicrobial populations, including both aerobic and anaerobic species. There is evidence, that the microfloras of these wounds play a role in the healing process, althrough there is still considerable debate as to the importance of individual species or microbial density in relation to healing and subsequently to chronic wound management. Recent evidence suggest that Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasing problem amongst patients with chronic wounds. This bacteria can cause serious wound infection. Clinical microbiology laboratories still depend on phenotypic assays for both identification and antibiotic resistance testing of organisms. Currently it requires 24h to 48h to definitively identify an MRSA culture by conventional methods. The rapid and accurate identification of MRSA in clinical specimens has important implications for the therapy and management of both colonised and infected patients. Numerous molecular approches that reduce the time for identification of MRSA has been described, however the molecular tests developed to date for the detection of MRSA cannot be applied for the direct detection from clinical specimens, without previous time consuming isolation, capture or enrichment of MRSA.

In environmental microbiology, denaturing gradient gel electrophoresis (DGGE) has been used as a tool for profiling complex microbial populations without the biases of cultural analysis for many years and the technique has now been applied to the study of a limited number of human microbial populations. The advantage of this approach is that it creates a genetic fingerprint or profile of total community diversity by separating mixed 16S rRNA PCR amplification products on the basis of their sequence melting behavior. In this study we will apply the use of DGGE and sequencing to the analysis of the chronic wound microflora. This will allow us to quickly and efficiently analyze the whole bacterial population of individual wounds by using a single PCR and to run samples alongside one another on the same gel for direct comparison. In this way, it will be possible to concurrently obtain considerable information about the species composition of multiple wounds (40 in all).

2. Study objectives:

The aim of this study is to develop quantitative RT-PCR assay for direct detection of MRSA from wound swabs and comparison of this method with quantitative conventional tests. Additionally, the diversity of microflora in chronic wounds will be assessed by developing the Denaturating Gradient Gel Electrophoresis (DGGE) sequencing method. Bacterial population analysis of wounds has been recently reported (James *et al*., 2008) but the bacterial diversity of wounds has not. The literature in this area is very limited, thus our work will contribute to a wider understanding of the microbial colonisation of wounds using both conventional and molecular techniques.

Trial Design:

3.1 Design

This will be a prospective, sequential trial in male and female patients.

3.2 Endpoints
The primary trial endpoint will be the successful collection of suitable microbiological samples by the clinician.

- **Patient population**

4.1 Number
A total of 40 adults aged 18-75 will be included in the study.

4.2 Patient Inclusion criteria
Patients aged over 18 years who have provided written informed consent.
Patients with variety of non-infected chronic wounds will be included in the study.

4.3 Patient Exclusion criteria
- Patients under the age of 18 years
- Patients unable to comply with study procedures
- Immunosuppressed patients
- In the opinion of the Investigator, a patient who is not likely to complete the trial for whatever reason

4.4 Withdrawal
Patients may withdraw their consent to participating in the trial at any time for any reason, without stating that reason or compromising the medical care they will receive.

The investigator may withdraw patients from the trial if they are not compliant with the trial protocol or to protect patients' safety and well-being.

5. Plan of Investigation

A total of 40 patients with chronic wounds will be recruited to the study from Manchester Diabetes Centre, the Manchester Foot Hospital or in-patients at the Manchester Royal Infimary.
The patients will be appropriately informed and consented for the trial.
In the first instance the wound will be cleaned with sterile saline. The number of superficial swabs will be taken by Sigma dry swab (Medical Wire &Equipment Co. Ltd., Corsham, England) using rolling the zigzag method.

- One swab will be tested using conventional microbiological culture technique.
- Second swab will be used for quantitative RT-PCR.
- Third swab will be used for bacteria population analysis DGGE, sequencing.

All samples will not have patients identifiers attached, only anonymous randomised numbers will be attached to the sample. A batch of samples will then be prepared for transport to the laboratory for testing.
The samples will be forwarded to the testing laboratory Manchester Metropolitan University Department of Microbiology for conventional culturing, RT-PCR and DGGE, sequencing testing. This will involve the detailed identification of the bacterial species present but will not involve the analysis of any patient derived human DNA.

6. AE, SAE and ADR Reporting

– Adverse events (Aes)

An adverse event (AE) is any untoward medical occurance in a volunteer or clinical subject to whom a pharmaceutical product has been administered, which does not necessarily have a casual relationship with this treatment. As no materials are being applied or administered to the patient no Aes are anticipated.

– Severity of Adverse Events

As no materials are being applied or administered to the patient no SAEs are anticipated.

7. Statistical Design

7.1 Sample size

40 patients will be recruited into the study.

7.2 Data handling

Trial data will be double data entered onto the password protected database. Data will be validated using electronic and manual checks.

8. Additional Information

8.1 Protocol Amendments

No changes to the protocol will be initiated without prior written approval of the relevant independent ethics committee of an appropriate amendment.The only exception being when the change is necessary to eliminate immediate hazards to the patients or when the change involves only logistical or administartive aspects of the trial.

9.Ethical and Legal Issues

9.1 Ethical Guidelines

This trial will be conducted according to the Declaration of Helsinki (1996), with local laws and regulations relevant to the use of new therapeutic agents in the country of conduct, and in strict compliance with this protocol.

9.2 Informed Consent

The written, informed consent of patients to parcitipate in the trials must be obtained before any trial specific procedures are undertaken. The information supplied to potential trial patients and the consent form will be in accordance with the requirements in ICH GCP and will have been approved by Independant Ethics Committee/ Institutional Review Board.

9.3 Patient Confidentiality

Patient's confidentiality will be respected at all times. Patients will not be referred to by name in any trial documentation outside of the Invesigator site. Patients will not be referred to by name in any trial report.

Appendix 3

Bacteriology of chronic wounds - Patient Information Sheet

Participant Information Sheet

Title of Study: "Bacteriology of chronic wounds"

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others. Please ask us if anything is not clear or if you would like to have more information and take time to decide whether or not you wish to take part. Thank you for reading this information sheet.

What is the purpose of this study?
The aim of the study is to develop ways of identifying Methicillin resistant *Staphylococcus aureus* (MRSA) in samples collected from open wounds (taken by wiping the wound with a foam swab) and to compare this test with other more conventional tests that are often used to detect MRSA. Additionally, the presence of different bacteria (Bugs) in chronic wounds will be assessed by developing novel bacterial identification methods.

Why have I been chosen?
You have been invited to take part in this study, because we require volunteers aged between 18-75 years with variety of chronic (long standing) wounds colonized with multiple bacteria.

Who is organising the study?
The study is being organised by Manchester Metropolitan University.

What will the study involve?
You will be involved in the research for one hour once you have given consent. During this visit the clinician will collect two samples (wound swabs) from your wound by wiping a very soft foam swab across the wound.
All methods will be demonstrated to you by the research team and clinician. This will take approximately 10 minutes.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be asked to sign a consent form acknowledging that you understand what is involved in taking part. If you take part you are still free to withdraw without giving a reason.

What are the possible RISKS/HARMS of taking part?
There are no risks or harms to you in taking part in this study. We collect the samples using very gentle and soft foam swab transport systems, which are routinely used in hospitals.

What are the possible benefits of taking part?
We cannot promise that the study will help you, but the information we get from this study may help to improve the detection of MRSA in the future.

Who will know about my taking part?
All information collected during this study will be kept confidential and will have your name removed so you cannot be identified. No hospital records will be accessed for this study.

Thank you for your time and interest in this study. If you require any further information please contact:

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Appendix 4

Bacteriology of chronic wounds - Consent form

Version 3 14 February 2011

Centre Number:

CONSENT FORM

Title of Project: Bacteriology of chronic wounds

Name of researcher: Monika Stuczen

Please initial box

1. I confirm that I have read and understand the information sheet dated 14 February 2011 (version 3) for the above study. ☐

2. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from Manchester Metropolitan University, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to take part in the above study. ☐

________________________ ______________ ______________________

Name of participant Date Signature

______________________ ______________ ______________________

Name of person taking consent Date Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 5

The Diversity of Microflora in Healthy Feet - Protocol

PROTOCOL

VERSION NUMBER: Issue 1

ISSUE DATE: 10 September 2010

TITLE: The diversity of microflora in healthy foot

SPONSOR:

CHIEF INVESTIGATOR: Professor Valerie Edwards-Jones
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PhD STUDENT: Monika Stuczen
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Department of Microbiology
Manchester Metropolitan University
John Dalton Building
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PROJECT START DATE: 15 October 2010

PROJECT FINISH DATE: 30 November 2010

Bacteriology of wounds

1. Background:

The skin is colonised by a variety of bacteria which form its natural microflora. Disruption to the normal barrier function of the skin may result in invasion of the dermis by opportunistic bacteria. To date, these organisms, which may contribute to the

chronicity of skin wounds, have been analysed solely by culture methods. It is increasingly realized that standard culture methods of analysis do not accurately reflect the full diversity of complex microflora. In this study we will apply the use of DGGE and sequencing to the analysis of the skin microflora. This will allow us to quickly and efficiently analyze the whole bacterial population of individual samples by using a single PCR and to run samples alongside one another on the same gel for direct comparison. In this way, it will be possible to concurrently obtain considerable information about the species composition of multiple samples. In environmental microbiology, denaturing gradient gel electrophoresis (DGGE) has been used as a tool for profiling complex microbial populations without the biases of cultural analysis for many years and the technique has now been applied to the study of a limited number of human microbial populations. The advantage of this approach is that it creates a genetic fingerprint or profile of total community diversity by separating mixed 16S rRNA PCR amplification products on the basis of their sequence melting behavior.
This study is a pilot study to develop and optimise methods for larger clinical trial on variety of chronic wounds.

2. Study objectives:

The aim of this study is to develop the Denaturating Gradient Gel Electrophoresis (DGGE) sequencing method (using samples collected from healthy volunteers) for a larger clinical trial involving NHS patients.

3. Trial Design:

3.1 Design

This will be a prospective, squential trial in male and female volunteers.

3.2 Endpoints

The primary trial endpoint will be the successful collection of suitable microbiological samples by the PhD student.

4. Patient population

4.1 Number

A total of 20 adults aged 18-75 will be included in the study.

4.2 Patient Inclusion criteria

Volunteers aged over 18 years who have provided written informed consent.
No clinical signs of infection distal to the medial and lateral mallali.

4.3 Patient Exclusion criteria

- the presence of foot ulceration
- fungal or bacterial conditions including tinea pedis (athlete's foot)
- antibiotic treatment
- immunosuppressed

4.4 Withdrawal

Volunteers may withdraw their consent to participating in the trial at any time for any reason, without stating that reason or compromising the medical care they will receive.

The investigator may withdraw volunteers from the trial if they are not compliant with the trial protocol or to protect volunteers' safety and well-being.

5. Plan of Investigation

A total of 20 healthy volunteers aged 18-75 years will be included in the study. The volunteers will be appropriately informed and consented for the trial. Four samples will be taken from each volunteer – two swabs from the sole of each foot. The sole of the foot will be initially cleaned using sterile saline and then swabbed using zig-zag method.
One swab from each foot will be processed using conventional microbiological methods. Bacterial DNA will be extracted from second swab and run through DGGE-sequencing method.
Qualitative microbiology will be performed by inoculating the samples onto a variety of selective media and identifying bacteria after incubation using standard microbiological methods.

The cultural method will be compared with molecular methods (DGGE, sequencing) population studies to study bacterial diversity in normal, intact skin.
All samles will not have volunteers identifiers attached, only anonymous randomised numbers will be attached to the sample. A batch of samples will then be prepared for transport to the laboratory for testing.

6. AE, SAE and ADR Reporting

6.1 Adverse events (Aes)

An adverse event (AE) is any untoward medical occurance in a volunteer or clinical subject to whom a pharmaceutical product has bee administered, which does not necessarily have a casual relationship with this treatment. As no materials are being applied or administered to the volunteers no Aes are anticipated.

6.2 Severity of Adverse Events

As no materials are being applied or administered to the volunteers no SAEs are anticipated.

7. Statystical Design

7.1 Sample size

20 volunteers will be recruited into the study.

7.2 Data handling

Trial data will be double data entered onto the password protected database. Data will be validated using electronic and manual checks.

8. Additional Information

8.1 Protocol Amendments

No changes to the protocol will be initiated without prior written approval of the relevant independent ethics committee of an appropriate amendment. The only exception being when the change is necessary to eliminate immediate hazards to the volunteers or when the change involves only logistical or administartive aspects of the trial.

9.Ethical and Legal Issues

9.1 Ethical Guidelines

This trial will be conducted according to the Declaration of Helsinki (1996), with local laws and regulations relevant to the use of new therapeutic agents in the country of conduct, and in strict compliance with this protocol.

9.2 Informed Consent

The written, informed consent of volunters to parcitipate in the trials must be obtained before any trial specific procedures are undertaken. The information supplied to potential trial volunteer and the consent form will be in accordance with the requirements in ICH GCP and will have been approved by Independant Ethics Committee/ Institutional Review Board.

9.3 Patient Confidentiality

Volunteer's confidentiality will be respected at all times. Volunteers will not be referred to by name in any trial documentation outside of the Invesigator site. Volunteers will not be referred to by name in any trial report.

Appendix 6

The Diveristy of Microflora in Healthy Feet - Participant Information Sheet

Participant Information Sheet

Title of Study: "The diversity of microflora in healthy feet"

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and discuss it with others. Please ask us if anything is not clear or if you would like to have more information and take time to decide whether or not you wish to take part. Thank you for reading this information sheet.

What is the purpose of this study?
The aim of this study is to investigate the diversity of normal microflora in healthy feet using conventional microbiological methods and molecular techniques (Denaturating Gradient Gel Electrophoresis, sequencing). Both techniques will be compared together to look for their accuracy in identifying bacteria.

Why have I been chosen?
You have been invited to take part in this study, because we require healthy volunteers aged 18-75 years without any foot problems. Exclusion criteria for the study include: active foot ulceration, bacterial or fungal conditions including athlete's foot, immunosuppression medication, recent or current antibiotic treatment. If any of these apply, we ask that you do not take part in this study. You do not have to give a reason. You may withdraw from the study at any time without reason.

Who is organising the study?
The study is being organised by Manchester Metropolitan University.

What will the study involve?
You will be involved in the research for one hour once you have given consent. During this visit you will be asked to provide 2 samples (superficial swabs) from the sole of each foot.
All methods will be demonstrated to you by the research team. This will take approximately 10 minutes.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be asked to sign a consent form. If you take part you are still free to withdraw without giving a reason.

What are the possible risks of taking part?

There are no risks to you in taking part in this study. We collect the samples using very gentle and soft foam swab transport systems, which are routinely used in hospitals.

What are the possible benefits of taking part?

The skin is colonized by a variety of bacteria which form its natural microflora. Disruption to the normal barrier function of the skin may result in invasion of the dermis by opportunistic bacteria. To date, these organisms, which may contribute to the chronicity of skin wounds, have been analysed solely by culture methods. In our study we are going to compare conventional culture methods routinely used in clinical laboratories with more complex molecular techniques. It is increasingly realized that standard culture methods of analysis do not accurately reflect the full diversity of complex foot microflora.

What do I have to do?

Should you decide to take part, you will be asked to remove your shoes and socks. The sole of the foot will be cleaned by sterile saline. Two samples will be taken from each foot by swabbing the sole of the foot.

After sampling the foot will be wiped using dry paper tissue.

Who will know about my taking part?

All information collected during this study will be kept confidential and will have your name removed so you cannot be identified. No hospital records will be accessed for this study.

Thank you for your time and interest in this study. If you require any further information please contact:

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Appendix 7

The Diversity of Microflora in Healthy Feet - Consent form

Participant Consent Form

Manchester Metropolitan University

Title of Study: “The diversity of microflora in healthy feet.”

Please initial box

- I confirm that I have read and understand the information sheet dated 7 September 2010 [version 1] for the above study. ☐

- I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

- I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

- I have received sufficient information about this study ☐

- I agree to take part in the above study. ☐

______________________	____________	____________________
Name of participant	Date	Signature
______________________	____________	____________________
Name of witness	Date	Signature

When completed, 1 copy for participant; 1 (original) for researcher site file

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