Sheffield Hallam University

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Title/Thesis Number: MOLECULAR MICROBIAL ECOLOGY 27592

Degree: PHD PHILOSOPHY

Year: 2013

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Molecular microbial ecology of hospital ward environments

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A thesis submitted in partial fulfilment of the requirements of

Sheffield Hallam University

for the degree of Doctor of Philosophy

August 2013

Abstract

Evidence indicates that the hospital environment plays a role in hospital acquired infections (HAI). Previous studies have focused on outbreaks and the role of specific culturable pathogens. There is still considerable debate about the role of the environment and the importance of cleaning, therefore more comprehensive culture-independent microbial ecology studies are required.

This study aimed to utilise culture-independent methods to characterise the microbial communities in an orthopaedic ward and theatre environment under normal operating conditions, to determine the distribution of different microorganisms, their viability and persistence following routine cleaning.

Culture was used to quantify culturable bacteria present and select bacterial populations of interest. Antibiotic-resistance gene specific PCRs were used to investigate the presence of resistance determinants. PCR-DGGE was used to resolve fragments of the 16S rRNA gene from total DNA extractions and the resulting sequence data was used to identify microorganisms present. Cell viability was assessed using RNA as the template for reverse transcriptase PCR-DGGE.

Quantification of bacteria on environmental surfaces using culture indicated that near patient surfaces rarely exceeded the recommended 2.5 CFU/cm^2 limit, providing evidence that current cleaning regimes employed on these wards are sufficient to keep microbial contamination low. PCR-DGGE showed that sequences similar to *Staphylococci* dominated the environment, particularly *S. hominis*, and *S. haemolyticus*, which were retrieved from the floor before and immediately after cleaning. Antibiotic gene specific PCRs were used to demonstrate the presence of the *mecA* gene, aminoglycoside modifying enzyme genes and *qac* genes in isolated environmental non-aureus *Staphylococci*. This data has highlighted the potential role of this environment as a reservoir of pathogenic *Staphylococci*.

Using PCR-DGGE the orthopaedic wards yielded sequences from DNA and RNA templates similar to species associated with human skin and large intestine. While *Staphylococci* were shown to dominate most environmental sites, *Kocuria* and *Corynebacterium* species were found to be specifically associated with the bed rails. Few Gram negative species were detected by molecular methods or selective culture in the ward environment. Sequences similar to *Cupriavidus* and *Chryseobacterium* species were detected in the operating theatre environment using PCR-DGGE, in contrast using culture; *Staphylococci* were readily isolated from the theatre environment. PCR-DGGE was also used to investigate the presence of fungi in sink drains on an orthopaedic and intensive care ward. While *Fusarium* species were widely distributed on both wards, sequences similar to *Candida* were only detected in samples from the ICU wards using PCR-DGGE. When culture was used *Candida* species were readily grown from both environments. The differences between the species detected using culture and PCR-DGGE suggest that the two techniques should be used together to provide complementary data.

Overall PCR-DGGE has been used for direct identification of the dominant viable bacteria and fungi in the hospital environment, to provide an estimate the relative abundance of species, and give an overview of the effect of routine ward cleaning on the bacterial community.

Acknowledgements

I would like to acknowledge Sheffield Teaching Hospitals Trust for their permission to access the wards and operating theatre. I would also like to thank the Healthcare Infection Society for funding this study.

I would like to thank Professor Tom Smith and Dr Karen Stanley for their excellent supervision and our hospital collaborators Mr Bob Kerry, Dr Rob Townsend and Clare Mason for their assistance.

I would also like to acknowledge everyone in the BMRC, particularly the microbiology group. I am grateful to Sara Dunn for always being there, cheering me up during the tough times and making my time in the BMRC more enjoyable and memorable.

I am also extremely grateful to my parents and brother for their love and support.

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Abbreviations

AAC	Aminglycoside N-acetyltransferases
ACC	Aerobic colony count
AME	Aminoglycoside modifying enzyme
APH	Aminoglycoside O-phosphotransferases
ANOVA	Analysis of variance
ANT	Aminoglycoside O-nucleotidyltransferases
AT	Annealing temperature
BLAST	Basic local alignment search tool
BSAC	British Society for Antimicrobial Chemotherapy
cDNA	Complementary DNA
CCR	Cassette chromosome recombinase
CFU	Colony forming units
CLED	Cysteine Lactose Electrolyte Deficient
CNS	Coagulase-negative *Staphylococci*
CRE	Carbapenem resistant Enterobacteriaceae
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribo Nucleic Acid

EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended Spectrum Beta-Lactamase
GI	Gastrointestinal
HAI	Hospital Acquired Infection
HEPA	High efficiency particulate air
HPA	Health Protection Agency
ICU	Intensive Care Unit
IS	Insersion sequence
MDE	Multidrug-resistant Enterobacteriacea
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus*
MR-CNS	Methicillin Resistant CNS
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NGH	Northern General Hospital
PBP	Penicillin Binding Protein
PBS	Phosphate buffered saline

PCR	Polymerase Chain reaction
QAC	Quaternary ammonium compounds
qPCR	Quantitative PCR
RHH	Royal Hallamshire Hospital
RNA	Ribo Nucleic Acid
rRNA	Ribosomal RNA
SCC*mec*	Staphylococcal cassette chromosome
Sp.	Species
Spp.	Species (plural)
SSI	Surgical site infection
STH	Sheffield Teaching Hospitals
TAE	Tris base, acetic acid, EDTA
Tn	Transposon
UTI	Urinary Tract Infection
VRE	Vancomycin-resistant *Enterococci*

1 Introduction

It has been suggested that the hospital environment including chairs, tables, beds, curtains, sinks and medical equipment plays a role in hospital acquired infections (HAI). Previous studies in the hospital environment have focused on the role of specific culturable pathogens, for example methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and *Clostridium difficile.* However more comprehensive studies of the total microbial ecology of hospital environments are required.

Patients undergoing orthopaedic surgery that involves the insertion of a foreign object (e.g. pins or joint prostheses) are at particular risk of infection at the surgical site. Postoperative deep wound infections are a particular problem for these patients as they can lengthen hospital stays for orthopaedic patients and sometimes necessitate surgical revision of joint replacements, causing significant impact on the patient and hospital resources.

Characterisation of the environmental reservoir of microorganisms in hospital wards is therefore needed and effective methods are required to characterise it and to inform the infection control measures that are critical to minimising infection risks to patients.

1.1 Evidence for the role of the hospital environment in HAI

Since effective cleaning regimes reduce the incidence of infections such as *Clostridium difficile*, VRE, norovirus, and *Acinetobacter* spp., the hospital environment must play an important role as a source of infective microorganisms (Backman *et al.*,2012, Bergstrom *et al.*,2012, Breathnach *et al.*,2012, Doan *et al.*,2012, Muzslay *et al.*,2012, Dancer *et al.*,2009, Boyce,2007). Previous studies have shown that the risk of a patient acquiring an antibiotic resistant pathogen is increased if the room was previously occupied by a patient positive for that organism, highlighting the important role of the environment in hospital acquired infection (Nseir *et al.*,2011, Huang, Datta and Platt,2006).

A review by Boyce (2007) demonstrated that the potential for contaminated surfaces to contribute to hospital acquired infections within the hospital setting is dependant on a) the ability of potential pathogens remain viable on surfaces, b) the frequency with which these surfaces are touched by patients and staff, and c) the level of contamination on that surface (Boyce,2007).

Genera known to be associated with HAI able to survive for months in the environment include *Staphylococcus* (including MRSA), *Enterococcus* (including VRE), *Pseudomonas, Klebsiella, Escherichia* and *Shigella*. Overall Gram negative bacteria have been reported to persist longer and factors including high humidity, low temperature and the presence of protein or serum have been shown to improve survival (Kramer, Schwebke and Kampf,2006).

Recently Muzslay *et al.* (2012) investigated the environment of a medical and surgical gastrointestinal ward over five months. They found that VRE could be recovered from 2.7% of the environmental surfaces sampled (n=2,046) within the bay and isolation rooms tested. Sampling was conducted around 74 different patients and it was shown that 80% of the VRE contamination sites were found in the near-patient environment of a single patient not known to be positive for VRE. Stool analysis of this patient later confirmed that the patient was colonised with the same clone that was contaminating their near-patient environment. This study highlighted the fact that unrecognised colonisation, combined with inadequate cleaning lead to persistent widespread contamination and resulted in significant risk for acquisition of VRE (Muzslay *et al.*,2012).

1.1.1 The role of near patient sites

Sites frequently touched by hands present the greatest risk of infection for patients, particularly those situated right beside the patient (Dancer,2009, Oelberg *et al.*,2000, Rheinbaben *et al.*,2000). Examples of these sites include beds, bed rails, bedside equipment hoists, bedside lockers, over bed tables and chairs.

A review by Creamer and Humphreys (2008) summarised the currently available literature surrounding evidence for the role of patient beds. They report that bed frames, mattresses, linen and pillows have all been shown to be

contaminated with potential pathogens, contribute to outbreaks and are often not properly decontaminated (Creamer and Humphreys,2008). In addition the process of bed making has previously been shown to contribute to dispersal of pathogens into the air and contamination of the surrounding environment (Shiomori *et al.*,2002).

Brady *et al.* (2007) demonstrated frequent microbial contamination of bed-control handsets on multiple sampling occasions. Potential pathogens including CNS, MRSA and *Enterococci* were isolated from approximately 40% (n=70) of swab samples (Brady *et al.*,2007).

Dancer *et al.* (2008) screened two surgical wards weekly over two consecutive six month periods. They frequently recovered methicillin susceptible and resistant *Staphylococcus aureus* (MSSA/MRSA) from beds, lockers and tables. It was also found that higher loading with culturable microorganisms was associated with higher bed occupancy (Dancer, White and Robertson,2008).

Bhalla *et al.* (2004) evaluated the frequency of acquisition of pathogens on hands following contact with bed rails in occupied and unoccupied patient rooms. Investigators were able to demonstrate acquisition of one or more pathogen in 53% of occupied rooms (n=64) and 24% of unoccupied rooms (n=25), with MRSA and VRE most frequently isolated (Bhalla *et al.*,2004). Similarly Stiefel *et al.* (2011) provided evidence that hand contamination was equally common following contact with both patient skin and their immediate

environment (bed rails, over bed table and telephone) (Stiefel *et al.*,2011). These studies provide evidence that the environment contributes to the contamination of health care worker hands.

A recent study demonstrated low level of hand hygiene compliance among healthcare workers in a UK hospital. Through covert observations they were able to demonstrate that compliance with hand hygiene protocols was only 25% and that healthcare workers frequently handled the patient and their environment without washing their hands before or after (Smith *et al.*,2012). In addition Creamer *et al.* (2010) were able to recover MRSA from healthcare workers hands after hand hygiene procedures were performed highlighting that even when performed hand hygiene can be ineffective at removing or killing pathogens (Creamer *et al.*,2010).

1.1.2 The role of other frequently touched environmental sites

Within the hospital ward environment there are many sites that are further away from the patient than the near-patient sites identidied above, but are still regularly touched by patients, healthcare staff and visitors.

Wojgani *et al.* (2012) recently investigated microbial contamination on hospital door handles and correlated this with frequency of movement through the door. They found a significant correlation between the degree of movement through a

door and microbial contamination (detected using culture techniques). In addition door handle design contributed to contamination levels with lever handles showing highest contamination followed by pull handles (Wojgani *et al.*,2012). Similarly Oie *et al.* (2002) highlighted extensive MSSA and MRSA contamination of hospital door handles (Oie, Hosokawa and Kamiya,2002).

Patient files are frequently handled by healthcare workers, these are used to record notes about patients or to enter details about procedures and therefore it seems likely that these could act as reservoirs. Panhotra *et al.* (2005) investigated the rate of contamination of patient files in an Intensive Care Unit (ICU); they found that over 85% of ICU patient files (n=102) were contaminated compared to only 25% of surgical ward files (n=89). Analysis confirmed that ICU files were predominantly contaminated with *Pseudomonas aeruginosa* (32%), while *Staphylococcus aureus* was more common among surgical ward files (11%) (Panhotra, Saxena and Al-Mulhim,2005).

Computer technology has become an essential part of patient management, but it is possible that these devices could play a role in hospital acquired infection. An investigation was carried out following the introduction of bedside computers in a critical care department; this study found that the contamination rate of computer keyboards and mice was approximately double that of the reference objects, with *Enterococcus* species and *Staphylococcus aureus* the most frequently isolated organisms (Hartmann *et al.*,2004). Similarly other studies

have reported that keypads, desktops and portable computers are frequently contaminated with MRSA (Wilson *et al.*,2008) and toxin producing *Clostridium difficile* (Dumford *et al.*,2009). In addition lanolin and emollient from alcohol based gels increases the risk of bacterial transfer to surfaces and may promote the survival of microorganisms for extended periods on the plastic surfaces of computers (Wilson *et al.*,2008, Hartmann *et al.*,2004).

1.2 Evidence for the role of the operating theatre environment

It is estimated that only ten colony forming units are required to enter a wound during surgery for a deep infection to occur (Gosden, MacGowan and Bannister,1998, Lidwell *et al.*,1982, Charnley and Eftekhar,1969). Bacteria in the operating room may reach the open wound via contact with contaminated instruments, environmental surfaces or airbourne bacteria may settle on the wound or environmental surfaces (Gosden, MacGowan and Bannister,1998).

The majority of previous studies have focused on the role of operating theatre air in causing surgical site infection. Laminar air flow systems (Figure 1.1) that utilise high efficiency particulate air (HEPA) filters were introduced to orthopedic operating theatres in the 1980s following a series of trials by Lidwell *et al.* (1982-1987) that suggested that operation in ultraclean air lead to reduced joint sepsis and the need for reoperation during total hip and knee replacements (Lidwell *et al.*,1987, Lidwell *et al.*,1985, Lidwell *et al.*,1984, Lidwell *et al.*,1983a, Lidwell *et al.*,1983b, Lidwell *et al.*,1983c, Whyte *et al.*,1983, Lidwell *et al.*,1982).

However later studies showed that the use of prophylactic antibiotics contributed more to preventing joint sepsis than the use of HEPA filters (Fitzgerald,1992, Marotte *et al.*,1987).

It is noted that once optimum air quality is achieved, further improvements in surgical site infection (SSI) rates are dependent on better aseptic techniques (sterile instruments and surgical techniques) (Holton and Ridgway,1993), positioning of surgical staff (Madeo,1996) and choice of surgical dress (Dharan and Pittet,2002).

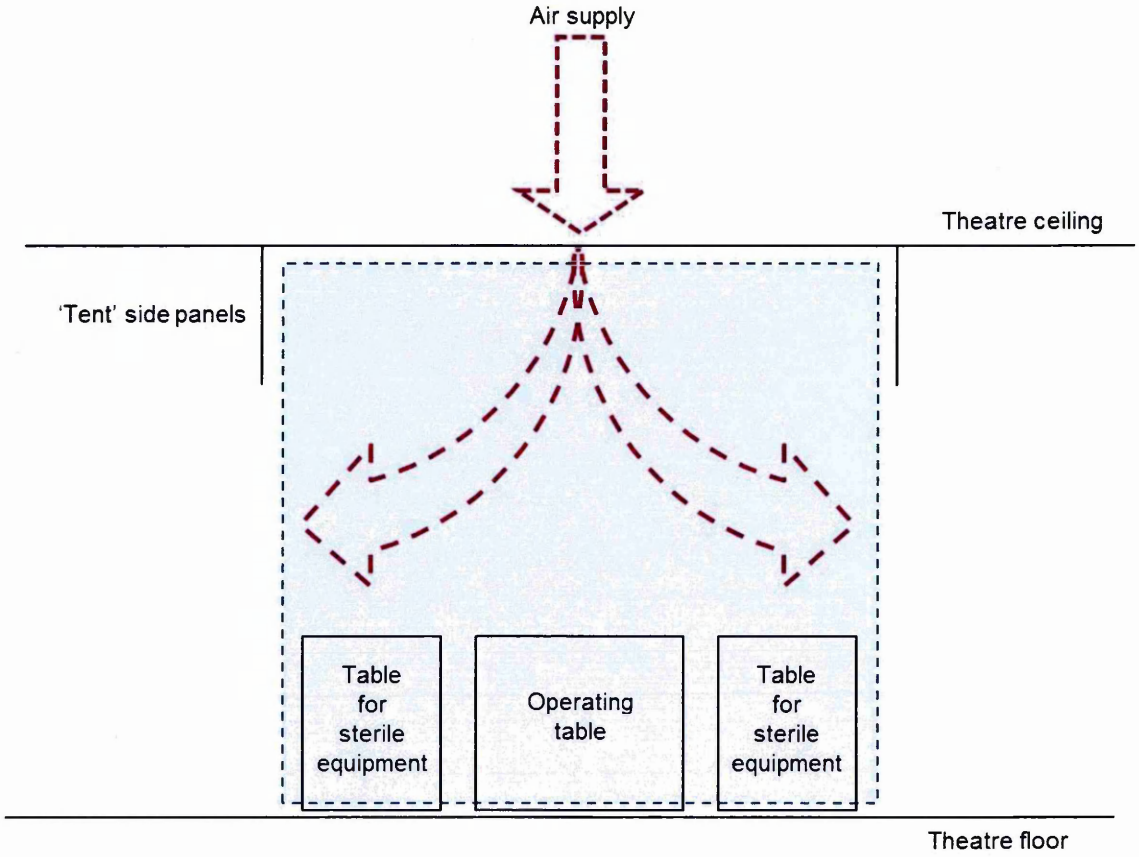

Figure 1.1 Laminar flow system

Laminar airflow systems consist of an air supply filtered through high efficiency particulate air (HEPA) filters that supply the room with clean air usually from the ceiling. This creates an "air curtain" that acts as a physical barrier between the operating field (shaded blue area) and the contaminated air. Return air grills are mounted at lower levels to exhaust the contaminated air.

1.2.1 Contribution of surgical staff

All humans shed bacteria and therefore in the operating theatre environment the surgical team are the main source of bacteria (Gosden, MacGowan and Bannister,1998). Bacteria on the skin surface may enter the air supply on skin particles or dust and be deposited in the environment by turbulent air currents (Dharan and Pittet,2002). Mills *et al.* (2000) investigated the effect of the sweating of surgeons on contamination of the operating theatre. They found a significant increase in colony forming units (CFU) when the surgeon is sweating compared with not. The organisms detected included *Staphylococcus epidermidis* and other normal skin commensals (Mills, Holland and Hardy,2000).

The choice of theatre dress is important to minimise the impact of surgical team shedding into the environment (Gosden, MacGowan and Bannister,1998). A reduction in contamination of the operating theatre environment by shedding has been reported when surgical staff wear masks and hats in addition to surgical gowns (Owers, James and Bannister,2004, Hubble *et al.*,1996).

1.2.2 Theatre equipment

Although the use of laminar flow systems has helped maintain ultraclean air and surgical dress minimises the risk of contamination by the surgical team, deep infections still cause major problems.

Previous studies have evaluated the level of contamination of different items of theatre equipment. It has been suggested that supports (Ahmad *et al.*,2011), jelly pads (Ranawat, Dowell and Teare,2004), theatre shoes (Amirfeyz *et al.*,2007), tourniquets (Brennan *et al.*,2009) and telephones (Nelson *et al.*,2006) are contaminated with potentially pathogenic microbes. In contrast convection warmers (Tumia and Ashcroft,2002), light handles (Hussein *et al.*,2001), protective lead garments (Grogan *et al.*,2011) and Bair Huggers (forced air warming systems) (Moretti *et al.*,2009) were reported to be of little clinical significance.

Equipment that is not 'single use' may become contaminated during surgery and if not effectively sterilised may represent a risk to the next patient. Ranawat *et al.* (2004) investigated pressure sore prevention pads in use in orthopaedic operating theatres as a potential risk. These 'jelly pads' used in the positioning of patients during joint replacement are required to be disinfected between cases and stored on a dedicated trolley. Pads were found to be contaminated with *Proteus, Acinetobacter, Enterococcus* and CNS. In addition many of the pads in use were damaged, rarely cleaned, frequently stored on the floor and transported between ultraclean and general theatres, all contrary to theatre protocol (Ranawat, Dowell and Teare,2004).

Fukada *et al.* (2008) investigated the level of microbial contamination on operating theatre computer keyboards. They observed that anaesthetists

frequently input data into the computer system using computer keyboards while still wearing dirty gloves and hand hygiene compliance was low. Swab samples taken from computer keyboards demonstrated high levels of microbial contamination with common skin commensals including CNS and *Corynebacterium*. MRSA was isolated from two keyboards in the operating theatre (Fukada, Iwakiri and Ozaki,2008).

These previous studies of the operating theatre environment have utilised cultural techniques to isolate culturable microorganisms, these techniques are biased by the ability of bacteria to grow on the agar selected and therefore may over- or under-estimate the bacterial diversity present.

1.3 The importance of environmental cleaning

Cleaning of hospital wards aims to remove contamination from environmental sites where potentially pathogenic microorganisms may reside. In addition to the removal of microorganisms cleaning serves to improve the level of patient confidence and reduce any concerns they may have (Dancer,1999). An increasing body of recent international literature suggests that hospital cleaning is of substantial importance in the control of HAIs such as MRSA, *Clostridium difficile*, vancomycin-resistant *Enterococci* (VRE), norovirus, and *Acinetobacter* spp (Backman *et al.*,2012, Bergstrom *et al.*,2012, Breathnach *et al.*,2012, Doan *et al.*,2012, Muzslay *et al.*,2012, Dancer *et al.*,2009, Boyce,2007).

The importance of cleaning the hospital environment as an infection control measure was highlighted by a study by Dancer *et al* (2009). The study demonstrated that by increasing the cleaning staff by one member produced a quantitatively measurable effect on MRSA colonisation of the clinical environment and a reduction in the incidence of hospital acquired MRSA (Dancer *et al.*,2009).

Cleaning is not regarded as an evidence-based practice and there are no scientific standards by which to measure the effectiveness of cleaning or environmental cleanliness. In the UK cleaning is routinely monitored by visual audit alone, but this is not a reliable assessment of the level of contamination with microorganisms or the risk of infection for an individual patient (Dancer,2009). A study by Griffith *et al.* (2000) compared the use of visual assessment, biochemical assessment (ATP bioluminescence) and microbiological assessment of cleanliness on a hospital ward. The results demonstrated that although areas were regarded as clean by visual assessment this was not actually the case when assessment was performed using biochemical and microbiological methods. It was found that only a quarter were free from organic soil (biochemical assessment) and less than half were free from microbes (microbiological assessment) (Griffith *et al.*,2000).

It has been suggested that hospitals could benefit from standards similar to those implemented by the food industry (Dancer *et al.*,2009, Dancer,2004). Here surfaces are regularly tested using a variety of techniques and isolation of

a pathogen warrants immediate action. In contrast hospital wards are only usually sampled during outbreak situations. Subjecting surfaces to more frequent routine screening may be sufficient to prevent an outbreak, as well as protecting patients may also reduce the financial impact of outbreak situations on hospitals (Dancer *et al.*,2009). The lack of microbiological standards for surface hygiene and regular screening in the hospital environment makes it difficult a) to assess the potential risk of an environment to patients, and b) to assess the efficacy of cleaning regimes used for these environments. It is suggested that total aerobic colony counts (ACC) on hand touch sites in hospitals should not exceed 2.5 CFU/cm^2 (Mulvey *et al.*,2011, Dancer,2004).

Although it is believed that sites frequently touched by hands present the greatest risk of infection for patients (Dancer,2009, Oelberg *et al.*,2000, Rheinbaben *et al.*,2000), domestic cleaning is usually restricted to the general surfaces and bathrooms, specifically floors and toilets (Dancer, White and Robertson,2008, White, Dancer and Robertson,2007). In addition responsibility of cleaning the hospital environment does not always rest with the ward cleaners; some of the responsibilities for cleaning clinical equipment lie with nurses. This inevitably leads to confusion due to the overlap of responsibilities which may ultimately lead to cleaning of certain items being reduced or abandoned completely (Dancer,2009).

Terminal cleaning refers to disinfection of a patient bay or entire ward following discharge of patients, either as part of routine cleaning or following an outbreak scenario. Detergents are not always effective at killing microorganisms and while disinfectants have been shown to be more effective, these are often expensive and can be damaging to the environment (Dancer,2011). In addition manual cleaning of furnishings and medical equipment with detergents and disinfectants is labour intensive and difficult (French *et al.*,2004). Alternatives including steam, ozone, hydrogen peroxide and ultra-violet (UV) light cleaning systems have recently been developed to overcome these problems (Dancer,2011).

Gaseous ozone has been shown to be effective against MRSA, *C. difficile* and *Acinetobacter baumannii* (Sharma and Hudson,2008). Similarly Hydrogen peroxide vapour has been shown to be effective against *C. difficile* (Doan *et al.*,2012) and MRSA (French *et al.*,2004). A disadvantage associated with the use of gaseous disinfection methods is that the area to be disinfected must be vacated and sealed for the duration of the process (Otter and French,2009, Otter *et al.*,2009).

1.4 Application of molecular techniques in understanding the microbial ecology of the hospital environment

Originally bacteria were characterised according to microscopic, morphological and biochemical properties, the development of molecular methods in the 1960s allowed determination of genetic relatedness among species resulting in changes in taxonomy; species were moved between genera and additional genera and species were suggested (Almeida and Araujo,2013).

Nucleic acid-based methods have significant advantages over conventional culture-based techniques because they offer easy identification of specific microorganisms, and are not biased by the ability of microorganisms to grow on laboratory media (Galvin *et al.*,2012). The main limitation of molecular methods is that they do not give an indication of viability of microorganisms detected on surfaces as DNA may persist in the environment following cell death (Galvin *et al.*,2012).

The polymerase chain reaction (PCR) allows amplification of DNA fragments, yielding large copy number for subsequent analysis. This is particularly useful for the analysis of environments where gene copy numbers may be low or for the detection of microorganisms that are not easily cultured (Galvin *et al.*,2012).

Molecular techniques also have the advantage that they are generally quicker than traditional culture. For example real time PCR can be used to quantify the amplified product in usually less than an hour while bacterial culture would take 24-48 hours (Espy *et al.*,2006). Real time PCR has previously been employed for the quantification of *Clostridium difficile* and *Acinetobacter baumannii* on hospital surfaces (McConnell *et al.*,2012, Mutters *et al.*,2009). However Otter *et al.* (2007) reported poor specificity of a real time PCR method for the detection of MRSA on hospital surfaces when compared to conventional culture (Otter, Havill and Boyce,2007).

1.4.1 The 16S rRNA gene

Ribosomes are responsible for the translation of mRNA into protein. They are composed of a large subunit, responsible for peptide chain formation and a small subunit, responsible for mRNA binding, translation and tRNA translocation (Schluenzen *et al.*,2000). In addition to proteins, chains of RNA, known as ribosomal RNA (rRNA), form part of the structure of the large (5S rRNA and 23S rRNA) and small (16S rRNA) bacterial ribosomal subunit (Mueller *et al.*,2000). These rRNA genes are transcribed from the ribosomal operon as 30S rRNA which is cleaved by RNase III to produce the 5S, 23S and 16S rRNA (Maidak *et al.*,1997). These are essential components of the bacterial ribosome and therefore the ribosomal operon is highly conserved among bacteria. The 16S rRNA gene is considered to be the most conserved of the three genes and therefore has been extensively investigated in studies of microbial phylogeny (Rajendhran and Gunasekaran,2011). Lane *et al.* (1985) first described the use

of 16S rRNA gene sequences for identification of uncultured bacteria. Since the 1980s 16S rRNA sequencing has been widely used for the identification of bacteria grown in pure cultures and for the estimation of diversity in a population of uncultured bacteria (Rajendhran and Gunasekaran,2011).

The size of the 16S rRNA gene is approximately 1542bp (Figure 1.2), however studies regularly use partial-16S rRNA sequence (500-700bp) analysis for bacterial identification (Cai, Archambault and Prescott,2003). Microbial taxonomy has significantly benefited from the use of 16S rRNA gene analysis and compared to other conserved genes the 16S rRNA gene has a significantly greater number of sequences deposited in any database (Yarza *et al.*,2013). However there is no agreed percentage similarity value for which definitive species identification can be obtained (Rajendhran and Gunasekaran,2011). In addition many closely related species within a genus cannot be distinguished by 16S rRNA gene sequencing (Almeida and Araujo,2013). For example *Bacillus globisporus* and *Bacillus psychrophilus* exhibit greater than 99.5% 16S rRNA sequences similarity (Fox, Wisotzkey and Jurtshuk,1992).

Fragments of the 16S rRNA gene generated during studies are usually only partial sequences of approximately 700bp due to cost restraints and limitations of the techniques used. Therefore many researchers use only partial sequences to assign bacterial identity which may create uncertainty in the correct taxonomic assignment (Kim, Morrison and Yu,2011). There are several

factors that affect the usefulness of partial 16S sequences for bacterial identification; variance is not evenly distributed across the gene it is concentrated in nine hypervariable regions (Figure 1.2), some of these variable regions are longer, more variable and some are more useful for taxonomical assignment than others (Kim, Morrison and Yu,2011, Youssef *et al.*,2009, Stackebrandt and Goebel,1994).

Previous studies of the indoor environment have utilised different variable regions of the 16S rRNA gene for bacterial identification. Lee *et al.* (2007) targeted V1-V4 in order to identify bacteria found in the environment of a childcare facility. Pyrosequencing of V1-V2 region has been previously used to investigate offices (Hewitt *et al.*,2012) and hospital air (Kembel *et al.*,2012a). Amplification of the V3-V5 region used in the present study was also used to investigate the presence of potential pathogens on hospital flooring (Harris, Pacheco and Lindner,2010) and hospital water systems (Wellinghausen, Frost and Marre,2001). Rintala *et al.* (2008) targeted the full length of the gene in order to investigate seasonal changes in bacteria found in the indoor environment.

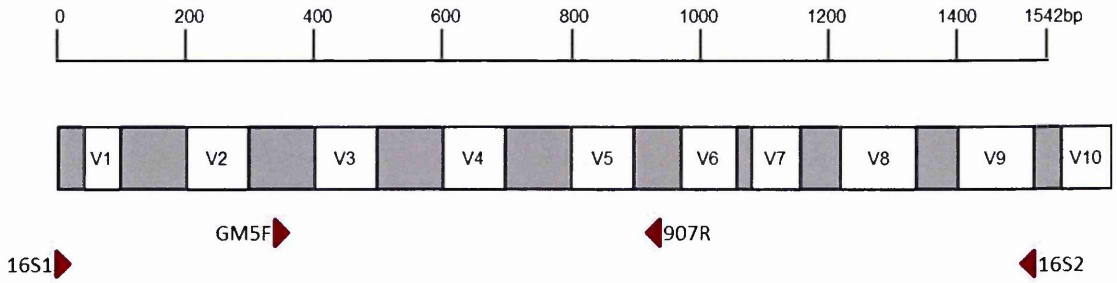

Figure 1.2 The 16S rRNA gene

Diagram of the 16S rRNA gene showing conserved (Grey) and variable (white) regions, adapted from (Xie, Hong and Goodfellow,2011, Cai, Archambault and Prescott,2003). Numbering based on the 16S rRNA gene sequence of *Escherichia coli* rrnB (GenBank JO1695; (Brosius *et al.*,1978))

Locations of primers used in this study are indicated by arrowheads (Brinkhoff *et al.*,1998, Bodrossy *et al.*,1997)

1.4.2 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR-DGGE of 16S rRNA gene amplicons is a useful tool for the study of complex microbial populations first described for the genetic profiling of microbial populations by Muyzer *et al* in 1993.

16S rRNA is an essential component of the bacterial ribosome and so is highly conserved among bacteria. As such universal primers directed towards highly conserved regions of the gene can be used to amplify the gene and confirm the presence of bacteria. In addition to highly conserved regions of the gene there are several variable regions that can be useful for bacterial identification (Rajendhran and Gunasekaran,2011, Maidak *et al.*,1997). Primers can be used to target variable regions and amplify specific fragments for further analysis.

Denaturing gradient gel electrophoresis can be used for the separation of double-stranded DNA fragments that are identical in length, but differ in sequence (Figure 1.3). A mixture of DNA fragments of different sequence are electrophoresed in an acrylamide gel containing a gradient of increasing DNA denaturants, in general, DNA fragments richer in GC are more stable and so remain double stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while the branching of partially denatured DNA molecule decreases the mobility of the fragment causing them to slow down or stop in the gel (Green, Leigh and Neufeld 2009, Sheffield *et al.*,1989). The addition of a 40bp GC rich region or GC clamp to one end of the fragment during PCR can improve the resolution of

DGGE to allow the detection of single base pair difference (Sheffield *et al.*,1989).

Muyzer *et al.* (1993) used PCR-DGGE to demonstrate that, by using primers directed to a variable region of the 16S rRNA gene, bacteria constituting as little as 1% of the population could be identified (Muyzer, de Waal and Uitterlinden,1993). This technique has since been applied to a number of microbial populations including soil and water communities (Felfoldi *et al.*,2010), dairy and brewing industries (Alegria *et al.*,2011) and animal and human colonisation (van Vliet *et al.*,2009, Gurtler, Barrie and Mayall,2002, Ley *et al.*,1997). More recently PCR-DGGE was applied to the hospital flooring environment to identify over 93% of microorganisms to the genus and species level (Harris, Pacheco and Lindner,2010).

PCR-DGGE was reccently utilised by Nelson *et al.* (2010) to identify medically relevant fungi in sputum samples from patients with cystic fibrosis. PCR-DGGE was used to confirm the presence of different species of *Candida* and *Aspergillus* (Nelson *et al.*,2010).

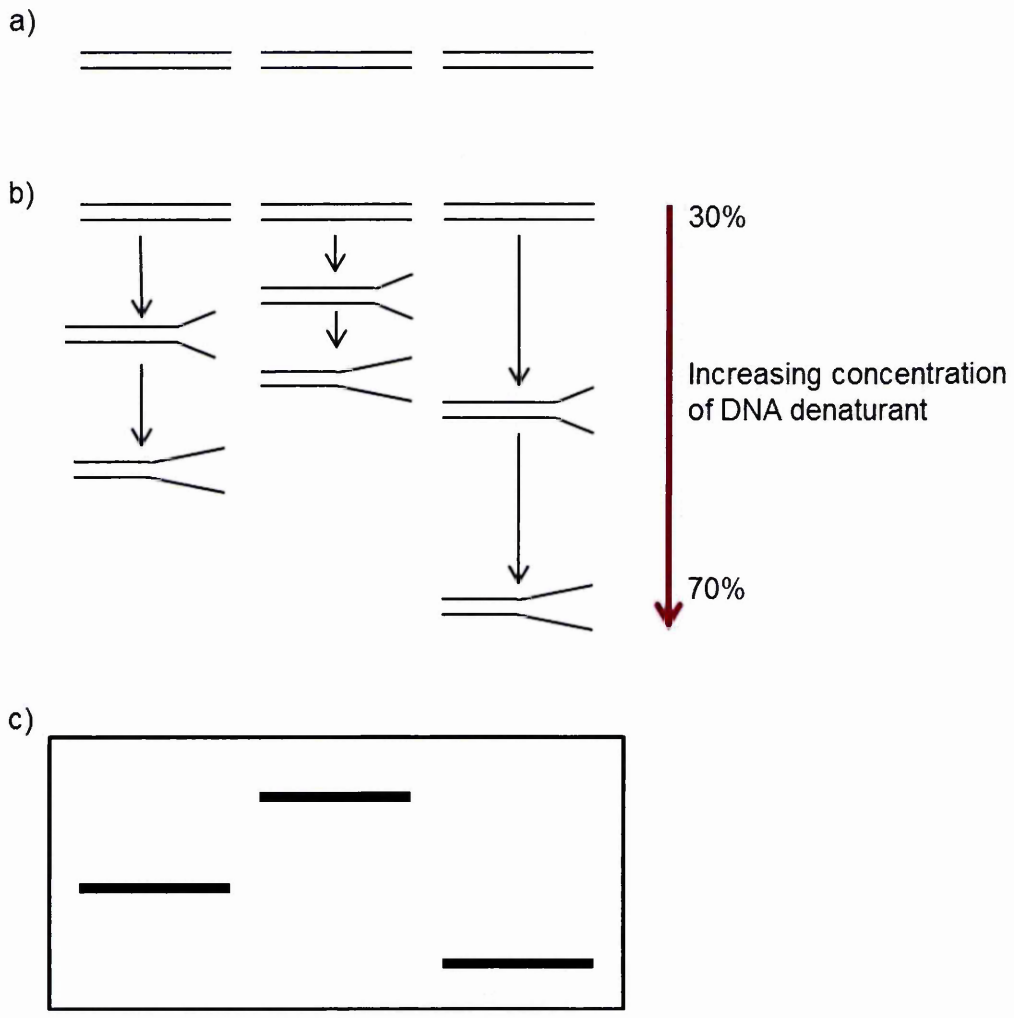

Figure 1.3 Principle of denaturing gradient gel electrophoresis (DGGE)

(a) Fragments of double stranded DNA are applied to a DGGE gel.

(b) Electrophoresis through an increasing concentration of DNA denaturants (30-70%) results in partial separation of strands dependent on sequence-specific denaturation characteristics. Branching of partially denatured DNA molecule decreases the mobility of the fragment. GC rich fragments remain more stable and denature at higher denaturant concentrations, therefore migrate further through the gel than AT rich fragments.

(c) When the acrylamide gel is visualised under UV light a single band is observed per sequence variant.

1.5 Pathogens involved in hospital acquired infections

Gram positive organisms are the dominant cause of orthopaedic infections. The Sixth report of the mandatory surveillance of surgical site infection in orthopaedic surgery states that *Staphylococcus aureus* is the most common cause of orthopaedic surgical site infection, responsible for around 40% of infections between 2004 and 2010. Coagulase negative *Staphylococci* and Gram negative *Enterobacteriaceae* are the next most common causes of surgical site infection in orthopaedics, followed by *Enterococci* (HPA,2010).

Gram positive pathogens such as *Staphylococcus aureus, Clostridium difficile* and *Enterococci* have previously been shown to survive on environmental dry surfaces within the hospital for extended periods (Otter and French,2009, Rohr *et al.*,2009, Hardy *et al.*,2006, Martirosian,2006, Asoh *et al.*,2005, Hirai,1991).

Pathogens involved in hospital acquired infections often carry resistance genes. This firstly makes them difficult to treat when they cause an infection in patients, but also may contribute to their survival in the environment. Antibiotic resistance genes are usually transferred between microorganisms where antibiotics are used intensively and the rise in antibiotic usage has promoted the emergence and spread of these genes (Hawkey,2008). Antibiotic resistance genes may be carried on mobile genetic elements such as plasmids that allow transfer between bacteria (Russell,1997). Bacteria present in the environment of the hospital may act as a reservoir for antibiotic resistance genes and

therefore the understanding of the distribution of antibiotic resistance genes in the hospital environment is important.

Clinical isolates causing infection in patients are routinely screened for antibiotic sensitivity, however environmental isolates are not and these may represent a potential reservoir for the accumulation and spread of resistance genes in an environment where antibiotics are relied on.

1.5.1 *Staphylococcus* species

Staphylococci are Gram positive bacteria usually associated with human and animal skin and mucus membranes. *Staphylococcus aureus* is the most invasive species of the genus and is a prominent cause of skin and soft tissue infections, septicaemia, food poisoning, endocarditis and pneumonia (Malachowa and DeLeo,2010). Coagulase-negative *Staphylococci* (CNS) are less invasive, but are frequently responsible for foreign body (e.g. catheters and joint prosthesis) associated infections (Kloos and Bannerman,1994).

Penicillin was the first antibiotic to be introduced for use to treat human bacterial infections. Resistance to penicillin was first reported in 1944 (Kirby,1944) and now it is estimated that over 90% of clinical *Staphylococcus aureus* are resistant (Olsen, Christensen and Aarestrup,2006). Resistance to beta-lactams is often the result of hydrolysis of the beta-lactam ring by the enzyme beta-lactamase; in *Staphylococci* this is encoded by the *blaZ* gene (Olsen,

Christensen and Aarestrup,2006). Methicillin resistant *Staphylococcus aureus* (MRSA) was first reported in 1962 following the introduction of methicillin for the treatment of penicillin-resistant *S. aureus* infections (Malachowa and DeLeo,2010). MRSA is able to cause difficult-to-treat infections due to resistance to many of the commonly used antibiotics. Resistance is due to an altered penicillin binding protein (PBP2a), which is encoded by the *mecA* gene (Duran *et al.*,2012, Zapun, Contreras-Martel and Vernet,2008, Livermore,2000).

It has been reported that there is also a high incidence of methicillin resistance among clinical isolates of CNS (MR-CNS) (Zingg *et al.*,2009, Widerstrom *et al.*,2006) . A high level of sequence similarity has been observed between the *mecA* of MRSA and MR-CNS (Suzuki, Hiramatsu and Yokota,1992, Murakami *et al.*,1991, Ubukata *et al.*,1990, Froggatt *et al.*,1989).

The *mecA* gene is carried as part of the mec complex along with its regulatory genes *mecI* and *mecR1*. Subclasses of the mec complex have been described according to polymorphisms in these genes (Katayama, Ito and Hiramatsu,2001, Kobayashi, Alam and Urasawa,2001b). The mec complex is carried on a mobile genetic element of variable size (21-67kb) called the Staphylococcal cassette chromosome mec (SCC*mec*), that inserts into the *orfX* gene near the chromosomal origin of replication (Malachowa and DeLeo,2010, Katayama, Ito and Hiramatsu,2000). SCC*mec* also contains a ccr complex composed of the cassette chromosome recombinase (ccr) genes responsible

for integration and excision of SCC*mec* (Turlej, Hryniewicz and Empel,2011, Katayama, Ito and Hiramatsu,2000). In addition to the mec and ccr complex, SCC*mec* contains three joining regions or J regions (J1-J3) (Figure 1.4). The classification of SCC*mec* is based on a combination of mec complex class and ccr allotype (IWG-SCC,2009, Ito *et al.*,2001).

To date eleven types of SCC*mec* have been characterised in *Staphylococcus aureus* according to structural variations in the mec and crr complexes and the J regions present (Oliveira, Milheirico and de Lencastre,2006, Ito *et al.*,2004, Ma *et al.*,2002, Ito *et al.*,2001). Type I, II and III are reported to be most common in hospital acquired MRSA, while type IV and V are frequently found in community acquired MRSA (IWG-SCC,2009). It has been suggested that SCC*mec* elements are more diverse in CNS (Zong, Peng and Lu,2011, Ruppe *et al.*,2009, Descloux, Rossano and Perreten,2008, Ibrahem *et al.*,2008, Miragaia *et al.*,2007, Miragaia, Couto and de Lencastre,2005).

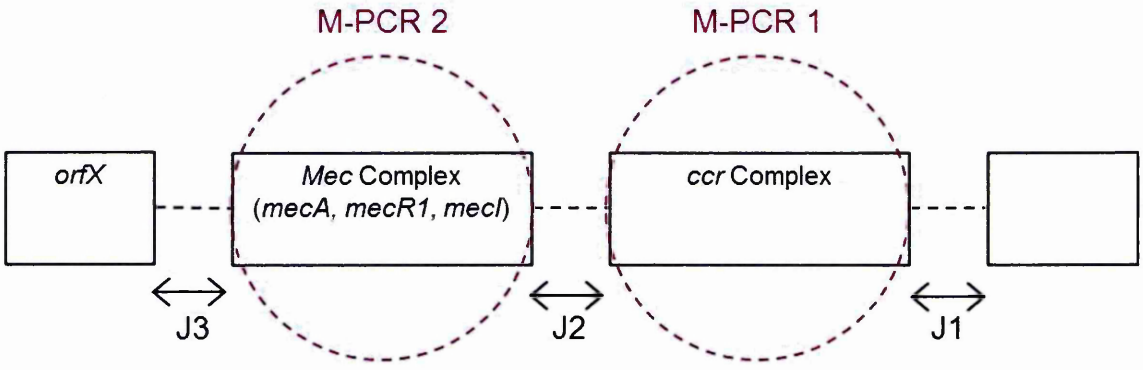

Figure 1.4 Basic schematic structure of the Staphylococcal cassette chromosome *mec*

The SCC*mec* is a mobile genetic element that inserts into the *orfX* gene near the chromosomal origin of replication. The SCC*mec* consists of the *mec* complex, *ccr* complex and three joining regions (J1-J3), adapted from (IWG-SCC,2009, Kondo *et al.*,2007). The regions targeted by primers used for the amplification of the *ccr* genes (M-PCR 1) and *mec* genes (M-PCR 2) are indicated by circles (Kondo *et al.*,2007).

Additional antibiotic resistance, heavy metal resistance and virulence determinants may be incorporated into the joining regions of SCC*mec* through insertion sequences (IS), plasmids or transposons (Tn). As such SCC*mec* enables the emergence and spread of mutli-resistant *Staphylococci* (Turlej, Hryniewicz and Empel,2011). Examples of additional mobile elements located within the SCC*mec* joining regions include transposon Tn4001 encoding a bifunctional aminoglycoside modifying enzyme (*aac(6')-aph2"*) located within the J region of SCC*mec* type IVc (Turlej, Hryniewicz and Empel,2011, Hanssen and Ericson Sollid,2006). As such, Methicillin resistance in *Staphylococci* is often accompanied by resistance to other antibiotics for example aminoglycosides (Ardic *et al.*,2006).

Aminoglycoside are broad spectrum antibiotics used to treat a variety of infections. Gentamicin is an aminoglycoside widely used in orthopaedics. Aminoglycoside antibiotics act by binding to the bacterial ribosome, preventing protein synthesis. The most important resistance mechanism is enzymatic modification, these enzymes act by chemically modifying the aminoglycoside so that it loses its affinity for the bacterial ribosome and therefore is unable to prevent protein synthesis (Fluit, Visser and Schmitz,2001). There are three groups of aminoglycoside modifying enzymes (AMEs); aminoglycoside O-nucleotidyltransferases (ANT), aminglycoside N-acetyltransferases (AAC) and aminoglycoside O-phosphotransferases (APH) (Frase, Toth and Vakulenko,2012). These are named according to the type of modification they

perform and numbered according to the modification position on the aminoglycoside substrate (Fluit, Visser and Schmitz,2001).

The bifunctional enzyme AAC(6')-APH(2") is responsible for high level resistance to nearly all aminoglycosides (with the exception of Streptomycin) (Leclercq *et al.*,1992) in *Enterococcus* and *Staphylococcus*, encoded by the *acc(6')-aph(2")* gene (Ferretti, Gilmore and Courvalin,1986). This enzyme contains two domains that may function independently to inactivate aminoglycosides through acetylation or phosphorylation (Daigle, Hughes and Wright,1999).

Staphylococci may also carry detergent resistance genes that promote their survival in the environment. Quaternary ammonium compounds are frequently used to disinfect the hospital environment and it has been suggested that this may apply a selective pressure that promotes the emergence of resistance (Russell,2000). Resistance in *Staphylococci* is usually encoded by *qacA, qacB* or *qacC*. The *qacA/B* resistance genes have previously been reported to be the most prevalent among clinical *Staphylococci* (Ben Saida *et al.*,2009, Sidhu *et al.*,2002, Leelaporn *et al.*,1994) . The *qacA* gene encodes a broad range of resistance to compounds including intercalating dyes (e.g. ethidium bromide), diamidines, biguanidines (e.g. chlorhexidine) and quaternary ammonium compounds. The *qacB* gene confers resistance to a narrower range of compounds including quaternary ammonium compounds and intercalating dyes

but is almost identical in nucleotide sequence to *qacA* (Leelaporn *et al.*,1994, Littlejohn *et al.*,1992).

Environmental CNS are considered to be of epidemiological importance as they may act as a reservoir of resistance genes, that can be transferred to other *Staphylococci* (Hanssen and Ericson Sollid,2006). *Staphylococcus haemolyticus* is known to be the most virulent of the group and is known to carry multiple drug resistances (Berglund and Soderquist,2008).

The recent increased recognition of CNS as important pathogens has led to the need for an improved identification and discrimination of CNS species. Traditional culture based methods are time consuming and not considered to offer sufficient discriminatory power and therefore molecular based detection are of interest, for example 16S rRNA gene and *tuf* gene sequencing (Heikens *et al.*,2005).

The *tuf* gene encodes the elongation factor Tu (EF-Tu) which is involved with peptide chain formation and so is an essential part of the ribosome. EF-Tu loads amino-acyl tRNA moleculaes onto the ribosome during translation (Ventura *et al.*,2003). Several studies have reported that the *tuf* gene may be useful for the identification of genera of gram positive cocci including;

Staphylococci (Li *et al.*,2012, Heikens *et al.*,2005, Martineau *et al.*,2001), *Enterococci* (Ke *et al.*,1999) and *Streptococci* (Picard *et al.*,2004).

1.5.2 Enterobacteriaceae

Enterobacteriaceae are a large family of gram negative rods that includes many pathogenic genera; for example *Escherichia*, *Klebsiella, Enterobacter, Salmonella* and *Shigella*. *Enterobacteriaceae* including, *Escherichia coli* and *Klebsiella pneumoniae,* are the dominant cause of many hospital acquired infections including septicaemia (Daikos *et al.*,2012), abdominal sepsis (Chen and Hsueh,2012), ventilator associated pneumonia (Barbier *et al.*,2013) and urinary tract infections (Jansaker *et al.*,2013). Enterobacteriaceae are easily spread by contact, may contaminate food and water sources and frequently acquire antibiotic resistance genes through horizontal gene transfer (Nordmann, Dortet and Poirel,2012).

Enterobacteriacea producing TEM and SHV beta-lactamase enzymes became prevelant following the intoduction of ampicillin in the 1960s (Hawkey,2008). In recent years hospital acquired infections due to Gram negative *Escherichia coli* and other Enterobacteriaceae have increased, many of which produce extended spectrum beta-lactamase (ESBL) enzymes that hydrolyse penicillins and most cephalosporins (Khan, Dancer and Humphreys,2012, Falagas and Karageorgopoulos,2009). More recently carbapenem resistant Enterobacteriaceae (CRE) have emerged (Cantón *et al.*,2012). Multi-resistant

Enterobacteriaceae have been shown to contaminate the environment surrounding hospitalised patients and be able to persist for extended periods in these environments (Kramer, Schwebke and Kampf,2006, Lemmen *et al.*,2004). In addition a link between local antibiotic prescription and resistance in environmental Enterobacteriaceae has been observed (Dancer *et al.*,2006).

1.5.3 *Enterococci*

The *Enterococci* are normal residents of the human gastrointestinal tract that may help aid digestion; however they are increasingly being recognised as important causes of opportunistic human infection. *E. faecalis* and *E. faecium* have been implicated in a variety of HAI including; urinary tract infections, endocarditis, bacteraemia and abdominal infections (Byappanahalli *et al.*,2012, Moellering,1992).

Enterococci are known to have a number of intrinsic resistance mechanisms, which offer low level resistance to beta-lactams, aminoglycosides and cephalosporins (Hollenbeck and Rice,2012). Beta-lactam antibiotics bind to penicillin binding proteins (PBP) in the bacterial cell wall and prevent synthesis. Intrinsic low level resistance to beta-lactams in *Enterococci* is due to the expression of PBPs with low affinity for beta-lactams (PBP5 *E. faecium* and PBP4 *E. faecalis*), resulting in minimum inhibitory concentrations (MIC) that are higher than other related species (e.g. *Streptococci*) (Sifaoui *et al.*,2001, Murray,1992). Aminoglycoside antibiotics act by binding to the bacterial

ribosome, preventing protein synthesis. Intrinsic resistance to aminoglycoside antibiotics in *E. faecalis* is due to the inability of aminoglycosides to enter the bacterial cell and exert their effect on the bacterial ribosome. The use of a cell wall synthesis inhibitor (Penicillin) in combination with aminoglycoside antibiotics has been shown to improve aminoglycoside uptake by the cell and bactericidal activity (Moellering,1992, Zimmermann, Moellering and Weinberg,1971).

In addition to intrinsic resistance mechanisms *Enterococci* may acquire mobile genetic elements containing resistance genes. The acquisition of resistance mechanisms through horizontal gene transfer has also been described for beta-lactamase genes (*bla* genes) (Murray and Mederski-Samaroj,1983), aminoglycoside modifying enzyme genes (*acc(6')-Ie-aph(2")-Ia* gene) (Mederski-Samoraj and Murray,1983), glycopeptide resistance genes (*Van* genes) (Gold,2001, Arthur and Courvalin,1993) and others (Hollenbeck and Rice,2012).

1.5.4 Opportunistic Fungi

Fungal hospital acquired infections are an important cause of morbidity and mortality in hospitalised patients. Although fungi are not frequently responsibly for surgical site infections in orthopaedic patients, they are more common and responsible for more devastating infections in intensive care unit patients (Blumberg *et al.*,2001). *Aspergillus* and *Candida* spp. are known to account for

the majority of fungal HAI (Groll and Walsh,2001). *Candida albicans*, a significant fungal pathogen and can survive for up to four months on dry surfaces within the hospital environment (Kramer, Schwebke and Kampf,2006). However the non-albicans *Candida* have also been reported as important pathogens; *C. tropicalis* and *C. glabrata* are associated with complications including endocarditis and osteomyelitis, and *C. parapsilosis* is associated with increasing antifungal resistance (Nguyen *et al.*,1996).

Fungi other than *Candida* are also becoming increasingly recognised as potential pathogens in hospitalised patients. *Fusarium* and *Trichosporon* spp. are considered emerging opportunistic pathogens responsible for disseminated infections and high morbidity particularly in immunocompromised patients (Groll and Walsh,2001). *Trichosporon sp*ecies are considered to be medically relevant yeast responsible for deep seated trichosporonosis, with poor prognosis (Sugita *et al.*,1999).

1.6 Scope of the Present Study

Previous studies of the hospital environment have focused largely on outbreak situations; however it is also essential to understand the hospital environment under normal operating conditions. In addition the majority of previous studies have relied on the use of traditional microbiological methods to study specific culturable pathogens in the hospital environment. This study aimed to use molecular techniques to provide a more coprehensive study of the total

microbial ecology of the hospital environment. These methods have advantages over conventional culture-based techniques because they offer easy identification of specific microorganisms and their genes, and are not biased by the ability of microorganisms to grow on laboratory media.

The environment of the orthopaedic department of the Northern General and Royal Hallamshire Hospitals, Sheffield was selected for investigation. The project investigated contrasting areas of the orthopaedic department: (1) a pre and post-operative elective ward where patients are screened for MRSA prior to admittance, (2) a trauma ward where patients are admitted without screening and may have pre-existing infections or complications and (3) an operating theatre where the environment is ultra-controlled to minimise the risk of infection during surgery.

The overall aim of this study was to characterise the total microbial ecology of the orthopaedic department environment under non outbreak conditions using PCR-DGGE in order to characterise the microbial burden of this environment, provide baseline data and evaluate the use of molecular techniques.

The objectives were to:

- Retrieve and identify bacterial 16S rRNA gene and fungal 28S rRNA gene fragments from the environment using PCR-DGGE (Molecular techniques)

- Compare the environmental distribution of these retrieved sequences at different sampling sites in the orthopaedic department
- Investigate the viability of bacteria in the environment using PCR-DGGE to separate fragments of the 16S rRNA gene amplified from total RNA extractions
- Enumerate, isolate and identify microorganisms from the environment using cultural techniques
- Compare the change in microbial load and bacterial species present over a 24 hour period following routine cleaning
- Investigate the presence of antibiotic resistance genes in total DNA extracted directly from environmental swab samples
- Investigate the presence of antibiotic resistance genes in phenotypically resistant isolates selected from the environment using antibiotic containing agars
- Identify bacterial species carrying these antibiotic resistance genes

2 Methods

2.1 Ethical Approval

Ethical approval was gained from the Sheffield Research Ethics committee on 27th January 2011 (Ref. no. 10/H1308/78). R & D authorisation was also obtained from Sheffield Teaching Hospitals NHS Foundation trust on 22nd March 2011 (STH Ref 15907).

2.2 Study Wards

Two orthopaedic wards A and B each contained 34 beds in a 1100 bed UK teaching hospital; the Northern General hospital, Sheffield. Each ward had the same layout: five bays each containing six beds on one side of the main corridor and four separate side rooms, wash and utility rooms on the other side (Fig 2.1). Ward A is an elective 'MRSA-free' ward where patients are screened before to admission and a strict admittance criterion is applied (patients are only admitted if they are MRSA negaitive). Ward B is a trauma ward where patients are admitted without prior MRSA screening. During the period of sampling ward A was closed and relocated to another hospital in the same city; the Royal Hallamshire hospital (Ward C). Sampling was performed on ward C after refitting and before it opened as an orthopaedic ward and after six months use as an orthopaedic ward.

All wards are cleaned to the same specifications by domestic services. For the floors this consists of a daily vacuum to remove dust and mop. Doors, switches

and sockets are cleaned daily. Nurses are responsible for the cleaning of commodes, manual handling equipment, medical equipment, notes and drugs trolleys and patients' personal items.

The orthopaedic operating suite consisted of a single preparation room, two operating theatres and adjoining anaesthetic rooms. The preparation room is used for surgical staff preparation prior to surgery and storage of sterile instruments and equipment for the day operating list. Both theatres are used for orthopaedic surgical procedures. Both adjoining anaesthetic rooms are used for patient preparation prior to surgery (Anaesthetisation and surgical site preparation) (Fig 2.2).

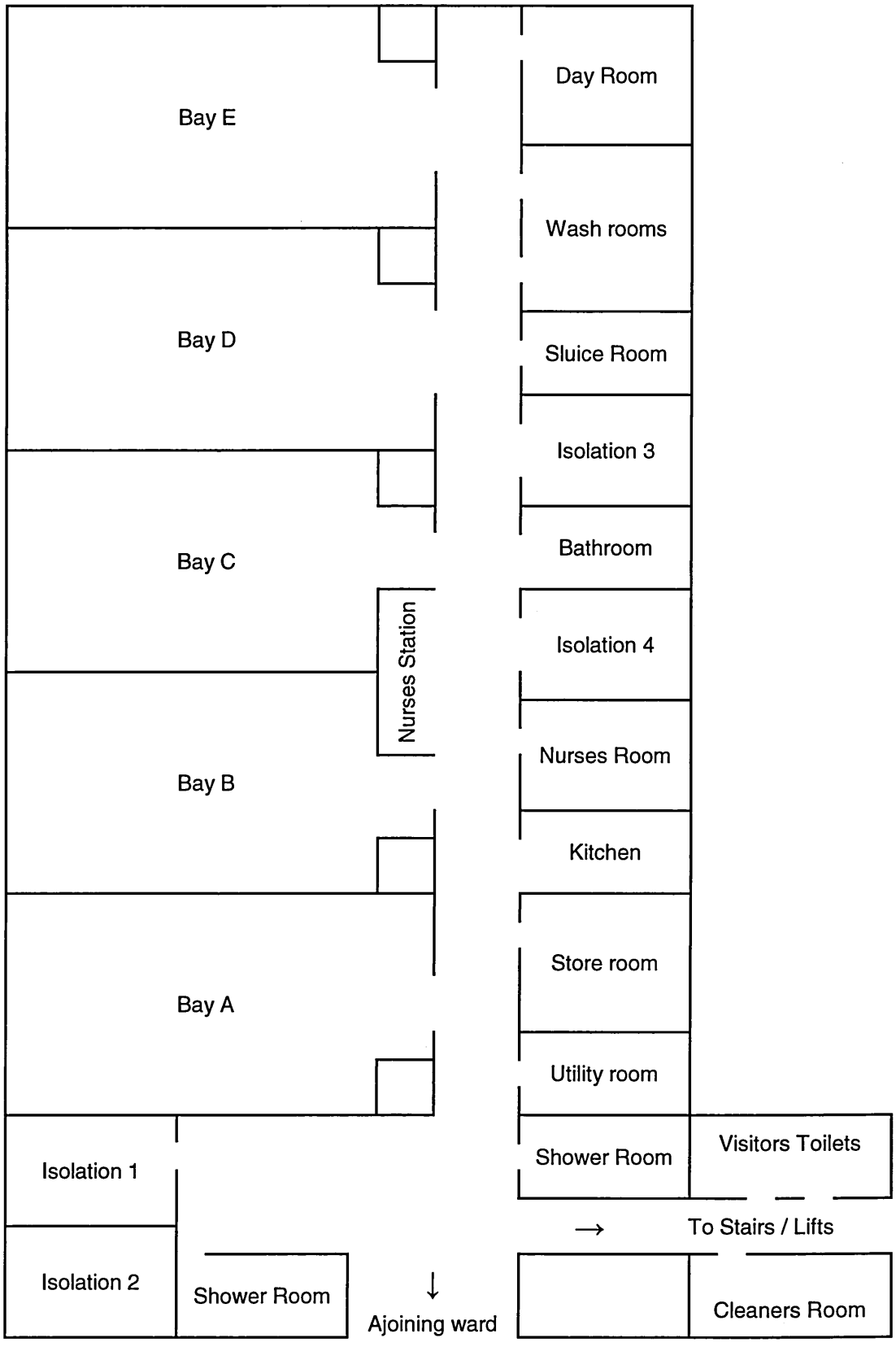

Figure 2.1 Ward A and B floor plan

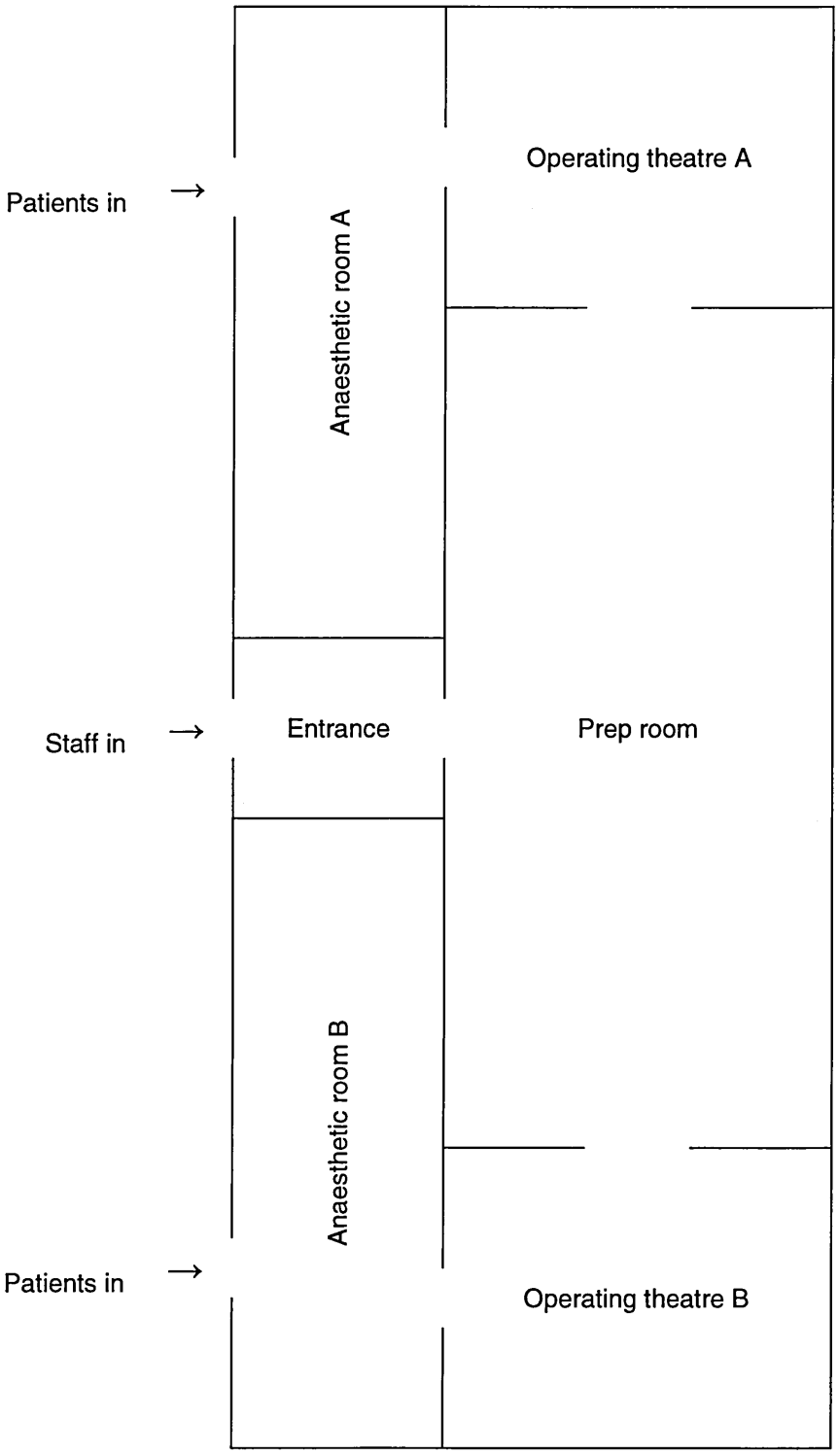

Figure 2.2 Orthopaedic operating theatre floor plan

2.3 Environmental screening

Environmental samples were taken on 12 occasions between March 2011 and March 2012 from wards A and B. Samples were taken on a single occasion in December 2011 on ward C after refitting and before opening as an orthopaedic ward and on two occasions in June 2012 after six months as an orthopaedic ward. Samples were taken from the floors at the start (approximately 1 hour after daily clean) and end (approximately 24 hours after daily clean, i.e. immediately before next clean) of the day (total area swabbed for each sample was 1250 cm^2). Near patient sites, namely bed rails (240 cm^2), patient chairs (seat 1175 cm^2 and arms 192 cm^2) and top and underside of over bed tables (1488 cm^2 each) were also sampled (Figure 2.3). Floors were selected as sampling sites as to explore the possiblilty that these may be useful as an indicator for the environmental microflora of the room as a whole. Near patient sites including patient bed rails, over bed tables and chairs were selected as these are frequent hand touch sites for patients, staff and visitors.

Environmental samples were taken on one occasion in December 2012 from the operating suite during a routine hip replacement operation. On this occasion, samples were taken from the floors of the preparation room, theatres and anaesthetic rooms (area 1012 cm^2). Swab samples were also taken from the operating theatre phone, instrument keyboard, equipment shelves and the footwear of theatre staff (Figure 2.4).

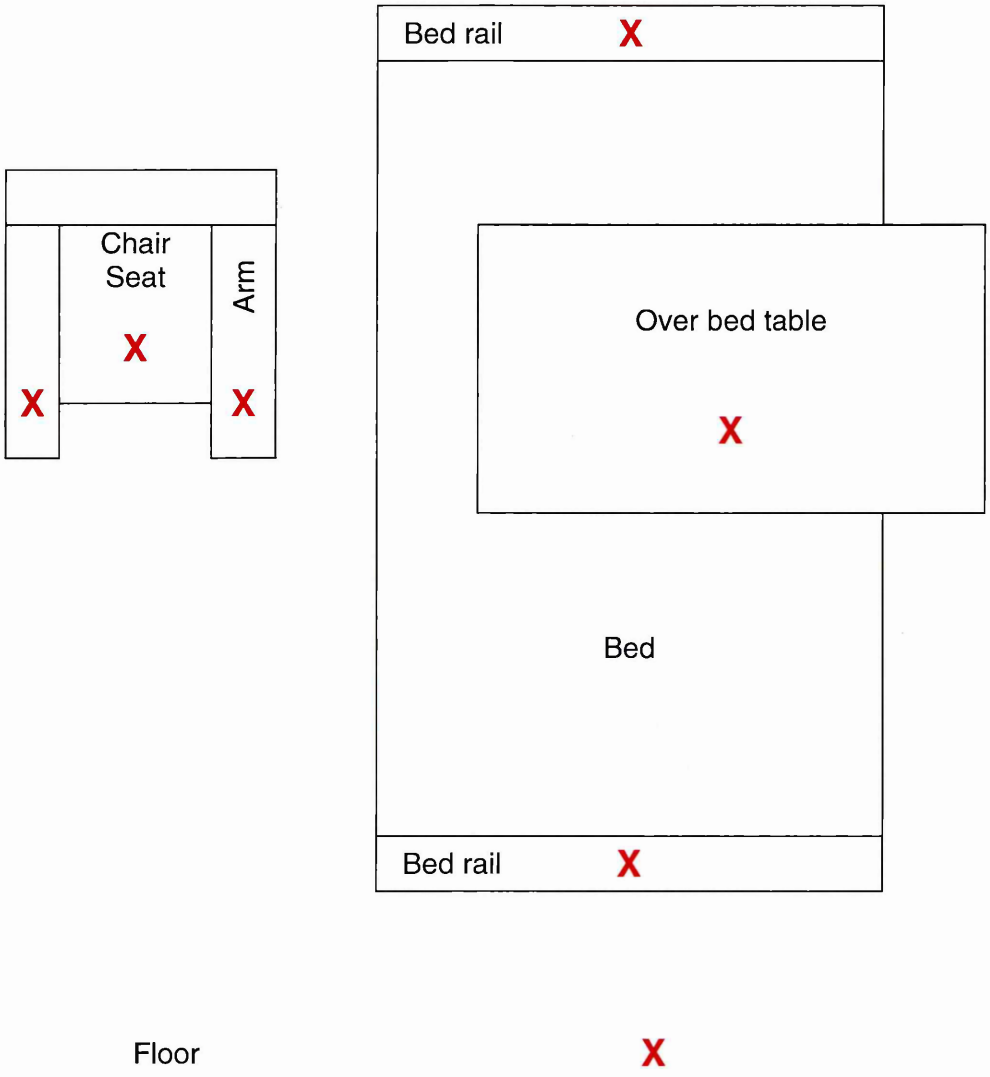

Figure 2.3 Sites sampled on orthopaedic wards A and B

Sites on the ward where swab samples were collected using pre-moistened

marked with a red X

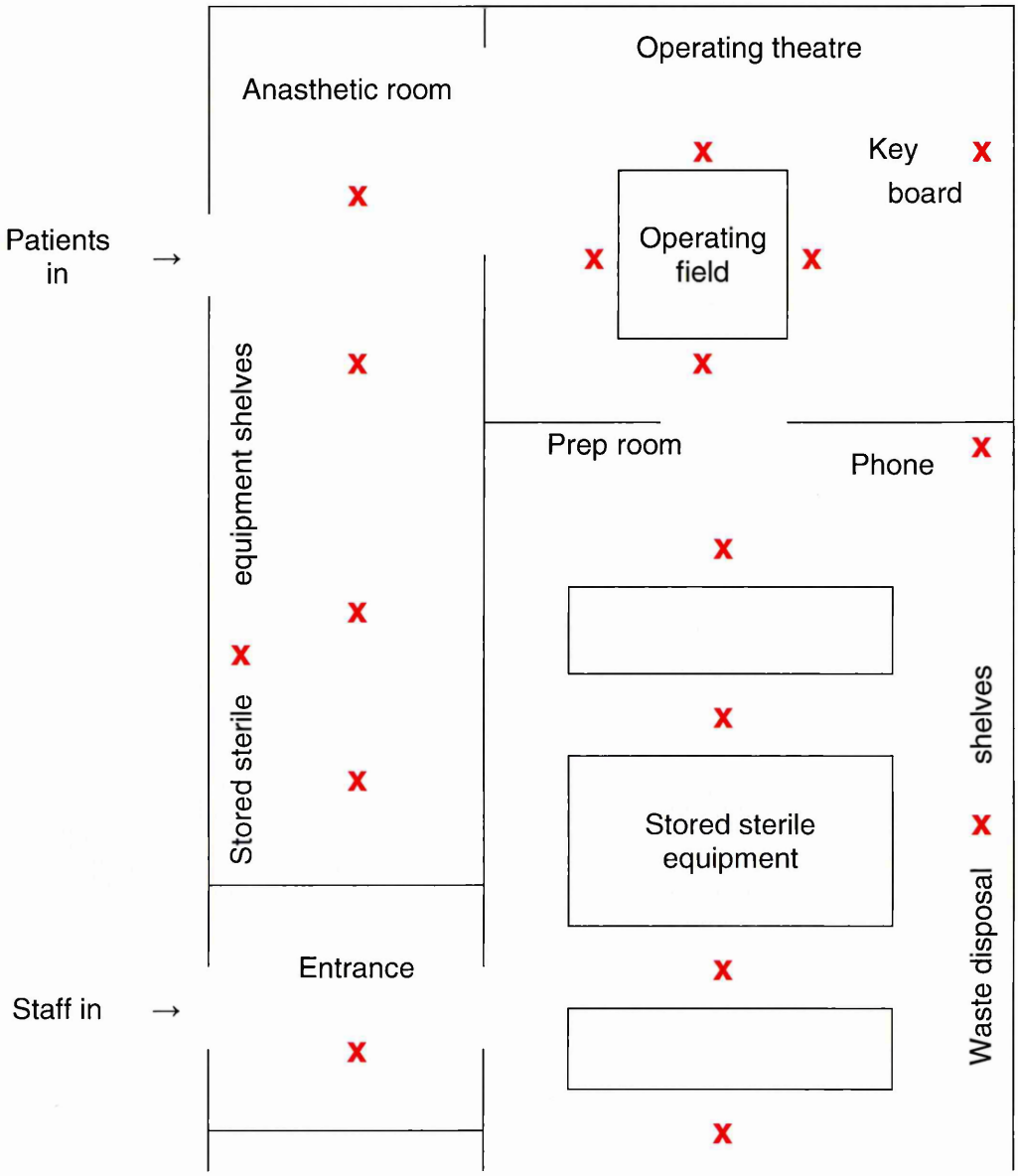

Figure 2.4 Sites sampled in the orthopaedic operating theatre

Sites in the orthopaedic operating theatre where swab samples were collected using pre-moistened swabs marked with a red X

An area was entirely swabbed by rolling the Steriswab (Medical wire), pre-moistened in phosphate buffer saline (PBS), in a zigzag pattern two times over a defined area. The swab was then inserted into a sterile tube containing 500 µl of sterile PBS, for transport. Material collected with each swab was divided so that both microbiological and molecular analysis could be performed on each swab sample taken.

Swabs were agitated on an orbital shaker in PBS for 1 hour to release microorganisms. The swab was then discarded. Microbial cells were pelleted by centrifugation and the supernatant discarded. The pellet was resuspended in PBS and used to produce log serial dilutions to 10^{-5}. The remaining sample in PBS was saved for DNA and RNA extraction.

2.4 Microbiology

2.4.1 Environmental Isolates

Enumeration of bacteria was performed on Mueller-Hinton, Blood, CLED, MacConkey's, Colorex-MRSA, *Burkholderia cepacia*, *Pseudomonas* selective agars and antibiotic containing agars (gentamicin). Briefly, serial dilutions were plated onto a plate of each type of agar in triplicate. Following 24 hours incubation at 37°C the number of colonies was recorded, converted to CFU/ml, divided by total area sampled and expressed as colony forming units (CFU) per unit area (cm^2).

Average aerobic colony counts (ACC) were compared using analysis of variance (ANOVA) performed using Microsoft Excel, differences were considered significant if $P < 0.05$. Where ACC was plotted graphically, error bars were included to show the standard error (Cumming, Fidler and Vaux,2007).

Representative colonies were subcultured from each agar plate; one colony was selected from each observed morphology type and identified by sequencing of the 16S rRNA gene amplified by colony PCR using primers 16S1 (AGA GTT TGA TCM TGG CTC AG) and 16S2 (TAC GGY TAC CTT GTT ACG ACT T) primers (Bodrossy *et al.*,1997). PCR was carried out in a total volume of 50 µl containing 20 mM Tris-HC (pH 8.4), 50 mM KCl, 0.2 mM dNTPS, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1U of *Taq* DNA Polymerase, (Life Technologies). Amplification conditions were: 95°C for 10 minutes, 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds, 72°C for 90 seconds, with a final extension of 72°C for 15 minutes. Amplification products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol and sent to Eurofins MWG Operon for sequencing. Sequences were compared to those in the GenBank database, similarity scores according to BLAST were used to identify bacteria to the genus and species level. A sequence similarity of ≥97% was sufficient to identify to the genus level, while a similarity score of ≥99% was sufficient to identify to the species level (Drancourt *et al.*,2000).

Eluted microorganisms from swab samples were also inoculated on Colorex *Candida* and Sabouraud dextrose (containing 50mg/l Chloramphenicol) agars. Representative fungal colonies were subcultured from each plate and identified by sequencing of the 28S rRNA gene using primers U1 (GAA ATT GTT GAA AGG GAA) and U2 (GAC TCC TTG GTC CGT GTT) (Nelson *et al.*,2010).

2.4.2 Clinical isolates

Clinical isolates were obtained from the STH clinical microbiology laboratory throughout the study. These isolated were recovered by biomedical staff at the clinical laboratory as part of routine patient diagnosis and treatment. A list was generated using the STH computer system of all the isolates cultured from clinical specimens (Blood, tissue, wound swabs and urine samples) of patients present on both ward A and B during the time that environmental sampling was being performed on these wards as part of this study.

All isolates obtained from the clinical laboratory were maintained on blood agar. Clinical isolates from patient tissue or wound cultures were identified by sequencing of the 16S rRNA gene amplified using PCR primers 16S1 and 2 (Section 2.4.1).

2.5 Total DNA extraction from environmental samples

DNA was extracted directly from swab samples following the Qiagen QIAamp DNA mini kit, adapted according to recommendations by Lee *et al* (2007). Briefly samples were incubated with 300 µl of enzymatic lysis buffer (20 mM Tris, 2 mM EDTA pH 8.0, 25 mg/ml lysozyme, 20 µg/ml lysostaphin) at 37˚C for 2 hours (Lee, Tin and Kelley,2007). Following lysis the sample was processed according to the Qiagen kit instructions. The eluted DNA (in 2 x 1.5 ml microcentrifuge tubes) was stored at -20˚C.

2.6 Total RNA extraction from environmental samples and cDNA synthesis

RNA was extracted directly from swab samples following the Qiagen RNeasy Mini Kit in combination with the Qiagen RNAprotect Bacteria Reagent to stabilise RNA prior to isolation according to the Qiagen kit instructions. The eluted RNA solution was stored at -20°C.

Following total RNA extraction, cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen) that enables effective removal of contaminating genomic DNA and cDNA synthesis from all RNA present. The resulting cDNA solution was stored at -20°C.

2.7 Bacterial 16S rRNA gene analysis via PCR-DGGE using total DNA and cDNA templates

Primers were identified from the literature to amplify a 550bp fragment of the 16S rRNA gene containing variable regions that enable bacterial identification following separation of metagenomic DNA using DGGE; primer GM5F containing a GC rich region, or GC clamp (in bold), (**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G**CC TAC GGG AGG CAG CAG) and primer 907R (CCG TCA ATT CMT TTG AGT TT) (Brinkhoff *et al.*,1998, Muyzer, de Waal and Uitterlinden,1993). PCR was carried out in a total volume of 50 µl containing 20 mM Tris-HC (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1U of *Taq* DNA Polymerase, (Life Technologies). Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad) with an 8% (wt/vol) polyacrylamide gel, denaturing range 30 - 70% (100% defined by Muyzer *et al.* (1993) as 7 M Urea 40% vol/vol formamide). Electrophoresis was performed at 75 V for 18 hours in 1 X TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at 60°C. Bands were visualised by staining with 1 X Sybr Gold (Life Technologies) diluted in TAE for 40 minutes. Following visualisation bands of interest were excised from the gel using a sterile scalpel and incubated in sterile water at 4°C overnight. Where bands

appeared at the same position in several lanes of the gel, multiple bands were excised in order to confirm identify of the species present.

Following overnight incubation in sterile water, eluted DNA from excised bands was reamplified using the above thermocycler conditions and GM5F (without GC clamp) and 907R primers. PCR products were purified in preparation for sequencing (Eurofins MWG Operon). Sequences were compared to those in the GenBank database using BLAST (Altschul *et al.*,1990) and percent identity scores were used to identify bacteria where a sequence similarity of ≥97% and ≥99% were used as criteria for identification to genus and species respectively (Drancourt *et al.*,2000) .

As previously described an assumption was made that PCR amplicons that migrated the same distance in different lanes all had the same sequence (Kassem, Esseili and Sigler,2011, Harris, Pacheco and Lindner,2010, van Vliet *et al.*,2009). The assumption was validated by sequencing multiple bands, from different lanes, that had migrated the same distance. Where sequences matched, that sequence was then assumed for all bands at that position in a given gel.

2.8 Fungal 28S rRNA gene analysis via PCR-DGGE using total DNA template

Primer pair U1GC containing a GC rich region (in bold) (**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G**GT GAA ATT GTT GAA AGG GAA) and U2 (GAC TCC TTG GTC CGT GTT) were used to amplify a 260bp fragment of the 28S rRNA gene containing variable regions that enable fungal identification (Nelson *et al.*,2010). PCR was carried out in a total volume of 50 µl containing 20 mM Tris-HC (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1U of Taq DNA Polymerase, (Life Technologies). Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad) with a 12% (wt/vol) polyacrylamide gel, denaturing range 25 - 55%. Electrophoresis was performed at 75 V for 20 hours in 1 X TAE buffer at 60°C. Bands were visualised by staining with Sybr Gold (Life Technologies) for 40 minutes. Following visualisation bands of interest were excised from the gel using a sterile scalpel and incubated in sterile water at 4°C overnight. Where bands appeared at the same position in several lanes of the gel, multiple bands were excised in order to confirm identify of the species present.

Following overnight incubation in sterile water, eluted DNA from excised bands was reamplified using the above thermocycler conditions and U1 (without GC

clamp) and U2 primers. PCR products were purified in preparation for sequencing (Eurofins MWG Operon). Sequences were compared to those in the GenBank database using BLAST (Altschul *et al.*,1990) and percent identity scores were used to identify fungi as described above.

2.9 Antibiotic resistance gene detection in total DNA using PCR

All primers were optimised using total DNA extracted as described above from MRSA (mecA), *E. coli* (*bla_{CTX-M}, bla_{SHV}, and bla_{TEM}*) and *Staphylococci* (AME). All antibiotic resistance gene specific PCRs were carried out in a total volume of 50 µl containing 20 mM Tris-HC (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1U of *Taq* DNA Polymerase, (Life Technologies). All PCR reactions were performed using total DNA extracted directly from swab samples as described above.

2.9.1 Extended spectrum beta-lactamase enzyme genes in total DNA extractions

Primers were identified from the literature to amplify regions of the extended spectrum beta-lactamase genes (*bla_{CTX-M}, bla_{SHV}, and bla_{TEM}*) (Table 2.i). Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, annealing temp for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

2.9.2 *mecA* gene in total DNA extractions

Primers pair MECA-1 and MECA-2, which amplify a 533bp region of the *mecA* gene, were identified from the literature (Murakami *et al.*,1991) (Table 2.i). PCR was performed using total DNA extracted directly from swab samples. Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

2.9.3 Aminoglycoside modifying enzyme genes in total DNA extractions

Primers were identified from the literature that had been previously used to identify aminoglycoside modifying enzyme genes from Gram positive bacteria (Table 2.i). The selected primers had previously been used for environmental bacteria (Heuer *et al.*,2002). PCR was performed using total DNA extracted directly from swab samples. Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, annealing temperature (AT) for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

Target gene	Primer name and sequence	Annealing temperature (AT)	Expected amplicon size	Reference
bla$_{CTX-M}$	**CTX-M F** GCGTGATACCACTTCACCTC	55°C	540bp	(Weill *et al.*,2004)
	CTX-M R TGAAGTAAGTGACCAGAATC			
bla$_{SHV}$	**SHV-F** CTTTACTCGCCTTTATCG	55°C	827bp	(Naiemi *et al.*,2005)
	SHV-R TCCCGCAGATAAATCACC			
bla$_{TEM}$	**TEM-F** ATGAGTATTCAACATTTCCG	50°C	862bp	(Naiemi *et al.*,2005)
	TEM-R GACAGTTACCAATGCTTAATCA			
mecA	**MECA-1** AAAATCGATGGTAAAGGTTGGC	60	533bp	(Murakami *et al.*,1991)
	MECA-2 AGTTCTGCAGTACCGGATTT			
aph(2")-Ia	**Fapha** GCCACAAATGTTAAGGCAATGA	50°C	644bp	(Heuer *et al.*,2002)
	Raph GAATCTCCAAAATCRATWATKCC			
aph(2")-Ic	**Faphc** CCCAAGAGTCAACAAGGTGCAGA	55°C	527bp	(Heuer *et al.*,2002)
	Raph GAATCTCCAAAATCRATWATKCC			
aph(2")-Id	**Faphd** GCGAATGACTGTATTGCATATGA	50°C	572bp	(Heuer *et al.*,2002)
	Raph GAATCTCCAAAATCRATWATKCC			

Table 2.i Primer pairs used to amplifiy fragments of the beta-lactamase, *mecA* and aminoglycoside modifying enzyme (AME) genes from total DNA extractions

2.10 Identification of antibiotic resistance determinant genes from environmental and clinical isolates using PCR

All primers were optimised using total DNA extracted as described above. All antibiotic resistance gene specific PCRs were carried out in a total volume of 50 µl containing 20 mM Tris-HC (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of each primer and 1U of *Taq* DNA Polymerase (Life Technologies), unless otherwise stated. All colony PCR reactions were performed as follows. First a single colony was diluted in 20 µl of sterile water and incubated at 95°C for 20 minutes to ensure lysis. Following lysis 2 µl of the lysate was used as template for PCR reactions.

2.10.1 *mecA* gene in environmental and clinical isolates

The presence of the *mecA* gene in environmental and clinical culturable isolates was investigated using a multiplex colony PCR. The primers selected allow a multiplex PCR to be performed; primers X (GGA ATT CAA AKG AAT TGA CGG GGG C) and Y (CGG GAT CCC AGG CCC GGG AAC GTA TTC AC) target the 16S rRNA gene, giving rise to a 479bp product. Primers Z1 (GTA GAA ATG ACT GAA CGT CCG ATA A) and Z2 (CCA ATT CCA CAT TGT TTC GGT CTA A) amplify a 310bp region of the *mecA* gene (Geha *et al.*,1994). This multiplex PCR can be used to confirm successful lysis of bacterial colonies in the PCR, since true *mecA* negative isolates that had lysed effectively would still give rise to a 479bp product (representing the 16S rRNA gene). Amplification conditions

were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

2.10.2 Aminoglycoside modifying enzyme genes in environmental isolates

The presence of aminoglycoside modifying enzyme (AME) genes was investigated in environmental and clinical *Staphylococci* using a multiplex colony PCR (Table 2.ii). Primers were identified from the literature that amplify of regions of the three AME genes most commonly carried by *Staphylococci* (Ardic et al.,2006). Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

2.10.3 Quaternary ammonium compound resistance genes in environmental isolates

The presence of quaternary ammonium compound resistance (*qac)* genes was investigated using a colony PCR. Primers qacA/B-F (GCT GCA TTT ATG ACA ATG TTT G) and qacA/B-R (AAT CCC ACC TAC TAA AGC AG) amplify a 628bp fragment of the *qac*A/B gene (Ben Saida *et al.*,2009). Amplification conditions were: 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 40°C for 30 seconds, 72°C for 60 seconds, with a final extension of 72°C for 10 minutes.

Target gene	Primer Sequence	Expected amplicon size
aac(6')/aph(2")	GAA GTA CGC AGA AGA GA	491 bp
	ACA TGG CAA GCT CTA GGA	
aph(3')-IIIa	AAA TAC CGC TGC GTA	242 bp
	CAT ACT CTT CCG AGC AA	
ant(4')-Ia	AAT CGG TAG AAG CCC AA	135 bp
	GCA CCT GCC ATT GCT A	

Table 2.ii Primer pairs used to amplifiy fragments of the aminoglycoside modifying enzyme genes from staphylococcal isolates

(Ardic et al.,2006)

2.11 Staphylococcal cassette chromosome typing

The staphylococcal cassette chromosome *mec* (SCC*mec*) was typed using PCR in isolates shown to carry the *mecA* gene. Total DNA was first extracted as described above (Section 2.5) from an overnight culture of the *Staphylococcus* strain isolated on Colorex-MRSA agar. Primers were identified from the literature that amplify fragments of the *mec* and *ccr* complex of the chromosome using two separate multiplex PCR reactions (Kondo *et al.*,2007). Primer pairs, gene targets and expected amplicon size are shown below (Table 2.iii). Both PCRs performed on each sample were carried out in a total volume of 25 µl containing 1x MyTaq DNA polymerase (contamining 0.5 mM dNTPs), 5 pmol of each primer and 2U of MyTaq DNA Polymerase (Bioline). The concentration of $MgCl_2$ in MPCR1 was 3.2 mM and 2 mM in MPCR2. Amplification conditions were: 95°C for 3 minutes, 35 cycles of 95°C for 30 seconds, 57°C for 15 seconds, 72°C for 90 seconds, with a final extension of 72°C for 5 minutes.

The size of the resulting fragments was then used to determine which genes or alleles were present for each complex. The results from the two PCRs were then used to assign SCC*mec* type, where possible, according to the current guidelines (Table 2.iv) (IWG-SCC,2009).

Primer	Target gene(s) or allele(s)	Primer sequence	Expected amplicon size	Type
MPCR 1				
mA1	mecA	TGCTATCCACCCTCAAACAGG	286bp	
mA2		AACGTTGTAACCACCCCAAGA		
α1	ccrA1-ccrB	AACCTATATCATCAATCAGTACGT	695bp	1
α2	ccrA2-ccrB	TAAAGGCATCAATGCACAAACACT	937bp	2
α3	ccrA3-ccrB	AGCTCAAAAGCAAGCAATAGAAT	1791bp	3
βc	ccrA4-ccrB4	ATTGCCTTGATAATAGCCITCT	1287bp	4
α4.2		GTATCAATGCACCAGAACTT		
β4.2		TTGCGACTCTCTTGGCGTTT		
γR	ccrC	CCTTTATAGACTGGATTATTCAAAATAT	518bp	5
γF		CGTCTATTACAAGATGTTAAGGATAAT		
MPCR 2				
mI6	mecA-mecI	CATAACTTCCCATTCTGCAGATG	1963bp	A
IS7	mecA-IS1272	ATGCTTAATGATAGCATCCGAATG	2827bp	B
IS2	mecA-IS431	TGAGGTTATTCAGATATTTCGATGT	804bp	C
mA7		ATATACCAAACCCGACAACTACA		

Table 2.iii Primer pairs used to amplifiy fragments of the ccr (MPCR 1) and mec (MPCR 2) complex from DNA extracted from staphylococcal isolates

(Kondo *et al.*,2007)

SCC*mec* Type	ccr complex	mec complex
I	1	B
II	2	A
III	3	A
IV	2	B
V	5	C2
VI	4	B
VII	5	C1
VIII	4	A

Table 2.iv Classification system for designation of SCC*mec* type

(IWG-SCC,2009)

2.12 Identification of *Staphylococcal* isolates using *tuf* gene PCR and sequencing

The *tuf* gene of isolated environmental *Staphylococci* was amplified using a gene specific PCR and sequenced. Total DNA was first extracted as described above (Section 2.5) from an overnight culture of the *Staphylococcus* strain isolated. Primers tuf_32_FW (CCAATGCCACAAACTCGTGA) and tuf_512_RV (CAGCTTCAGCGTAGTCTAATAATTTACG) amplify a 480bp fragment of the *tuf* gene (Loonen *et al.*,2012, Loonen *et al.*,2011). Amplification products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol and sent to Eurofins MWG Operon for sequencing. Sequences were compared to those in the GenBank database, similarity scores according to BLAST were used to identify bacteria to the genus and species level.

3 Investigation of the microbial ecology of the orthopaedic ward environment under non-outbreak conditions using PCR-DGGE

3.1 Background and Aims

It is clear that the hospital environment plays an important role as a source of microorganisms (Backman *et al.*,2012, Breathnach *et al.*,2012, Doan *et al.*,2012, Muzslay *et al.*,2012, Dancer *et al.*,2009, Boyce,2007). However most previous studies of the environment have focused on the detection of specific culturable pathogens (Kembel *et al.*,2012a) and little is known about the accumulation of microorganisms within the hospital environment over time or their persistence between cleaning cycles. A more comprehensive study of the total microbial population is therefore needed.

The aim of this study was to apply PCR-DGGE to characterise the microbial ecology of the environment of two orthopaedic wards under normal operating conditions, i.e. in the absence of outbreaks.

The objectives were to:

- Characterise bacteria present in the orthopaedic ward environment using PCR-DGGE
- Determine the environmental distribution of bacterial species

- Investigate the accumulation of bacteria within the environment following routine cleaning
- Characterise bacteria present in the environment of a new orthopaedic ward before and after opening
- Characterise the metabolically active proportion of the environmental bacterial community using total RNA and PCR-DGGE
- Enumerate microorganisms present at different sampling sites
- Isolate and identify culturable microorganims in the environment

3.2 Method summary

Environmental samples were taken from ward A and B on 12 separate occasions from the ward floor, bed rails, patient chairs and underside and tops of over bed tables. Sampling was performed on ward C before it opened as an orthopaedic ward on a single occasion and on two occasions following 6 months in operation as an orthopaedic ward. Total DNA and RNA was extracted directly from microorganisms eluted from swabs and cDNA was generated from total RNA extracts. Fragment of the 16S rRNA gene were amplified and separated using PCR-DGGE. Retrieved sequences were used to identify bacteria to the genus and species level.

Enumeration of environmental bacteria was performed on on blood (7% sheep blood), C.L.E.D., MacConkey's, *Burkholderia cepacia* and *Pseudomonas* Sel C.N. agars (E&O Laboratories Ltd) media. Representative colonies were sub-cultured from each plate and identified by sequencing of the 16S rRNA gene.

3.3 Results

3.3.1 Species and Genera identified in the ward environment using PCR-DGGE

PCR-DGGE was used to characterise the microbial ecology of two orthopaedic ward environments. 16S rRNA gene fragments were amplified using PCR from total DNA extractions from environmental swab samples. Fragments were resolved using DGGE and retrieved fragments were sequenced and compared with those in the GenBank database in order to identified bacteria present in the environment.

Bands in the lower portion of the DGGE gels were well resolved, readily excised and their sequences used to identify bacteria to the genus and species level by comparison with the GenBank database. Multiple poorly resolved bands were observed in the upper portion of every DGGE gel. Sequencing of these bands revealed ambiguous sequences with low percentage match to species in the database. Sequences matching the same species were frequently retrieved from the lower section of the same gel with percentage similarity >97%. It appeared that multiple bands were retrieved from single species and that resolution of copies of these sequences in the upper section (lower denaturant concentration) of the gel was poor, resulting in sequences of poor quality. This was confirmed using DNA extracted from pure cultures of *Escherichia coli* DH5α, *Staphylococcus aureus* SH1000 and *Pseudomonas aeruginosa*. The

PCR-DGGE gels of the pure cultures showed two bands per species, one in the upper portion of the gel that co-migrates with different genera and another in the lower portion of the gel that shows better resolution and can be used for species identification (Figure 3.1). The upper and lower bands were sequenced, confirming a >99% match to each other and >99% match to the expected species. This indicated that the bands visible in the upper portion of the gel were artifacts possible due to the use of degenerate primers, the presence of multiple ribosomal RNA operons or loss of the GC clamp due to failure of the polymerase to synthesise completely to the end of the linear template strand after the first cycle of PCR. However as the same sequences could also be retrieved from the lower portion of the gel, these articfacts do not effect the ability to characterise the microbial population.

Poorly resolved bands in the upper portion of the gel from environmental total DNA extractions were sequenced and the resulting chromatograms used to confirm a mixture of sequences present, these sequences were then excluded from further analysis. Bands from the lower portion of the gel, with better resolution, were used for species identification (Figure 3.2).

A total of 150 samples were analysed using PCR-DGGE. From the DGGE gels 90 resolved bands (which represented the mobilities of 220 visible bands) were excised from the lower potion of the DGGE gels (An example gel is shown in Figure 3.2) and the resulting sequences were compared to those in the

GenBank database using BLAST. Only sequences with greater than 200 aligned residues were included and percentage identity scores were used as criteria for identification of bacteria to the genus (≥97%) and species (≥99%) level (Table 3.i).

In addition sequencing chromatograms were assessed for the quality of sequence. Sequences with less than 200 aligned residues or with similarity scores <97% were rejected. Chromatograms of rejected sequences often suggested mixed sequences possibly as a result of co-migration or insufficient resolution of the DGGE gel (Figure 3.3). All sequences passing the designated criteria were deposited in the GenBank database using Bankit and assigned an accession number (Table 3.i). The sequences of suitable quality were used to identify 90.5% (199/220) of the visible bands to genus and species level.

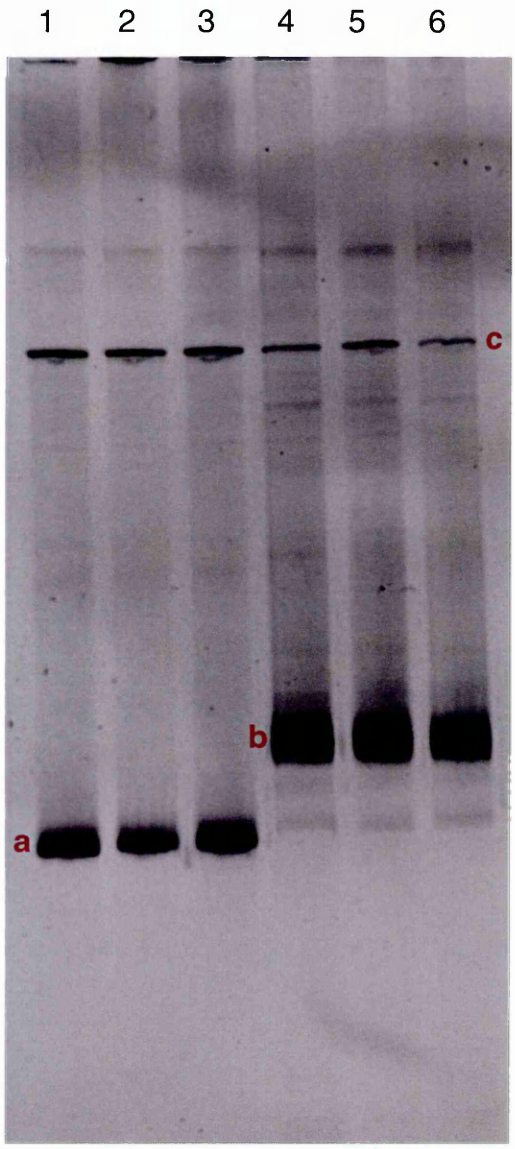

Figure 3.1 PCR-DGGE of pure culture of *Escherichia coli* DH5α (lanes 1-3) and *Staphylococcus aureus* SH1000 (lanes 4-6)

Two dominant bands are observed when PCR products from pure culture are resolved using DGGE; a) *E. coli* specific band, b) *S. aureus* specific band, c) co-migrating band observed for both *E. coli* and *S. aureus*

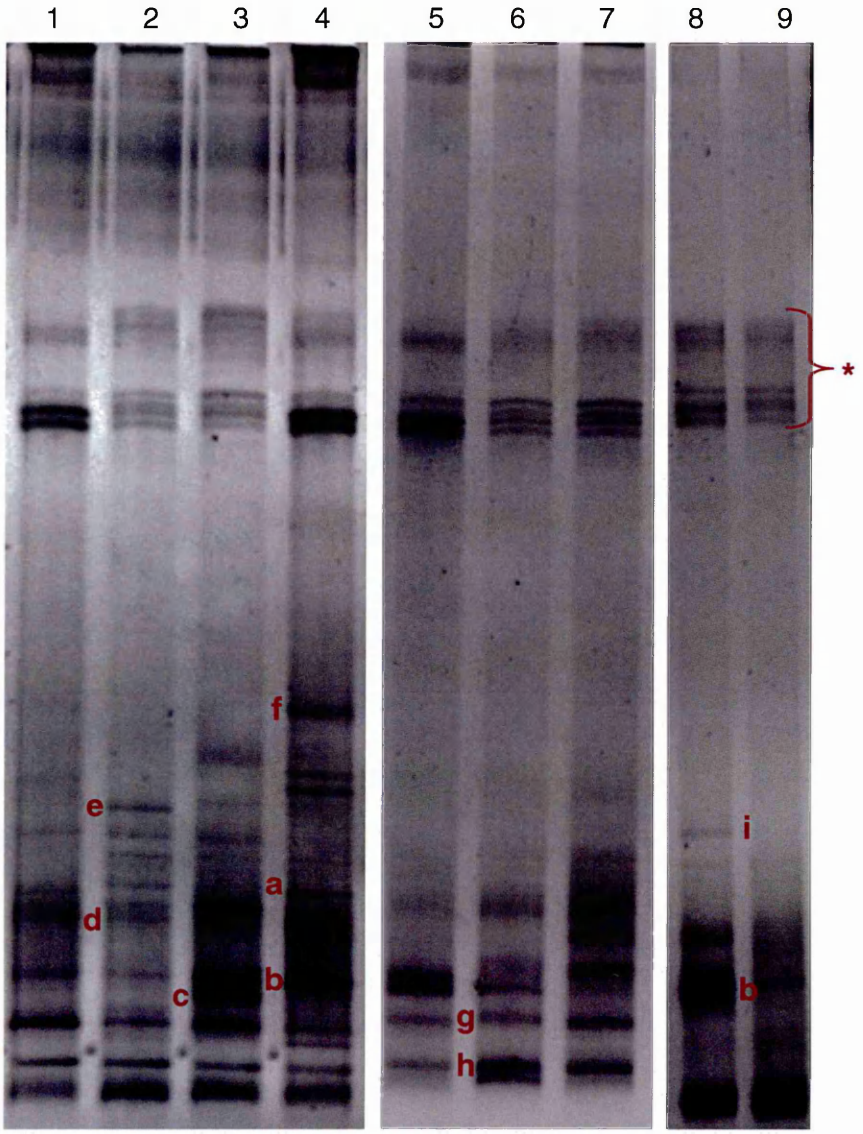

Figure 3.2 An example of PCR-DGGE showing bacterial diversity in orthopaedic ward floor (lanes 1-4), bedrail (lanes 5-7) and chair (lanes 8-9) samples

Dominant bands were identified as corresponding to a) *Staphylococcus epidermidis,* b) *Staphylococcus hominis*, c) *Staphylococcus saprophyticus,* d) *Ruminococcus gnavus*, e) *Clostridium* sp., f) *Chryseobacterium* sp. g) *Corynebacterium* sp., h) *Kocuria rosea*, i) *Faecalibacterium* sp. * Poorly resolved PCR product observed in upper portion of all gels.

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Floors	*Bacillus* sp	86%	277	n/a
Floors	*Blautia glucerasea*	99%	519	KC310435
Floors	*Chryseobacterium* sp	99%	452	KC310430
Floors	*Clostridium* sp	86%	63	n/a
Floors	*Clostridium* sp	96%	521	KC310431
Floors	*Cupriavidus* sp	95%	296	n/a
Floors	*Cupriavidus* sp	93%	333	n/a
Floors	*Enterobacter* sp	87%	268	n/a
Floors	*Enterococcus* sp	97%	390	KC540709
Floors	*Faecalibacterium prausnitzii*	99%	518	KC540707
Floors	*Faecalibacterium prausnitzii*	99%	515	KC310442
Floors	*Faecalibacterium prausnitzii*	99%	361	KC684876
Floors	*Faecalibacterium prausnitzii*	99%	267	KC310434
Floors	*Faecalibacterium* sp	98%	444	KC684874
Floors	*Lactococcus lactis*	97%	186	n/a
Floors	*Lactococcus lactis*	96%	360	n/a
Floors	*Marinococcus halophilus*	99%	511	KC310427
Floors	*Massilia dura*	99%	542	KC310443
Floors	*Massilia dura*	99%	537	KC540706
Floors	*Oscillibacter* sp	95%	508	n/a
Floors	*Ruminococcus gnavus*	100%	510	KC310428
Floors	*Ruminococcus gnavus*	99%	520	KC310432
Floors	*Ruminococcus gnavus*	99%	493	KC310438
Floors	*Ruminococcus gnavus*	99%	426	KC310436
Floors	*Ruminococcus* sp	97%	519	KC310440
Floors	*Ruminococcus* sp	80%	287	n/a
Floors	*Sphingobacterium* sp	100%	83	n/a
Floors	*Staphylococcus aureus*	99%	552	KC310441
Floors	*Staphylococcus epidermidis*	99%	365	KC310429
Floors	*Staphylococcus epidermidis*	99%	224	KC310433
Floors	*Staphylococcus haemolyticus*	99%	528	KC540705
Floors	*Staphylococcus hominis*	100%	532	KC310426
Floors	*Staphylococcus hominis*	99%	547	KC310437
Floors	*Staphylococcus hominis*	99%	539	KC310444
Floors	*Staphylococcus hominis*	99%	394	KC540704

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Floors	*Staphylococcus saprophyticus*	100%	472	JX846615
Floors	*Staphylococcus* sp	99%	237	KC684875
Floors	*Staphylococcus* sp	97%	568	KC684877
Floors	*Staphylococcus* sp	97%	305	KC540710
Floors	*Staphylococcus* sp	97%	344	KC540708
Floors	*Staphylococcus* sp	97%	377	KF512011
Floors	*Staphylococcus* sp	96%	300	n/a
Floors	*Staphylococcus* sp	96%	297	n/a
Floors	*Staphylococcus* sp	91%	347	n/a
Floors	*Staphylococuss* sp	95%	301	n/a
Floors	*Swine fecal bacterium*	76%	279	n/a
Chairs	*Clostridium nexile*	99%	507	KC540736
Chairs	*Clostridium* sp	96%	50	n/a
Chairs	*Corynebacterium tuberculostearicum*	100%	521	KC540729
Chairs	*Corynebacterium tuberculostearicum*	99%	502	KC540732
Chairs	*Corynebacterium tuberculostearicum*	100%	495	KC540730
Chairs	*Cupriavidus metallidurans*	94%	373	n/a
Chairs	*Faecalibacterium prausnitzii*	99%	452	KC540733
Chairs	*Faecalibacterium prausnitzii*	97%	66	n/a
Chairs	*Faecalibacterium* sp	97%	452	KC684880
Chairs	*Faecalibacterium* sp	98%	371	KC540739
Chairs	*Ruminococcus bromii*	99%	70	n/a
Chairs	*Ruminococcus* sp	95%	225	n/a
Chairs	*Staphylococcus haemolyticus*	99%	530	KC540734
Chairs	*Staphylococcus hominis*	99%	543	KC540741
Chairs	*Staphylococcus hominis*	99%	542	KC540740
Chairs	*Staphylococcus hominis*	99%	537	KC540738
Chairs	*Staphylococcus hominis*	99%	215	KC540731
Chairs	*Staphylococcus pettenkoferi*	99%	531	KC540737
Chairs	*Streptococcus* sp	98%	561	KF512010
Chairs	*Streptococcus parasanguinis*	99%	221	KC540735
Over bed tables	*Bacillus* sp	93%	174	n/a
Over bed tables	*Capnocytophaga leadbetteri*	99%	519	KC540748
Over bed tables	*Escherichia coli*	100%	542	KC540745
Over bed tables	*Kocuria rosea*	99%	524	KC540744

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Over bed tables	*Leptotrichia* sp	98%	517	KC540746
Over bed tables	*Neisseria meningitidis*	96%	361	n/a
Over bed tables	*Staphylococcus haemolyticus*	99%	539	KC540743
Over bed tables	*Veillonella dispar*	99%	546	KC540747
Over bed tables	*Veillonella dispar*	99%	534	KC540742
Bed rails	*Brevundimonas* sp	100%	533	KC540727
Bed rails	*Corynebacterium amycolatum*	99%	488	KC540752
Bed rails	*Corynebacterium* sp	98%	405	KC540749
Bed rails	*Corynebacterium* sp	96%	441	n/a
Bed rails	*Corynebacterium tuberculostearicum*	100%	34	n/a
Bed rails	*Faecalibacterium prausnitzii*	91%	180	n/a
Bed rails	*Kocuria rosea*	100%	512	KC540750
Bed rails	*Kocuria rosea*	99%	484	KC540751
Bed rails	*Paracoccus* sp	99%	420	KC540728
Bed rails	*Staphylococcus capitis*	99%	523	KC540726
Bed rails	*Staphylococcus* sp	99%	537	KC684879
Bed rails	*Staphylococcus* sp	99%	422	KC684878
Bed rails	*Staphylococcus* sp	95%	296	n/a
Bed rails	*Staphylococcus* sp	92%	542	n/a

Table 3.i Sequences retrieved using PCR-DGGE and identified by comparison with the GenBank data base using BLAST.

Bacteria and genera identified to the genus (≥97%) and species (≥99%) level. Sequences with similarity scores <97% were rejected; some of which had migrated the same distance as successfully identified sequences (orange), other that had migrated different distances (red).

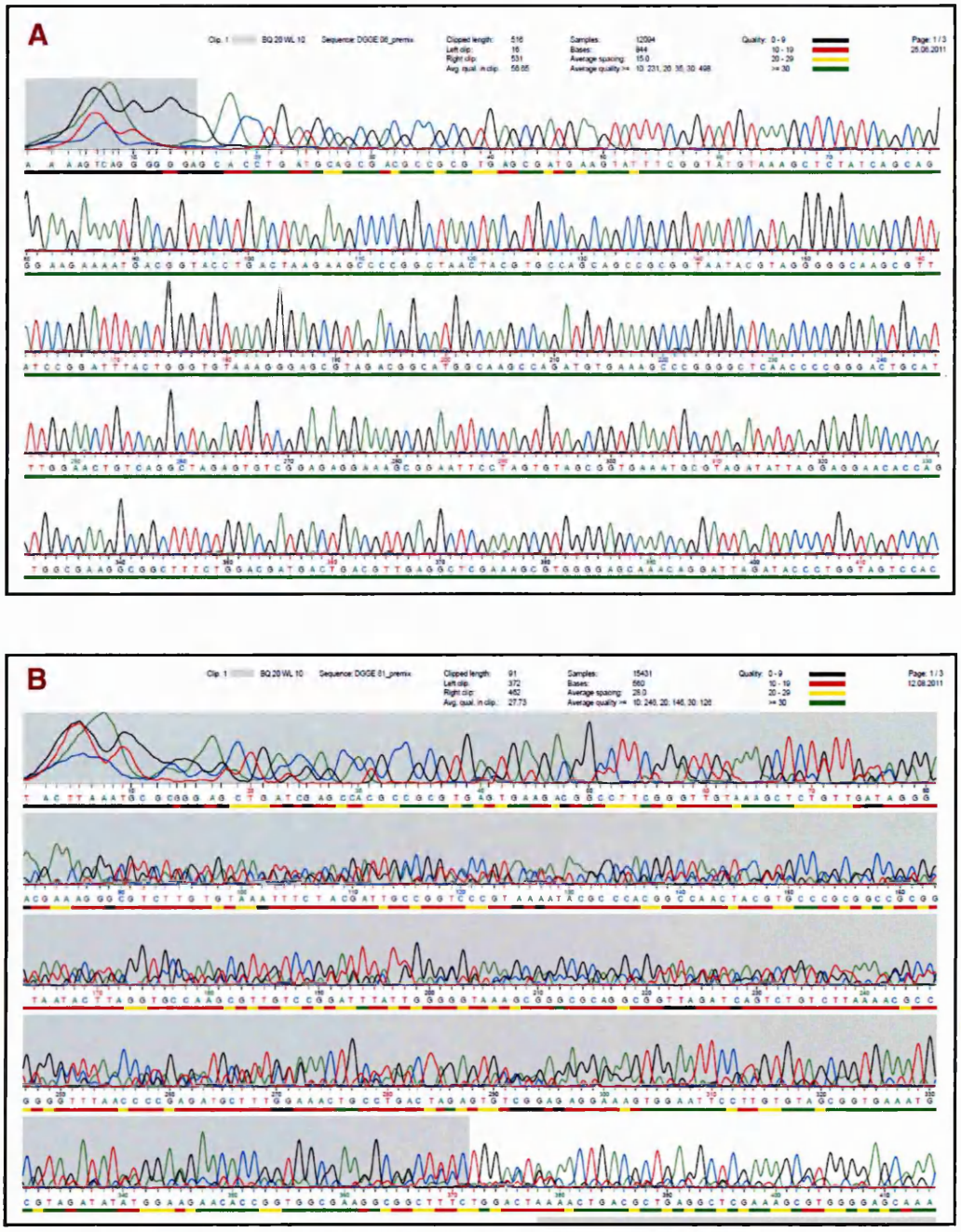

Figure 3.3 Representative sequencing chromatograms for DNA amplified from excised DGGE bands

A) Good quality sequence retrieved using PCR-DGGE, shown to be 99% match to *Ruminococcus gnavus,* B) Poor quality sequence from a poorly resolved band in upper portion of a DGGE gel, chromatogram suggests a mixture of sequences are present.

A total of 29 previously described species, representing 18 different genera, were identified by matching sequences from representative16S rRNA amplicons that were retrieved from total DNA extractions after PCR-DGGE and successfully identified using BLAST.

In order to characterise the environment, species identified by sequence comparison with the database were grouped according to their "likely origin", these are the sites where these species are typically known to be associated with, for example *Staphylococci* are typically associated with the skin.

Grouping of these 29 species according to their "likely origin", suggested that the environment is dominated by species typically associated with the human skin and human intestines including aerobic and strictly anaerobic species. A similar pattern was observed on both wards investigated; sequences similar to skin associated microbes accounted for 69.3 and 63.0% of the total sequences retried from ward A (n=101) and B (n=119) respectively. Intestinal microbe associated sequences were the second most commonly retrieved group of sequences accounting for 18.8 and 19.3%, respectively. While environmental (2.0% of ward A and 5.0% of ward B) and human oral cavity (2.0% of ward A and 1.7% of ward B) associated sequences were retrieved less frequently.

Since a similar pattern was seen on both wards the data was amalgamated for further analysis (Table 3.ii). Overall, species most frequently identified in the orthopaedic department ward environment using PCR-DGGE are typically associated with human skin (65.9% of all PCR amplicons, n=220) and human intestines (19.1%). Other, less frequently detected species are typically associated with the oral cavity (1.8%), or are typically considered to be of environmental origin (3.6%). A further 9.5% of the retrieved sequences (represented by 17 bands) yielded similarity scores <97% to known bacterial genera including *Lactococcus, Enterobacter* and *Cupriavidus* sp (similarity scores <94%) and so were excluded from Table 3.ii because they did not show strong similarity to well characterised bacteria.

Over two thirds (20/29) of the species detected in a total of 150 samples from dry surfaces of the environment were Gram positive. This trend was observed on both wards. Overall, 84% (85/101) of the sequences retrieved from Ward A and 76% (91/119) of sequences retrieved on Ward B matched previously described Gram positive species.

The four species most frequently detected using PCR-DGGE were *S. hominis* (27.7% of identified PCR amplicons), *S. haemolyticus* (8.2%), *Kocuria rosea* (5.9%) and *Corynebacterium* sp. (5.5%). *Staphylococcus* was the most frequently detected genus. Six species of coagulase negative staphylococci accounted for 54.8% of all 16S rRNA fragments retrieved by PCR.

Species	Likely origin	Percentage of samples yielding 16S rRNA gene sequence at environmental sampling sites				
		Floor 1 hours after clean (n=12)	Floor 23 hours after clean (n=48)	Bed Rails (n=18)	Over bed Tables (n=36)	Patient Chairs (n=36)
Staphylococcus hominis	Skin Flora	33	85	-	-	44
Staphylococcus haemolyticus		25	8	-	6	25
Kocuria rosea		-	-	67	3	-
Corynebacterium sp		-	-	67	-	-
Staphylococcus pettenkoferi		-	-	-	-	28
Staphylococcus sp		33	13	-	-	-
Corynebacterium tuberculostearicum		-	-	-	-	17
Staphylococcus epidermidis		-	10	-	-	-
Staphylococcus capitis		-	-	17	-	-
Staphylococcus aureus		-	6	-	-	-
Staphylococcus saprophyticus		-	4	-	-	-
Corynebacterium amycolatum		-	-	11	-	-
Ruminococcus gnavus	Intestinal Flora	-	21	-	-	-
Faecalibacterium prausnitzii		8	13	-	-	3
Enterococcus sp		8	10	-	-	-
Faecalibacterium sp		-	4	-	-	9
Blautia glucerasea		-	8	-	-	-
Clostridium nexile		-	-	-	-	8
Veillonella dispar		-	-	-	8	-
Escherichia coli		-	-	-	6	-
Clostridium sp		-	2	-	-	-
Streptococcus parasanguinis	Oral Flora	-	-	-	-	6
Capnocytophaga leadbetteri		-	-	-	3	-
Leptotrichia sp		-	-	-	3	-
Chrysteobacterium sp	Environment associated	-	2	-	-	-
Marinococcus halophilus		-	2	-	-	-
Massilia dura		8	2	-	-	-
Brevundimonas sp		-	-	11	-	-
Paracoccus sp		-	-	11	-	-

Table 3.ii Species identified from partial 16S rRNA sequences retrieved from environmental samples

Only 11.8% of the PCR fragments yielded sequences that matched Gram negative species. Some of which including *Escherichia coli*, *Capnocytophaga leadbetteri*, and *Leptotrichia* sp were species that are associated with the human body. Of the 9 Gram negative species detected, 3 were detected only once during the sampling programme (Appendix 1).

3.3.2 Environmental distribution of bacterial species identified using PCR

Specific sequences were associated with different sample sites in the orthopaedic ward environment. *Kocuria rosea* and *Corynebacterium* sp. were identified in 67% of bed rail samples (n=18). These species were shown to be recovered repeatedly from the bed rails on different sampling occasions (Appendix 1).

Species associated with skin were frequently detected in patient chair samples, including *Staphylococcus hominis* and *Staphylococcus haemolyticus* identified in 44% and 25% of chair samples respectively (n=36). Species typically associated with human intestines, including two non-pathogenic *Clostridia* spp. were also detected on patient chairs (Table 3.ii).

Samples from over bed tables yielded sequences that matched six different species typically associated with the oral cavity, skin and human intestines (e.g. Gram-negative *Escherichia* coli and *Veillonella* sp.) (Table 3.ii). *Escherichia* coli, *Veillonella* sp., *Capnocytophaga leadbetteri* and *Leptotrichia* sp. Were all detected in a single swab sample (Appendix 1).

Samples from ward floors, taken 23 hours after cleaning, yielded sequences that matched six *Staphylococcus* spp., the most frequently detected being *S. hominis* detected in 85% of floor samples taken 23 hours after cleaning (n=48). Over 24% of the total sequences from floors (n=98) matched non-pathogenic bacteria of the order Clostridiales that were classified as associated with human intestines (Table 3.ii) including *Faecalibacterium* sp. and *Ruminococcus gnavus,* and *Clostridium* spp..

3.3.3 Change in microbial species and genera identified in the ward environment over a 24 hour period

In order to understand the effect of routine floor cleaning on the microbial community, a series of 12 samples were taken from floors (each on a different sampling occasion) during the hour immediately after cleaning and compared with those results from samples taken 22 hours later (n=48). Samples taken just after cleaning yielded a lower diversity of species than those taken 22 hours later. The most frequently retrieved sequences matched *Staphylococcus*, specifically *S. hominis* and *S. haemolyticus*. Occasionally, sequences matching *Faecalibacterium prausnitzii* and *Enterococcus* were retrieved (Fig 3.4). Gram-negative strains such as *Cupriavidus sp*, were also retrieved but these had low similarity scores and so were excluded from Figure 3.4.

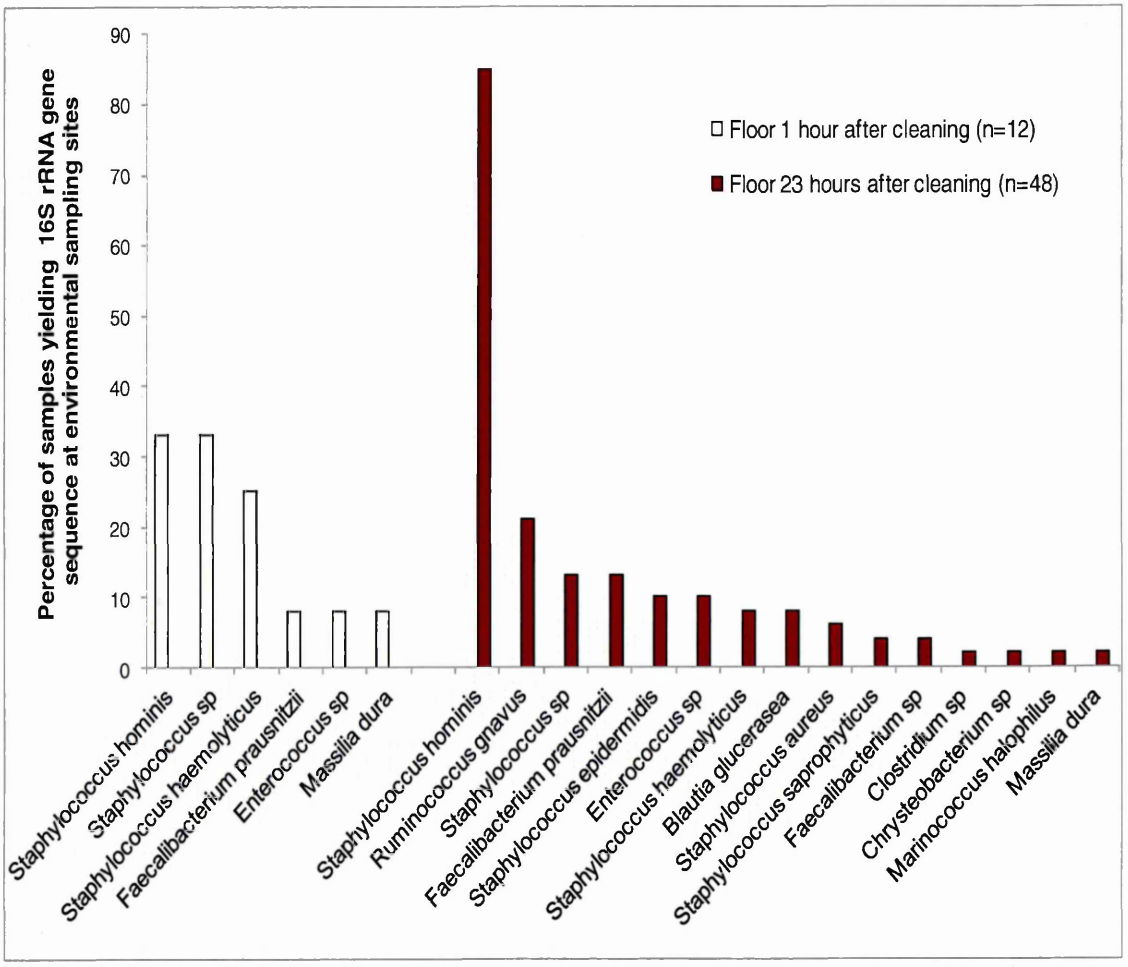

Figure 3.4 Frequency of observed bacterial species on the floors of the orthopaedic department at 1 and 23 hours after cleaning identified using PCR-DGGE

3.3.4 Contamination of a new orthopaedic ward following patient admission

Swab samples (n=5) were taken from ward C prior to opening as an orthopaedic ward and six months later (n=10). PCR-DGGE indicated that there was little bacterial diversity in this environment as only a few bands were visible (Fig 3.5) Sequences similar to the genus *Comamonas* were detected using PCR-DGGE prior to ward opening (Table 3.iii). A sequence similar to *Oxalobacteraceae* was also retrieved however only 83bp of the sequence aligned to the database sequence and so this was excluded.

Sequences similar to a range of microorganisms associated with human flora were detected at six months into use as an orthopaedic ward, these included Coagulase negative *Staphylococci, Streptococcus* spp, *Corynebacterium tuberculostearicum* and *Anaerococcus prevotii* (Table 3.iv). However the *Corynebacterium, Staphylococcus* and *Streptococcus* sequences showed <97% match with previously characterised bacteria in the database.

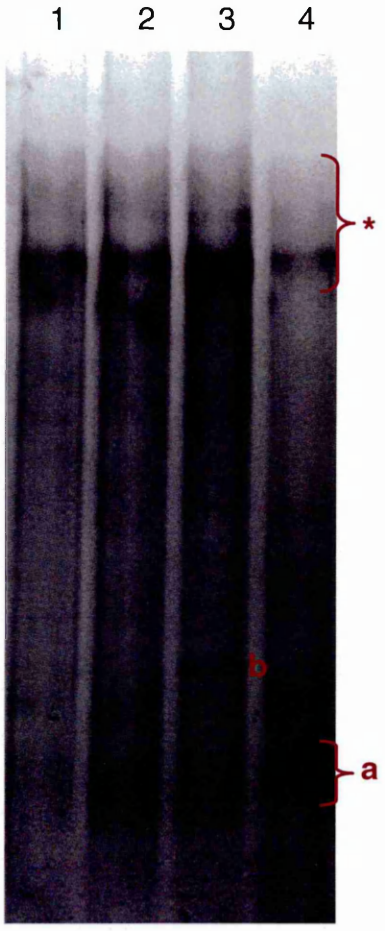

Figure 3.5 PCR-DGGE showing bacterial diversity in an orthopaedic ward prior to opening

a) *Comamonas* spp. , b) *Oxalobacteraceae* (<200 aligned residues), * Cluster of poorly resolved PCR product observed in upper portion of all gels

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Floors	*Oxalobacteraceae bacterium*	98%	83	n/a
Floors	*Comamonas aquatica*	99%	533	KC540711
Floors	*Comamonas aquatica*	99%	509	KC540712
Floors	*Comamonas sp*	98%	513	KF356692
Floors	*Comamonas sp*	98%	510	KF356693

Table 3.iii Sequences retrieved using PCR-DGGE from ward C prior to opening as an orthopaedic ward.

Identified by comparison with the GenBank data base using BLAST to the genus (≥97%) and species (≥99%) level. Sequences with similarity scores <97% were rejected (red).

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Floors	*Anaerococcus prevotii*	99%	505	KF356694
Floors	*Corynebacterium sp*	95%	360	n/a
Floors	*Staphylococcus sp*	85%	360	n/a
Floors	*Streptococcus sp*	97%	376	KF356695
Floors	*Streptococcus sp*	89%	352	n/a

Table 3.iv Sequences retrieved using PCR-DGGE from ward C 6 months after opening as an orthopaedic ward.

Identified by comparison with the GenBank data base using BLAST to the genus (≥97%) and species (≥99%) level. Sequences with similarity scores <97% were rejected (red).

3.3.5 Culture-independent RNA analysis of bacteria in the ward environment

In order to obtain an indication of whether the organisms detected were viable, cDNA templates were generated by reverse transcription of total RNA extracts of six floor samples and 16S rRNA sequences retrieved by PCR-DGGE of cDNA templates were compared with the corresponding gene sequences obtained using total DNA as a template (Fig 3.6 and Table 3.v). Sequences retrieved using DNA and cDNA (derived from RNA) matched the same taxonomic groups, namely *Staphylococcus aureus*, *Micrococcus luteus*, *Clostridium* sp., *Escherichia* sp, *Faecalibacterium* sp, *Veillonella* sp. and *Paracoccus yeei* (Table 3.vi).

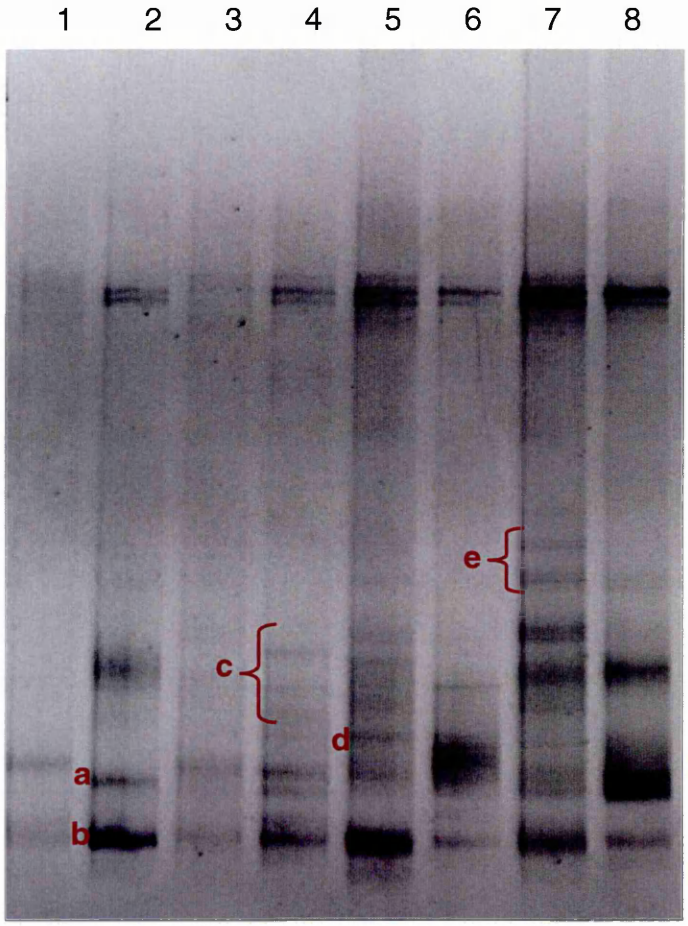

Figure 3.6 PCR-DGGE showing bacterial diversity in an orthopaedic ward using total DNA and RNA as templates

Total DNA extraction template, lanes 1, 3, 5 and 7, total RNA extraction template, lanes 2, 4, 6 and 8

Dominant bands were identified as corresponding to a) *Paracoccus yeei*, b) *Micrococcus luteus*, c) *Staphylococcus* spp, d) *Veillonella* sp, e) *Faecalibacterium* sp

PCR-DGGE template	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
cDNA	*Clostridium sp*	99%	400	KC540724
cDNA	*Clostridium sp*	99%	339	KC540725
cDNA	*Clostridium sp*	99%	341	KC540717
cDNA	*Clostridium sp*	92%	303	n/a
cDNA	*Clostridium sp*	94%	228	n/a
cDNA	*Escherichia sp (coli)*	98%	360	KC540722
gDNA	*Faecalibacterium sp*	98%	397	KC540719
gDNA	*Faecalibacterium prausnitzii*	94%	407	n/a
cDNA	*Micrococcus luteus*	100%	528	KC540715
cDNA	*Moraxella osloensis*	99%	488	KC540723
cDNA	*Paracoccus yeei*	100%	517	KC540714
cDNA	*Paracoccus sp*	98%	420	KC540721
cDNA	*Paracoccus sp*	84%	162	n/a
cDNA	*Paracoccus sp*	86%	330	n/a
gDNA	*Ruminococcus sp*	94%	158	n/a
cDNA	*Staphyloccoccus hominis*	99%	472	KC540716
cDNA	*Staphylococcus aureus*	99%	534	KC540720
cDNA	*Staphylococcus epidermidis*	99%	514	KC540713
cDNA	*Staphylococcus sp*	98%	362	KF512012
cDNA	*Staphylococcus sp*	87%	260	n/a
cDNA	*Veillonella sp (dispar)*	98%	480	KC540718

Table 3.v Sequences retrieved using PCR-DGGE from floor total DNA and RNA extractions and identified by comparison with the GenBank data base using BLAST.

Bacteria and genera identified to the genus (≥97%) and species (≥99%) level. Sequences with similarity scores <97% were rejected; some of which had migrated the same distance as successfully identified sequences (orange), other that had migrated difference distances (red).

Species identified from partial 16S rRNA sequence	Likely origin	PCR-DGGE template	
		Total DNA (n=6)	Complementary DNA derived from total RNA (n=6)
Faecalibacterium sp	Intestinal Flora	17%	17%
Clostridium sp		83%	83%
Escherichia sp		33%	33%
Veillonella sp		33%	33%
Staphylococcus aureus	Skin Flora	50%	67%
Staphylococcus hominis		50%	50%
Staphylococcus epidermidis		-	16%
Micrococcus luteus		83%	100%
Paracoccus yeei	Environmental	83%	100%
Moraxella osloensis		-	16%

Table 3.vi Comparison of percentage of samples positive for retrieved sequences using total DNA vs. RNA as templates for PCR-DGGE

3.3.6 Enumeration of viable microorganisms in the ward environment

The samples that yielded the highest viable counts of bacteria on defined agars per unit area were those taken from ward floors. When using blood agar, the average count of viable bacteria on floors immediately after cleaning was 1.02 CFU/cm^2 (n=18). When samples were taken at 23 hours after cleaning this increased to 4.03 CFU/cm^2 (n=18). Similar trends were observed when using CLED and Mueller Hinton agar but not when using MacConkey's agar, when significantly lower counts were observed (Figure 3.7).

Average viable counts from bed rails, patient chairs and over bed tables were significantly lower ($p<0.05$) compared to floors. Average counts obtained using blood and CLED agar varied between 0.04 CFU/cm^2 (underside of over bed table, ward B n=6) and 5.5 CFU/cm^2 (bed rails, ward B n=6). On ward B, average counts near patient sampling sites more frequently exceeded 2.5 CFU/cm^2 compared to ward A on Mueller-Hinton, blood and CLED agars (Table 3.vii).

No significant difference was observed between CFU counts on ward A and B using Mueller-Hinton ($p=0.31$), Blood ($p=0.47$), CLED ($p=0.67$), or MacConkey's ($p=0.16$) agars.

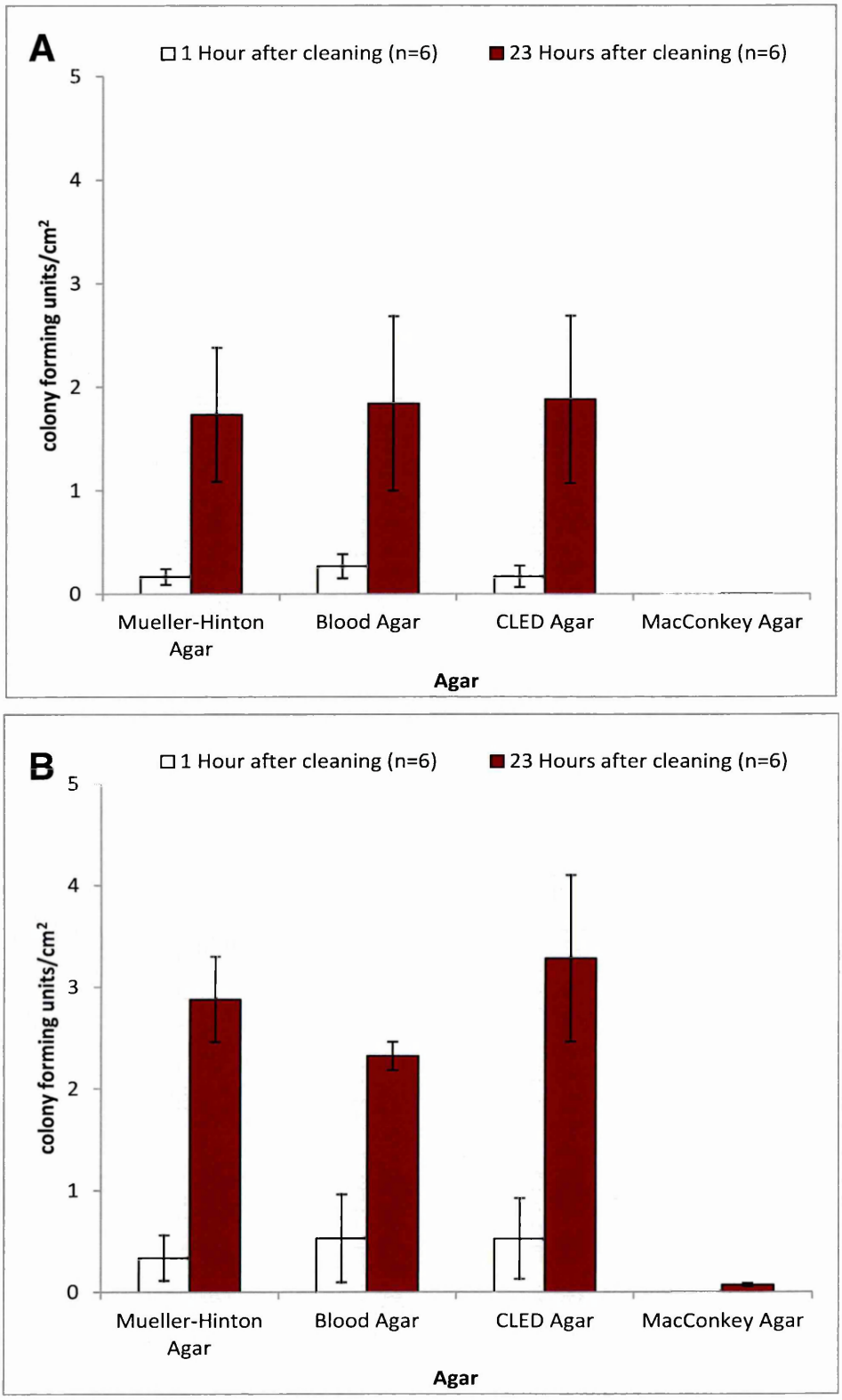

Figure 3.7 Average aerobic colony counts (ACC) from orthopaedic ward floors 1h and 23 h after cleaning on ward A (A) and ward B (B)

Site		Average CFU/cm^2				Number of samples exceeding 2.5 CFU/cm^2			
		MH (n=12)	Blood (n=6)	CLED (n=3)	MC (n=3)	MH (n=12)	Blood (n=6)	CLED (n=3)	MC (n=3)
Ward A	Bed Rails	0.774 (±0.50)	1.464 (±0.25)	1.160 (±0.23)	0.014 (±0.02)	-	-	-	-
	Table Top	1.199 (±3.16)	0.416 (±0.26)	0.478 (±0.12)	0.018 (±0.02)	1	-	-	-
	Table Underside	0.037 (±0.04)	0.054 (±0.03)	0.084 (±0.05)	0.000 (±0.00)	-	-	-	-
	Chair Seat	0.470 (±0.44)	0.865 (±0.54)	1.121 (±0.45)	0.003 (±0.00)	-	-	-	-
	Chair Arm	0.822 (±0.70)	1.228 (±1.19)	0.463 (±0.29)	0.000 (±0.00)	1	1	-	-
Ward B	Bed Rails	2.605 (±1.75)	4.390 (±2.42)	5.486 (±5.04)	0.000 (±0.00)	5	4	2	-
	Table Top	0.284 (±0.24)	0.518 (±0.37)	0.614 (±0.43)	0.000 (±0.00)	-	-	-	-
	Table Underside	0.026 (±0.04)	0.044 (±0.07)	0.076 (±0.10)	0.000 (±0.00)	-	-	-	-
	Chair Seat	6.160 (±12.1)	1.832 (±0.65)	1.092 (±0.15)	0.000 (±0.00)	4	1	-	-
	Chair Arm	2.314 (±2.49)	1.386 (±1.31)	0.935 (±0.29)	0.000 (±0.00)	5	1	-	-

Table 3.vii Average aerobic colony counts (AAC) on near patient sampling sites and the number that exceed the suggested 2.5 CFU/cm^2 standard

3.3.7 Detection of Gram negative bacteria using cultural techniques

The results of PCR-DGGE suggested that Gram-positive cocci dominated dry surfaces. Representative isolates subcultured from blood and CLED agar plates were Gram stained (n=172). Blood is a general purpose medium used for the isolation of most organisms. CLED (cysteine lactose electrolyte deficient) agar is usually used for the identification of urinary pathogens in the clinical laboratory. Gram staining confirmed the dominance of Gram positive cocci (Table 3.viii). Sequencing of the 16S rRNA gene of the 96 Gram positive isolates with cluster of grapes morphology confirmed the presence of Coagulase negative *Staphylococci* in the environment. *Bacillus* species were identified by sequencing five of the Gram positive rods cultured.

Gram Stain	Cocci / Rods	Cell arrangement	Percentage
Positive	Cocci	Cluster of grapes	55.8%
Positive	Cocci	Tetrads	16.9%
Positive	Rod	Individual	14.5%
Negative	Rod	Individual	7.0%
Positive	Cocci	Individual	5.8%

Table 3.viii Gram staining of culturable bacteria isolated using blood and CLED agars (n=172)

In order to investigate whether gram negative species could be detected via cultural techniques, MacConkey, *Pseudomonas* and *Burkholderia cepacia* selective agars were used. MacConkey's agar contains bile salts for the isolation of bile tolerant typically Gram negative organisms. The floors of the orthopaedic department yielded low numbers of colonies on these media; 0.07 CFU/cm^2 on MacConkeys Agar, 0.02 CFU/cm^2 on *Pseudomonas* Sel C.N. agar and 0.04 CFU/cm^2 on *Burkholderia cepacia* agar on ward A. Ward B yielded no isolates on *Pseudomonas* Sel C.N. or *Burkholderia cepacia* agar and only 0.001 CFU/cm^2 on MacConkey's agar. Sequencing of 17 isolates revealed the presence of *Acinetobacter* and *Pseudomonas* on MacConkey and *Pseudomonas* Sel C.N. agars. Isolates obtained using *Burkholderia cepacia* agar were not *B. cepacia* but Gram positive cocci, subsequently identified by 16S rRNA gene sequencing as *Staphylococcus* and *Enterococcus* spp..

3.4 Discussion

Previously traditional cultural techniques have been used to study specific pathogens in the hospital environment. The aim of this study was to characterise the microbial ecology of orthopaedic ward environments under normal operating conditions using PCR-DGGE.

The ward floors, which were cleaned every 24 hours, were found to yield the highest number of viable organisms between cleaning cycles. Using PCR-DGGE and sequencing this study revealed that Gram positive species typically associated with human skin and intestinal flora dominate the environment in both wards of the orthopaedic department. The over bed tables used by patients also revealed the presence of microorganisms associated with the oral cavity.

The bed rails sampled on both wards revealed the specific association of *Kocuria rosea* and *Corynebacterium* species. Both species are generally considered non-pathogenic commensal of human skin and mucosa but have previously been reported to cause opportunistic infection in some cases (Altuntas *et al.*,2004, Knox and Holmes,2002). Skin associated microorganisms were also detected at other sampling sites on the ward; however at other sites *Staphylococci* typically dominate.

Although it is already well recognised that ward environments are contaminated with bacteria from patient skin, here, PCR-DGGE readily yielded information about the relative abundance of different staphylococcal species as well as the specific association of *Kocuria* and *Corynebacteria* with patient bed rails. While *S. hominis* and *S. epidermidis* are known to be the most abundant species on skin (Otto,2010), *S. haemolyticus* was the second most frequently detected sequence in this study. A study by Larson *et al* (1986) showed that significantly more *S. haemolyticus* organisms were isolated from the skin of hospital patients compared to non-patient controls. The authors went on to conclude that S. *haemolyticus* was nosocomially acquired or selected for in the hospital environment (Larson *et al.*,1986). Accurate identification of CNS isolates to species level is difficult and relatively expensive to perform. One of the few studies carried out under non-outbreak conditions by Agvald-Ohman *et al* investigated dissemination of coagulase negative *Staphylococci* (CNS) in a multidisciplinary intensive care unit (ICU). By genotyping nearly 200 isolates collected from colonised patients, these workers concluded that a high proportion of the patients who carried CNS had acquired them whilst on the ward (Agvald-Ohman, Lund and Edlund,2004). These results highlight otherwise invisible deficiencies in infection control measures and the need for research into the role of the environment in causing CNS infections. The application of molecular techniques, such as PCR-DGGE, to study the hospital environment may facilitate such investigations.

The incidence of *S. aureus* and MRSA infections is low amongst patients on wards A and B. Importantly, this study further shows that under normal operating conditions, in the absence of outbreaks, in a low MRSA setting, neither *S. aureus* DNA, nor cultivable *S. aureus* are readily detectable in samples from near patient sites such as bed rails and patient chairs, or from floors.

The use of PCR-DGGE further revealed the frequent and widespread distribution of DNA of Gram positive anaerobic species known to numerically dominate the lower GI tract (Manson, Rauch and Gilmore,2008, Rajilic-Stojanovic, Smidt and de Vos,2007, Zoetendal, Vaughan and de Vos,2006). *Ruminococcus, Faecalibacterium, Enterococcus, Blautia* and *Clostridia* were detected on the floors and chairs; in addition *Escherichia coli* and *Veillonella dispar* were detected on overbed tables. As these species are known to be part of normal human intestinal flora, they may be considered possible indicators of faecal contamination in the environment however these sequences were retrieved in the absence of an outbreak of diarrhoea on either ward. Laborde *et al.* (1993) demonstrated that the presence of faecal associated microorganisms on hand touch sites is a risk factor for the spread of diarrhoeal disease (Laborde *et al.*,1993). Most of the Gram positive anaerobic species were only detected in samples taken just before cleaning. Given the sensitivity of the non-sporulating species (e.g. *Ruminococcus*) to oxygen and detergents and their unlikely persistence in the environment, these data suggest that the environment is recontaminated with such species on a daily basis.

The highest diversity of microorganisms and highest viable counts were observed on the ward floors. The role of the floor environment in HAI is unclear; however, it is safe to assume that the floor represents a potential reservoir for pathogenic microorganisms (Ayliffe, Collins and Lowbury,1966). Evidence from previous studies suggests that activities such as walking may aid dispersal of bacteria present on the floor into the air (Hambraeus, Bengtsson and Laurell,1978).

In addition to understanding the microbial ecology of the floor environment, in this study sampling of the orthopaedic ward floors allowed the investigation of the effect of routine cleaning and change in microbial load and diversity over time. This study found that at both 1 and 23 hours after cleaning *Staphylococci*, particularly *S. haemolyticus* and *S. hominis* dominate the environment. Similarly Kassem *et al.* used PCR-DGGE to show that clinical surfaces remained contaminated with a range of *Staphylococci,* including *S. hominis* and *S. haemolyticus* after cleaning (Kassem, Esseili and Sigler,2011).

A significant change in microbial load was observed over time following routine cleaning, demonstrating that throughout the daily running of the ward the floors become contaminated and that daily cleaning is effective at removing a significant proportion of these microbes.

There are currently no microbiological standards for surface hygiene in the hospital environment. This makes it difficult to assess the efficacy of cleaning regimes used for these environments. Previous studies have suggested that standards similar to those employed in the food industry should be applied to the hospital environment and have suggested that total aerobic colony counts (ACC) on hand touch sites should not exceed 5 CFU/cm^2 (Mulvey *et al.*,2011, Dancer,2004). This study found that the ACC only exceeded this limit on two occasions; the average chair seat count on Mueller-Hinton agar and average bed rail count on CLED agar, both on ward B. However, recently it was suggested that the limit for surface hygiene in the hospital environment should be set at the lower limit of 2.5 CFU/cm^2 (Lewis *et al.*,2008, White *et al.*,2008). If the lower standard is used, swab samples taken from bed rail, over bed table and patient chairs frequently exceeded this on both wards. Genet *et al.* reported even higher ACC (34 CFU/cm^2) for table tops on both a surgical ward and an operating theatre (Genet, Kibru and Hemalatha,2012).

There is debate about the most appropriate method for the evaluation of cleanliness and the assessment of risk in the hospital environment. Other studies have evaluated the use of ATP bioluminescence for the detection of organic matter contamination in the environment. While the use of ATP bioluminescence is more expensive than traditional culture, it is a more rapid technique (Sherlock *et al.*,2009). However as results from the two techniques

do not often correlate with each other it has been suggested that a combination of the two would be a better approach to monitor hospital cleanliness (White, Dancer and Robertson,2007). ATP bioluminescence and ACC measurements do not however give a clear indication of infection risk to patients, although it is assumed in general environmental sites with greater overall contamination present a greater risk. The isolation of indicator organisms e.g. MRSA and VRE may therefore be a more reliable method for determining the risk of acquiring a specific pathogen (Dancer,2008, Goodman *et al.*,2008).

In this study both molecular and conventional culture were used to characterise the microbial population present in the environment. In the orthopaedic ward environment the results showed that there was good agreement in the result of detection of *Staphylococci* by both culture and PCR-DGGE. However intestinal associated genera such as *Clostridia* and *Ruminococcus* were only detected using PCR-DGGE. Many of these are considered to be anaerobic bacteria and therefore would require more specialised techniques in order to culture. PCR-DGGE therefore offers an advantage as the presence of both aerobic and anaerobic bacteria can be identified without the use of specialised media and growth conditions.

The results from the present study are generally in accordance with those of Lemman *et al* (2004) who, using cultural methods demonstrated the dominance of Gram positive species in samples from dry surfaces of ICU and general ward

environments. However Lemman *et al.* (2004) and others also detected a higher proportion of Gram negative organisms such as *Pseudomonas aeruginosa, Escherichia coli* and *Acinetobacter* spp, even on dry surface environments (Weber *et al.*,2010, White, Dancer and Robertson,2007, Lemmen *et al.*,2004, Getchell-White, Donowitz and Groschel,1989, MacArthur *et al.*,1988). During the present study, fast growing, aerobic species such as *Acinetobacter* and *Pseudomonas* were only detected in low numbers using cultural methods.

Here PCR-DGGE was used semi quantitatively to estimate relative abundance of different species. A major criticism of the use of molecular detection methods has been that DNA templates are detectable from non-viable cells. In this study reverse transcription was used to generate template for PCR-DGGE from total RNA extractions. The rRNA content of a bacterial cell has been reported to show a linear relationship with bacterial growth (Poulsen, Ballard and Stahl,1993) and a number of studies have used reverse transcription of bacterial rRNA from total RNA extractions to characterise metabolically active populations in different environments (Hoshino and Inagaki,2013, Inagaki *et al.*,2002). In terms of PCR-DGGE, the use of total DNA as a template can be used to determine the overall microbial diversity of a sample, while the use of reverse transcribed total RNA as a template can provide an indication of the proportion of metabolically active populations (Bassin *et al.*,2011, Dar *et al.*,2007). The retrieval of sequences similar to the same species using both DNA and RNA as a template for PCR-DGGE in this study suggests that these cells were metabolically active at the time of sampling.

qPCR is an alternative method that offers the opportunity to quantify specific pathogens. Mutter *et al* (2009) demonstrated the effectiveness of qPCR for the detection of *Clostridium difficile;* the technique is rapid and allows absolute quantification of specific bacterial contamination (Mutters *et al.*,2009). A review by Espy *et al* (2006) highlighted the benefits of increased sensitivity compared with culture for the detection of antibiotic resistant pathogens including MRSA and Vancomycin resistant Entrococci (VRE) (Espy *et al.*,2006). In the present study PCR-DGGE was chosen over qPCR as it allows semi quantitative characterisation of the total microbial diversity rather than focusing on the quantification of a specific pathogen.

Despite the advantages of molecular techniques over conventional culture PCR-DGGE does have limitations. Reported problems include PCR bias (Suzuki and Giovannoni,1996), artifacts (Kusar and Avgustin,2012), co-migration of species (Gafan and Spratt,2005), variation in GC clamp synthesis (Rettedal, Clay and Brozel,2010), and hetroduplex formation (Muyzer and Smalla,1998, Jensen and Straus,1993). In addition the technique can be time consuming and technically difficult to optimise and perform (Green, Leigh and Neufeld 2009).

Use of universal primers in this study may have resulted in bias in the amplification of sequences. At each stage, there is loss in sampling, DNA extraction, where cells are resistant to breaking open by lysozyme and

lysostaphin, DNA is lost to downstream analysis. It is therefore assumed that this method is likely to yield sequences that are numerically dominant in the sample and this may be the reason why results from PCR-DGGE and culture vary so much. Suzuki and Giovannoni suggested a kinetic PCR bias model whereby in a PCR reaction with a mixed template the final concentration of each tended towards a 1:1 ratio irrespective of starting concentration, which would allow for under and over estimation of some species. However they also suggest this is unlikely to have an effect on environmental samples where the template DNA is diverse (Suzuki and Giovannoni,1996).

In this study multiple bands were observed for single species and poor resolution of the second band was observed. Kowalchuk *et al.* (1997) previously reported that double bands in DGGE gels were due to the use of degenerate primers where a mixture of PCR product are generated and then separated using DGGE (Kowalchuk *et al.*,1997). The reverse primer used in the present study is degenerate and therefore may enable amplification of multiple PCR products from the same sequence. Another explanation is the presence of multiple ribosomal RNA operons. Previous studies have reported multiple bands due to slight sequence variations between operons (Schabereiter-Gurtner *et al.*,2001, Nubel *et al.*,1996). In addition it has previously been shown that attachment of a GC rich region (GC clamp) during amplification results in improved resolution (Sheffield *et al.*,1989). Here failure to attach the GC clamp during PCR amplification of 16S rRNA gene fragments may be responsible for insufficient resolution.

A small proportion of the sequences retrieved were excluded from the study due to poor quality or poor percentage match to sequences in the database. It is possible therefore that this has prevented identification of some species present in this environment. Co-migration of DNA fragments is often responsible for preventing retrieval of clean sequences (Muyzer and Smalla,1998) and may explain some of the poor quality sequences retrieved where the chromatograms suggested mixed sequences. It has previously been reported that DGGE is unable to resolve PCR fragments of closely related species, where low polymorphism is observed for that species in the amplified region (Vallaeys *et al.*,1997). Another factor that may have limited analysis of the bacterial community in this study is that PCR-DGGE can only separate fragments of up to 500bp (Myers *et al.*,1985), which limits the amount of sequence information available for identification. This may, in this study, explain the number of sequences that were only identified to genus and not species level, as not enough sequence information was available to distinguish between similar species that vary little in the portion of the 16S rRNA gene sequence that was analysed.

Another disadvantage of the use of 16S rRNA sequences is that copy number varies greatly between bacteria and therefore can have an impact when attempting to estimate abundance (Case *et al.*,2007, Acinas *et al.*,2004). Kembel *et al.* (2012) demonstrated that assuming the abundance of 16S rRNA genes from a particular bacterial species is a measure of organism abundance

resulted in an underestimation of rare species and an overestimation of the most abundant species. They suggested a method to overcome this problem using a reference database where 16S rRNA gene copy number is known based on bacterial species with fully sequenced genomes. Copy number of an environmental 16S rRNA sequence can then be estimated using the nearest species copy number when it is placed into the reference phylogeny (Kembel *et al.*,2012b).

Although PCR-DGGE has its limitations, in this study it has been used to investigate the total microbial diversity of the hospital environment. Here it has demonstrated a dominance of skin associated microorganisms, in keeping with the results of conventional culture. PCR-DGGE has also highlighted the widespread distribution of intestinal associated typically anaerobic species, providing additional data about the environmental ecology not detected using culture. In addition the use of PCR-DGGE for the separation of cDNA fragments generated from total RNA extraction has been used to determine which species are active in this environment.

4 Investigation of the microbial ecology of the orthopaedic operating theatre environment

4.1 Background and Aims

Previous studies of the operating theatre environment have largely focused on the use of air sampling and cultural techniques. The collaborations established during the project provided the opportunity to undertake a one off sampling session in the orthopaedic operating theatres, in order to characterise the bacteria present on environmental surfaces using PCR-DGGE.

It has been suggested that surfaces and equipment within operating theatres may harbour potential pathogens (Alexander *et al.*,2013, Wahr and Abernathy,2013). A recent cultural study demonstrated that a low proportion of surfaces (< 5%) were contaminated with *Staphylococcus epidermidis,* coliforms and *Pseudomonas aeruginosa* (Ensayef, Al-Shalchi and Sabbar,2009). Bacteria in the operating room may reach the open wound via direct sedimentation of airbourne bacteria into the wound or via contact contaminated environmental surfaces, which are transferred to the wound via surgical instruments or the surgical teams gloved hands (Gosden, MacGowan and Bannister,1998).

This study aimed to use PCR-DGGE to characterise the microbial ecology of the orthopaedic operating theatre during a single morning in order to provide a snapshot of the microbial load and diversity of organisms present.

The objectives were to:

- Characterise bacteria present in the orthopaedic operating theatre environment using PCR-DGGE
- Characterise culturable microorganims in the environment
- Compare microbial load at different environmental sampling sites within the orthopaedic operating suite

4.2 Method summary

Environmental samples were taken on one occasion from the operating suite. Swab samples were taken from sections of the floor of the preparation room, theatres and anaesthetic rooms. Swab samples were also taken from the operating theatre phone, instrument keyboard, equipment shelves and the footwear of theatre staff. Total DNA was extracted directly from microorganisms eluted from swabs. Fragment of the 16S rRNA gene were amplified and separated using PCR-DGGE. Retrieved sequences were used to identify bacteria to the genus and species level.

Enumeration of environmental bacteria was performed on blood (7% sheep blood), C.L.E.D., MacConkey's, *Burkholderia cepacia* and *Pseudomonas* Sel

C.N. agars (E&O Laboratories Ltd). Representative colonies were sub-cultured from each plate and identified by sequencing of the 16S rRNA gene.

4.3 Results

4.3.1 Species and genera identified in the ward environment using PCR-DGGE

PCR-DGGE was used to characterise the microbial ecology of the orthopaedic operating theatre environment. A total of 12 samples were taken from the floors of the orthopaedic operating theatre. A single swab sample was taken from a nurse's clog, theatre phone, theatre keyboard, waste disposal shelf and the anaesthetic room shelves. 16S rRNA gene fragments were amplified using PCR from total DNA extractions from environmental swab samples. Fragments were resolved using DGGE and retrieved fragments were sequenced and compared with those in the GenBank database in order to identified bacteria present in the environment.

A total of 15 bands (representing a total of 31 visible bands) were excised from the resulting DGGE gels (Fig 4.1, Table 4.i). A total of five previously described species, representing five different genera, were identified by matching sequences from the fifteen 16S rRNA amplicons that were amplified from total DNA extractions after PCR-DGGE.

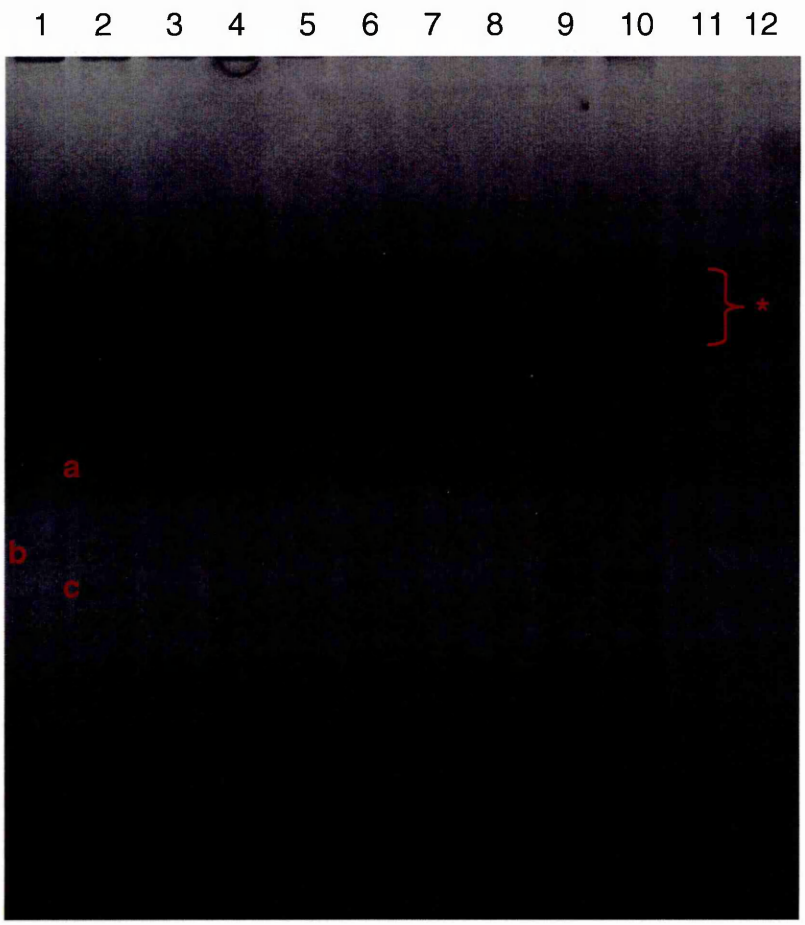

Figure 4.1 PCR-DGGE showing bacterial diversity in orthopaedic operating theatre and adjoining areas

Lanes 1-4 prep room floors, lanes 5-8 operating theatre floors, lanes 9-12 anaesthetic room floors

Dominant bands were identified as corresponding to a) *Chryseobacterium* sp., b) *Methylophilus* sp., c) *Cupriavidus* sp., * Poorly resolved PCR product observed in upper portion of all gels.

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 550)	Assigned Accession Number
Floors	*Chryseobacterium* sp.	99	527	KF356698
Floors	*Chryseobacterium* sp.	99	521	KF356700
Nurse's clog	*Chryseobacterium* sp.	99	236	KF356701
Theatre keyboard	*Chryseobacterium* sp.	99	283	KF356704
Theatre keyboard	*Chryseobacterium* sp.	99	531	KF356705
Floors	*Cupriavidus* sp.	99	536	KF356697
Theatre keyboard	*Cupriavidus* sp.	99	530	KF356703
Nurse's clog	*Cupriavidus* sp.	99	530	KF356706
Floors	*Cupriavidus* sp.	98	520	KF356699
Nurse's clog	*Massilia* sp.	99	278	KF356702
Floors	*Methylophilus* sp.	99	536	KF356696
Theatre keyboard	*Methyloversatilis* sp.	99	535	KF356707

Table 4.i Sequences retrieved from the orthopaedic operating theatres using PCR-DGGE and identified after sequencing by comparison with the GenBank data base using BLAST.

Bacteria and genera identified to the genus (≥97%) level.

Sequences similar to *Cupriavidus* spp. were identified in 72% (13/18) of samples taken from the orthopaedic operating theatre environment; found on the floors, nurse's clog, theatre phone and keyboard. *Chryseobacterium* spp. were also frequently retrieved (Fig 4.2 and Table 4.ii).

4.3.2 Quantification of culturable microorganisms in the orthopaedic operating theatre environment

When using blood agar, the average aerobic colony count (ACC) on floors ranged from 0.003 CFU/cm^2 (±0.002) on the prep room floor to 0.04 CFU/cm^2 (±0.033) on the operating theatre floor. Similar trends were observed when using CLED and Mueller Hinton agar but not when using MacConkey's agar, where no growth was observed for any of the sites sampled (Figure 4.3). The lack of growth on MacConkey's agar may therefore indicate that numbers of Gram negative bacteria are low in this environment.

A significant difference between the different sampling sites was observed using ANOVA. ACC were significantly higher on the floor of the operating theatre compared to the floors in the prep room and anaesthetic room ($p=0.017$).

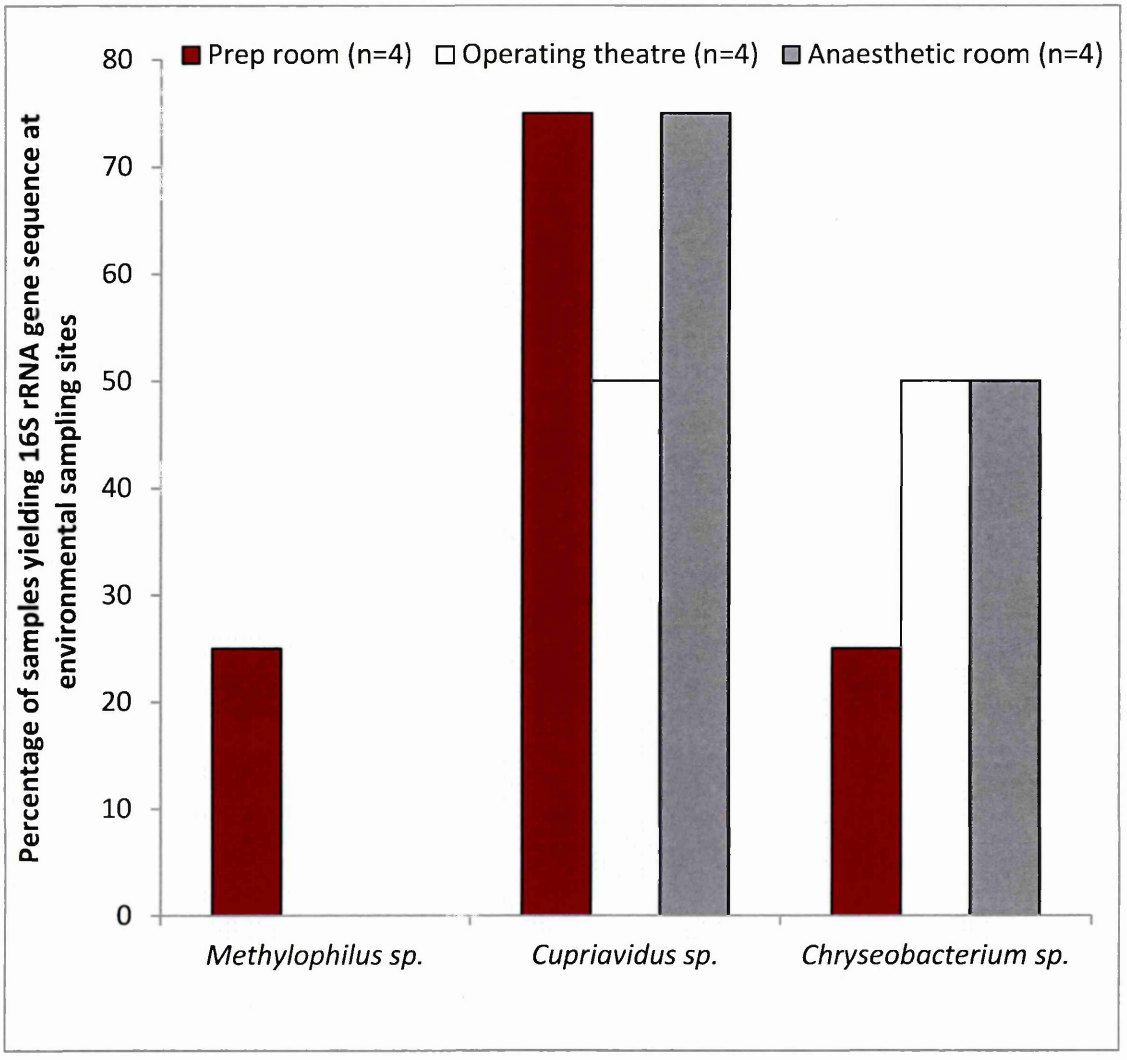

Figure 4.2 Frequency of observed bacterial 16S rRNA gene sequence on the floors of the orthopaedic operating theatre identified using PCR-DGGE

Species identified using 16S rRNA gene sequencing	Nurse's clog (n=1)	Floor – entrance (n=1)	Theatre keyboard (n=1)	Theatre phone (n=1)	Waste disposal shelves (n=1)	Anaesthetic room shelves(n=1)
Chryseobacterium sp.	✓	✓	✓	✓	✓	✓
Cupriavidus sp.	✓	✓	✓	✓	✓	
Methyloversatilis sp.	✓	✓	✓			
Massilia sp.	✓					

Table 4.ii 16S rRNA genes identified in the orthopaedic operating theatre environment using PCR-DGGE

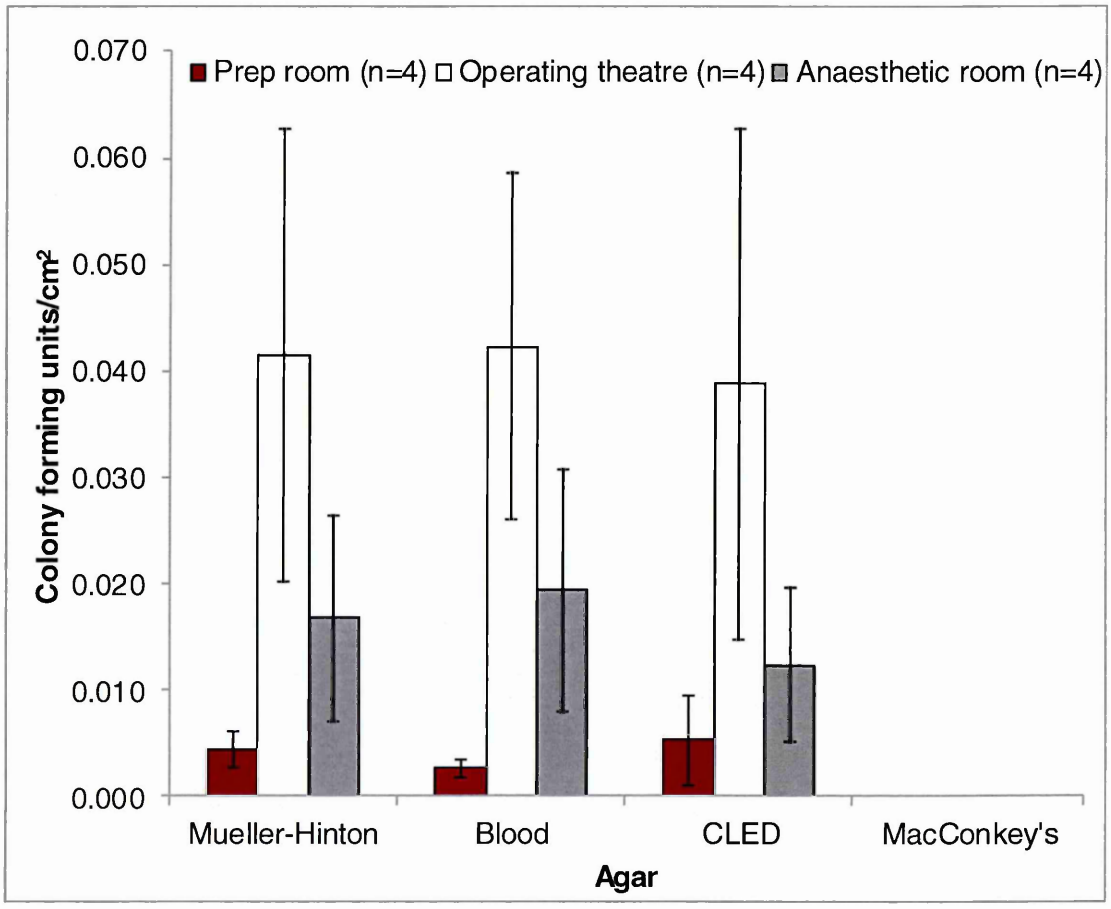

Figure 4.3 Average aerobic colony counts (ACC) from orthopaedic operating theatre floors

4.3.3 Species and genera isolated from the theatre environment using culture

All isolates cultured from the floors of the operating theatre were Gram positive bacteria. The genus *Staphylococcus* dominated the environment, a least one species of *Staphylococcus* was isolated from 92% (11/12) of the swab samples taken from the operating theatre floor. All were non-*aureus Staphylococci,* the most frequently isolated were *S. epidermidis*, *S. warneri* and *S. hominis*, isolated in 50%, 33% and 33% of floor samples respectively. Sample site specific associations were observed for other *Staphylococci; S. lugdunensis* was only recovered from the operating theatre floor and *S. pasteuri* and *S. haemolyticus* were only identified in the prep room. In addition bacteria of other genera including *Enterococcus* and *Bacillus* were only isolated from samples taken from the floor of the operating theatre (Table 4.iii).

Species identified by sequencing 16S rRNA gene	Percentage of samples positive for identified species		
	Prep room (n=4)	Operating theatre (n=4)	Anaesthetic room (n=4)
Staphylococcus epidermidis	75	50	25
Staphylococcus warneri	50	25	25
Staphylococcus hominis	25	25	50
Staphylococcus lugdunensis	-	50	-
Staphylococcus capitis	25	50	-
Staphylococcus pasteuri	25	-	-
Staphylococcus cohnii	25	-	25
Staphylococcus haemolyticus	25	-	-
Enterococcus faecalis	-	25	-
Bacillus cereus	-	25	-

Table 4.iii Frequency of isolated bacterial species on the floors of the orthopaedic operating theatre and adjoining areas identified by 16S rRNA gene sequencing of recovered isolates

4.4 Discussion

Previously, cultural techniques have been used to study the hospital environment. The aim of this study was to characterise the microbiology of the orthopaedic operating theatres using a combination of quantitative cultural microbiology and PCR-DGGE. Using PCR-DGGE and sequencing this study revealed that Gram negative species typically associated with environmental sources dominate. In contrast the use of culture indicated a dominance of gram positive species, predominantly *Staphylococci*.

PCR-DGGE demonstrated that environmental associated microorganisms such as *Cupriavidus* and *Chryseobacterium* spp. could be detected at the majority of sites sampled. To the author's knowledge this is the first study to observe these genera in the operating theatre environment.

The genus *Cupriavidus* is associated with chloroaromatic coumpound and heavy metal resistance (Li, Cai and Zhang,2013, Vandamme and Coenye,2004, Louie, Webster and Xun,2002). Biodegredation and heavy metal resistance genes are often carried on large plasmids (Monchy *et al.*,2007); these may offer *Cupriavidus* a survival advantage in the operating theatre environment which is cleaned on a more regular basis than other hospital environments.

Average ACC were shown to be significantly higher in swab samples taken from the floors of the operating theatre compared to the prep room or anaesthetic room. It has been suggested that the high speed instruments used during orthopaedic surgery can produce aerosols distributing bacterial, fungal or viral agents across the operating room (Nogler *et al.*,2003). Higher microbial counts in the operating theatre compared to the surrounding rooms may therefore reflect the dispersal of microorganisms throughout the operating theatre by the rigorous activity and instruments used during surgery.

Using culture *Staphylococcus* was the most frequently observed genus and could be detected in all of the sites sampled in the orthopaedic operating theatre. This is in accordance with other studies that have used cultural techniques to study operating theatre environments. Al Laham (2012) reported that 24% of swabs taken from the environment and equipment of a general theatre were contaminated with microorganisms. *Staphylococci* were the most frequent contaminant of environmental samples and around 90% of the *Staphylococci* detected were CNS (Al Laham,2012). Similarly Edmiston *et al* (2005) found that CNS could be isolated from 86% of air samples taken during vascular surgical procedures (Edmiston *et al.*,2005). Davis *et al.* reported that 76% of the organisms grown from swab samples of the orthopaedic operating theatre environment were CNS (Davis *et al.*,1999).

Staphylococcus epidermidis was the most frequently isolated species present at 50% of the sites sampled. Similarly Kiss *et al.* reported that *S. epidermidis* was the most frequent and *S. haemolyticus* the second most frequently isolated species in operating theatre air samples (Kiss, Sztroj and Szell,1996). In contrast the present study only found *S. haemolyticus* in a single swab sample taken from the theatre environment. The main difference between the methodology of the previous and present study is sampling method; here swab samples were collected from environmental surfaces, Kiss *et al.* used an air sampler.

The results obtained from cultural analysis in the anasthetic room are in keeping with previous reports by Loftus *et al.* who investigated the contamination of anaesthesia work area using culture. They reported that, during procedures, the environment became contaminated with *S. epidermidis, S. haemolyticus, Streptococcus* spp. and *Enterococcus* spp. (Loftus *et al.*,2012, Loftus *et al.*,2008).

In the present study PCR-DGGE revealed the presence of a number of genera not detected using culture and *vice versa*, genera detected using culture were not dectected using PCR-DGGE. Previous studies have reported that while some organisms detected using PCR-DGGE were not cultured, it does not mean that they are unculturable they may have been outcompeted by faster growing species better suited to the culture conditions or media used (Davies *et*

al.,2004, Schabereiter-Gurtner *et al.*,2001). The dominance of *Staphylococci* in culture is therefore likely to reflect the ability of these organisms to grow rapidly under the conditions used (Mueller-Hinton, Blood and CLED agars at 37°C).

Despite the advantages of PCR-DGGE over conventional culture, it does have reported problems. In general PCR-DGGE will only allow detection of the dominant species in a microbial community (Muyzer and Smalla,1998). However as *Staphylococci* were detectable using culture they must represent a proportion of the total microbial population, and so it seemed reasonable that it should have also been detected using PCR-DGGE.

The cell wall of *Staphylococci* (particularly *S. aureus*) makes them more difficult to lyse compared to other bacteria (Salton,1953). Previous studies have suggested that this means that culture is more sensitive than molecular methods for the detection of *Staphylococci*, as standard DNA extraction methods are not sufficient to release staphylococcal DNA, and therefore may be unable to detect or may underestimate their abundance (Zhao *et al.*,2012, Zemanick *et al.*,2010). The addition of an extra lysis step during the DNA extraction procedure, incubation with a lysis solution containing lysostaphin, has been shown to impove the lysis of *Staphylococci* and release of DNA (Zhao *et al.*,2012, Schindler and Schuhardt,1964). Improved lysis of staphylococcal cells during DNA extraction has been shown to help ensure they are detected and not underestimated by molecular methods. In the present study a lysozyme-

lysostaphin enzymatic lysis step was included in the DNA extraction procedure, however *Staphylococci* were still not detected using PCR-DGGE.

Genera including *Cupriavidus*, *Chryseobacterium* and *Methylophilus* were decteded using PCR-DGGE and not culture. The medium selected for cultural analysis in this study were general purpose media and growth conditions were at 37°C for 24 hours. Species of the genera *Methylophilus* have previously been shown to grow at temperatures 19-26°C, in addition some members of the genus are obligate methylotrophs and so can not grow in the absence of methanol (Gogleva *et al.*,2010). All *Chryseobacterium* have been shown to grow at 30°C (Vandamme *et al.*,1994). Therefore although it is likely that these genera numerically dominate the environment and their DNA is detectable using PCR-DGGE, they are not readily cultured under the conditions selected in this study.

The different result observed here using conventional culture and PCR-DGGE suggest that the two techniques provide complementary information and therefore should be used in combination to gain a full picture of the microbial diversity of the hospital environment.

5 The use of PCR-DGGE for the detection of emerging opportunistic fungal pathogens in hospital sinks

5.1 Background and aims

Sample taken as part of two other projects in the orthopaedic and ICU wards at Sheffield Teaching Hospitals (STH) provided the opportunity to characterise sink fungi using PCR-DGGE and to compare the results to data previously gathered from the STH ICU environment using culture. The first study by Kay (2008) investigated the microbial ecology of the intensive care environment and used culture to isolate fungi from environmental surfaces and drains. The second study by Alshamaki (Not yet complete) investigated the microbial ecology of sinks on the orthopaedic ward, focusing only on bacterial communities. The previous study of the STH ICU by Kay *et al.* (2010) demonstrated that medically relevant fungi could be isolated from nearly 50% of environmental surfaces using culture; these isolates were identified as predominantly non-*albicans Candida* using a 18S-25S/28S rRNA gene fragment specific-PCR. In addition they reported that sinks were most frequently contaminated (Kay 2010).

Reservoirs of pathogenic fungi within the hospital environment are not well characterised (Anaissie *et al.*,2001). Previous studies have focused largely on the evaluation of airborne fungi in hospital environments and the risk to patient health that they present (Perdelli *et al.*,2006, Fox *et al.*,1990). Wet indoor

environments, such as sinks and showers, provide a favourable ecological niche for fungi due to the characteristic continuous moisture, high pH and high level of organic matter contamination and therefore are currently receiving increasing attention as potential reservoirs for pathogenic fungi (Zalar *et al.*,2011).

Fungi are increasingly recognised as having an important role in the formation of biofilms. The ability of *Candida albicans* to form a biofilm and the resulting resistance to antimicrobials has been documented (Blankenship and Mitchell,2006, Douglas,2003). In addition evidence indicates that *Candida albicans* may be able to form mixed biofilms with bacteria for example *Streptococcus, Staphylococcus* and *Pseudomonas* (Douglas,2003, Adam, Baillie and Douglas,2002, Hogan and Kolter,2002).

Biofilms in sinks may act as a potential reservoir of pathogens as they are often missed by cleaning regimes, often located in difficult-to-access parts of the sink and the biofilm structure may prevent the action of cleaning agents and detergents if the biofilm is not physically removed during cleaning. Outbreaks of bacterial infections, for example infections due to *Pseudomonas* species, have frequently been linked to hand hygiene sinks drains where mixed species biofilms are found and replacement of sinks or plumbing is often required to eradicate the organism and terminate the outbreak (Hota *et al.*,2009, Muscarella,2004, Gillespie *et al.*,2000). More recent evidence indicates that

sinks and plumbing may harbour biofilms of potentially pathogenic fungi as well as bacteria (Short *et al.*,2011, Anaissie *et al.*,2001, Doggett,2000).

Many fungi are slow growing and difficult to culture, making them more difficult to study. In addition traditional cultural techniques may over- or under-estimate the abundance of a particular type of fungi depending on their ability to grow on laboratory media. Therefore molecular techniques that utilise the presence of fungal nucleic acid may be more appropriate for detection. Such methods are not biased by the ability of different species growth on selective media.

This study aimed to utilise PCR-DGGE for the characterisation of fungi within the hand washing sinks of both the ICU and orthopaedic department and patient shower room of the orthopaedic ward.

The objectives were to:

- Characterise fungi present in sink environments using PCR-DGGE
- Compare the diversity of fungi in ICU and orthopaedic hand washing sink drain environments
- Compare fungi identified in the orthopaedic shower room sink and shower drain in the same room
- Compare fungi identified in the taps and drain of the same sink unit

5.2 Method summary

Total DNA extractions were obtained for hand washing sink drains in the ICU and orthopaedic wards, and the taps and shower drain of the orthopaedic ward. Environmental samples were taken on 12 occasions from the intensive care unit (ICU) between March 2008 and December 2008 and from the orthopaedic department between March 2011 and June 2012. Samples were collected by two previous PhD students by inserting a pre-moistened Steriswab (Medical wire) into the plughole or tap and turning 3 to 4 times. DNA was then extracted directly from the swabs using the same method outlined in chapter 2.5.

Fragments of the 28S rRNA gene were amplified from total DNA extractions and resolved using PCR-DGGE. Retrieved sequences were used to identify fungi to the genus and species level.

Eluted microorganisms from swab samples were inoculated on Colorex *Candida* and Sabouraud dextrose (containing 50mg/l Chloramphenicol) agars. Representative colonies were subcultured from each plate and identified by sequencing of the 28S rRNA gene.

5.3 Results

5.3.1 Comparison of sequences retrieved from the ICU and orthopaedic hand washing sink drain using PCR-DGGE

PCR-DGGE was used to characterise the microbial ecology of the sinks of an ICU ward and an orthopaedic ward. Total DNA extractions from the handwashing sink drains (n=36) on the ICU ward, handwashing sink drains (n=36), taps (n=12), shower room skin drain (n=12) and shower drain (n=12) on the orthopaedic ward were obtained from two previous studies. 28S rRNA gene fragments were amplified using PCR from total DNA extractions. Fragments were resolved using DGGE and retrieved fragments were sequenced and compared with those in the GenBank database in order to identified fungi present in the environment.

A total of 57 bands (representing a total of 146 visible bands) were excised from the DGGE gels (an example gel is shown in Figure 5.1) and the resulting sequences were compared to those in the GenBank database using BLAST. Only sequences with greater than 150 aligned residues were included and percentage identity scores were used as criteria for identification of fungi to the genus (≥97%) and species (≥99%) level (Table 5.i). In addition sequencing chromatograms were assessed for the quality of sequence. Sequences with less than 150 aligned residues or with similarity scores <97% were rejected. All sequences passing the designated criteria were deposited in the GenBank database using Bankit and assigned an accession number. The sequences of

suitable quality were used to identify 91.7% (134/146) of the visible bands to genus and species level.

PCR-DGGE was used to identify fungi in environmental swab samples taken directly from taps, sink and shower drains. A total of 16 previously described species, of 7 different genera, were identified.

Sequences matching species of the genus *Candida* were frequently retrieved from swabs taken from hand washing sinks located on the ICU ward. All of these were sequences similar to non-*albicans Candida*, including *C. parapsilosis, C. tropicalis* and *C. glabrata* present in 61%, 28% and 14% of hand washing sink samples respectively (Figure 5.2). No sequences similar to *Candida* species were retrieved from the orthopaedic hand washing sinks.

Sequences similar to the genus *Exophiala* were the most widely distributed sequences in the orthopaedic department and were retrieved from 61% of hand washing sinks, 17% of shower room sinks and 17% of shower drains (Figure 5.2). No *Exophiala* sequences were retrieved from samples taken from the ICU.

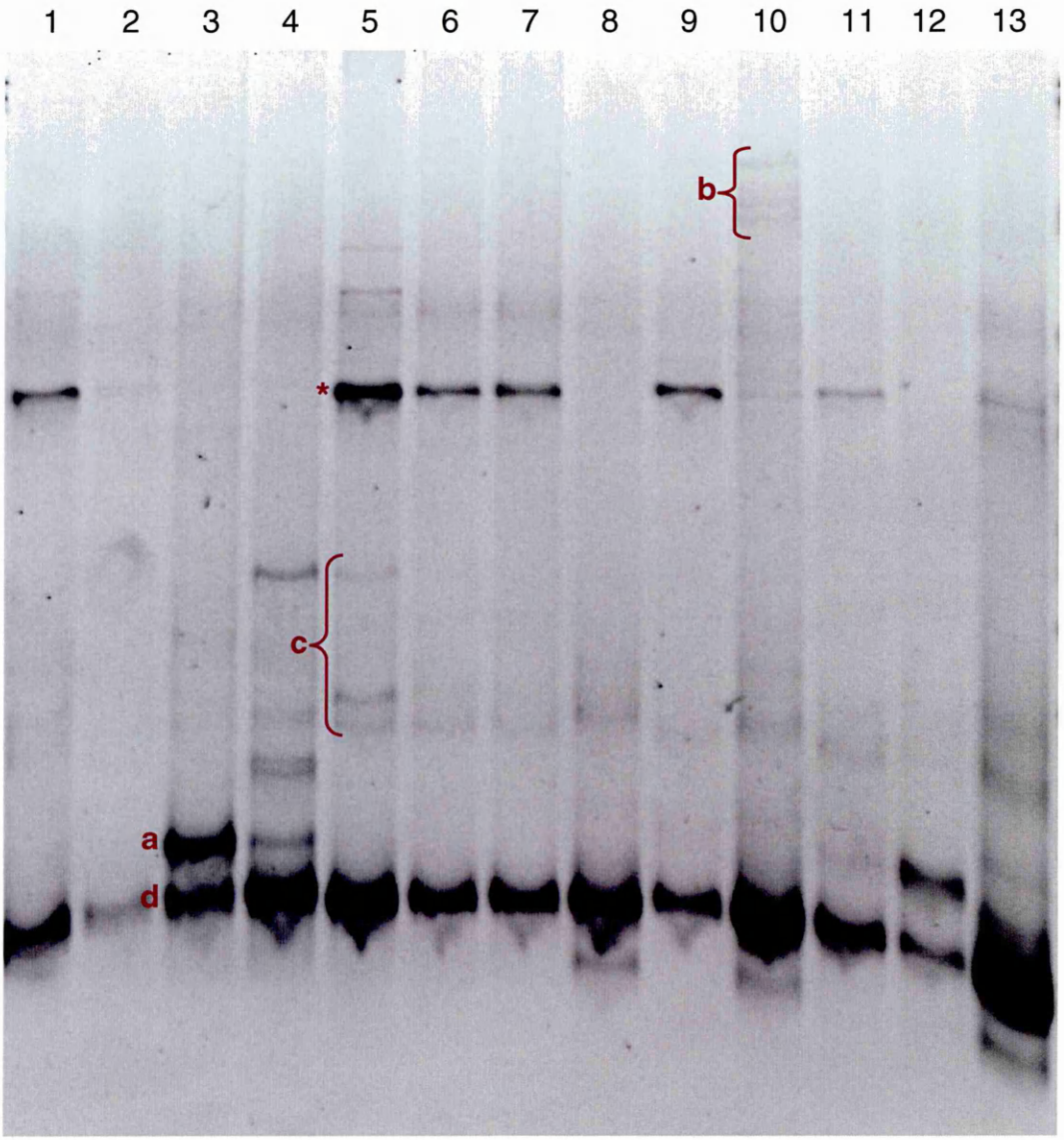

Figure 5.1 Fungal diversity in orthopaedic ward hand washing sinks (lanes 1-13) samples as determined using PCR-DGGE

Dominant bands were identified as corresponding to a) *Trichosporon mucoides*, b) *Fusarium* sp., c) *Exophiala* sp., d) *Exophiala lecanii-corni*.

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 260)	Assigned Accession Number
Ortho ward shower	*Candida albicans*	99	220	n/a
Ortho ward shower	*Fusarium oxysporum*	99	187	KF356720
Ortho ward shower	*Peyronellaea calorpreferens*	100	219	KF356719
Ortho ward shower	*Peyronellaea calorpreferens*	100	216	KF356718
Ortho ward shower	*Rhodotorula slooffiae*	99	240	n/a
Ortho ward sink	*Exophiala castellanii*	99	225	n/a
Ortho ward sink	*Exophiala lecanii-corni*	99	212	KF356712
Ortho ward sink	*Exophiala* sp	99	231	n/a
Ortho ward sink	*Exophiala* sp	99	226	n/a
Ortho ward sink	*Exophiala* sp	99	221	KF356708
Ortho ward sink	*Exophiala* sp	99	219	KF356721
Ortho ward sink	*Exophiala* sp	99	215	KF356714
Ortho ward sink	*Exophiala* sp	99	207	KF356715
Ortho ward sink	*Exophiala* sp	98	215	KF356725
Ortho ward sink	*Exophiala* sp	98	100	n/a
Ortho ward sink	*Exophiala* sp	98	82	n/a
Ortho ward sink	*Exophiala* sp	97	104	n/a
Ortho ward sink	*Exophiala* sp	96	110	n/a
Ortho ward sink	*Exophiala* sp	92	207	n/a
Ortho ward sink	*Fusarium dimerum*	100	213	KF356717
Ortho ward sink	*Fusarium dimerum*	100	213	KF356716
Ortho ward sink	*Fusarium dimerum*	99	176	KF356713
Ortho ward sink	*Fusarium dimerum*	88	196	n/a
Ortho ward sink	*Fusarium oxysporum*	100	218	KF356709
Ortho ward sink	*Fusarium oxysporum*	100	245	n/a
Ortho ward sink	*Fusarium oxysporum*	100	202	KF356710
Ortho ward sink	*Fusarium* sp	91	191	n/a
Ortho ward sink	*Trichosporon mucoides*	100	247	KF356722
Ortho ward sink	*Trichosporon mucoides*	99	234	KF356723
Ortho ward sink	*Trichosporon mucoides*	100	241	KF356711
Ortho ward sink	*Trichosporon mucoides*	100	232	KF356724
Ortho ward sink	*Trichosporon mucoides*	94	218	n/a
Ortho ward taps	*Exophiala* sp	100	98	n/a
Ortho ward taps	*Exophiala* sp	99	215	KF356726

Sampling sites	Sequence similar to	Percentage match	Number of aligned residues (max 260)	Assigned Accession Number
ICU ward sinks	*Cadophora* sp.	96	112	n/a
ICU ward sinks	*Cadophora* sp.	90	232	n/a
ICU ward sinks	*Candida albicans*	94	217	n/a
ICU ward sinks	*Candida glabrata*	99	180	KF356734
ICU ward sinks	*Candida parapsilosis*	99	206	KF356727
ICU ward sinks	*Candida parapsilosis*	99	88	n/a
ICU ward sinks	*Candida parapsilosis*	96	202	n/a
ICU ward sinks	*Candida parapsilosis*	92	207	n/a
ICU ward sinks	*Candida* sp	100	36	n/a
ICU ward sinks	*Candida* sp	97	194	KF356732
ICU ward sinks	*Candida tropicalis*	99	217	KF356728
ICU ward sinks	*Candida tropicalis*	95	202	n/a
ICU ward sinks	*Candida tropicalis*	95	201	n/a
ICU ward sinks	*Fusarium* sp	100	45	n/a
ICU ward sinks	*Fusarium* sp	100	41	n/a
ICU ward sinks	*Fusarium* sp	98	53	n/a
ICU ward sinks	*Fusarium* sp	97	229	KF356731
ICU ward sinks	*Kluyveromyces marxianus*	100	205	KF356730
ICU ward sinks	*Kluyveromyces* sp	98	201	n/a
ICU ward sinks	*Peyronellaea* sp	99	205	KF356735
ICU ward sinks	*Peyronellaea* sp	99	204	KF356736
ICU ward sinks	*Peyronellaea* sp	99	206	KF356733
ICU ward sinks	*Trichosporon mucoides*	99	231	KF356729

Table 5.i Fungal 28S rRNA gene sequences retrieved using PCR-DGGE and identified by comparison with the GenBank data base using BLAST.

Fungi identified to the genus (≥97%) and species (≥99%) level. Sequences with similarity scores <97% were rejected; some of which had migrated the same distance as successfully identified sequences (orange), other that had migrated difference distances (red). In addition some sequences had low sequence coverage and so could not be sumbitted to Bankit (Blue).

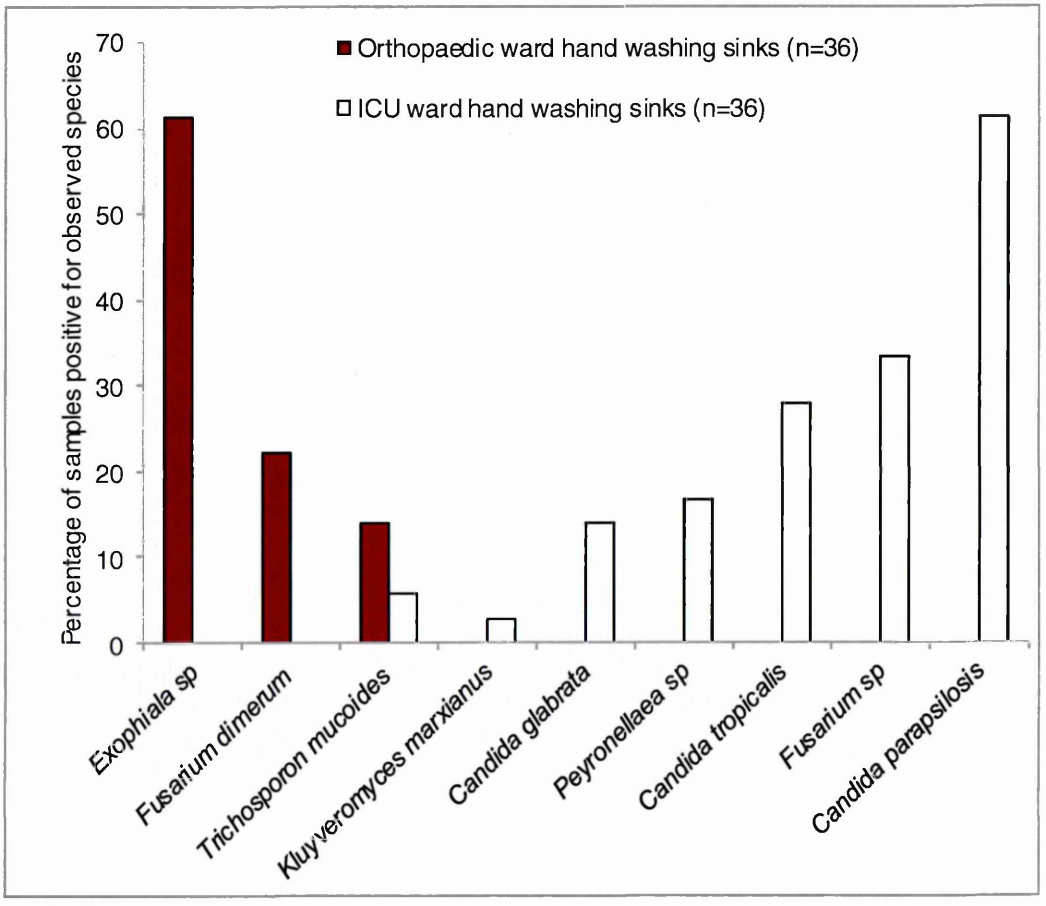

Figure 5.2 Comparison of fungal species identified in the orthopaedic ward and ICU ward hand washing sinks using PCR-DGGE

Sequences similar to the genus *Fusarium* were detected using PCR-DGGE on both the orthopaedic and ICU ward. Sequences similar to *Fusarium* sp were retrieved from 33% of ICU hand washing sinks, while *F. dimerum* was retrieved from 22% of orthopaedic hand washing sinks.

5.3.2 Comparison of sequences retrieved from the orthopaedic shower room

Candida albicans sequences were retrieved from the shower room sink drain on 3 separate occasions. *Rhodotorula slooffiae* sequences were also retrieved from the shower room sink. Sequences similar to *C. albicans* and *Rhodotorula* were not retrieved from the drain of the shower located in the same room (Figure 5.3).

Sequences similar to other genera were identified in both the shower room hand washing sink and shower drain including; *F. oxysporum* (8% and 17%), *Exophiala* (17% and 17%) and *Peyronellaea calorpreferens* (25% and 25%).

5.3.3 Comparison of sequences retrieved from the orthopaedic hand washing tap and drain using PCR-DGGE

PCR-DGGE showed that the taps of hand washing sinks in the orthopaedic ward harbour sequences similar to *Exophiala* and *Trichosporon*. Sequences similar to *Exophiala* were retrieved from 67% of taps and 50% of drains, while sequences similar to *Trichosporon mucoides* were retrieved from 50% of taps

and 50% of drains. Sequences similar to *Exophiala castellanii* were only retrieved from the sink taps (Figure 5.4).

5.3.4 Species and genera identified in the orthopaedic sinks using culture

Colorex *Candida* and Sabouraud dextrose agar were used to isolate culturable fungi from the orthopaedic sink drains. Isolated fungi were identified by sequencing the 28S rRNA gene. Sequencing revealed that culturable *Candida parapsilosis, Candida albicans, Tricosporon mucoides* and *Pichia guilliermondii* were isolated from the orthopaedic hand washing sinks. *Candida parapsilosis* was also isolated from the orthopaedic shower drain.

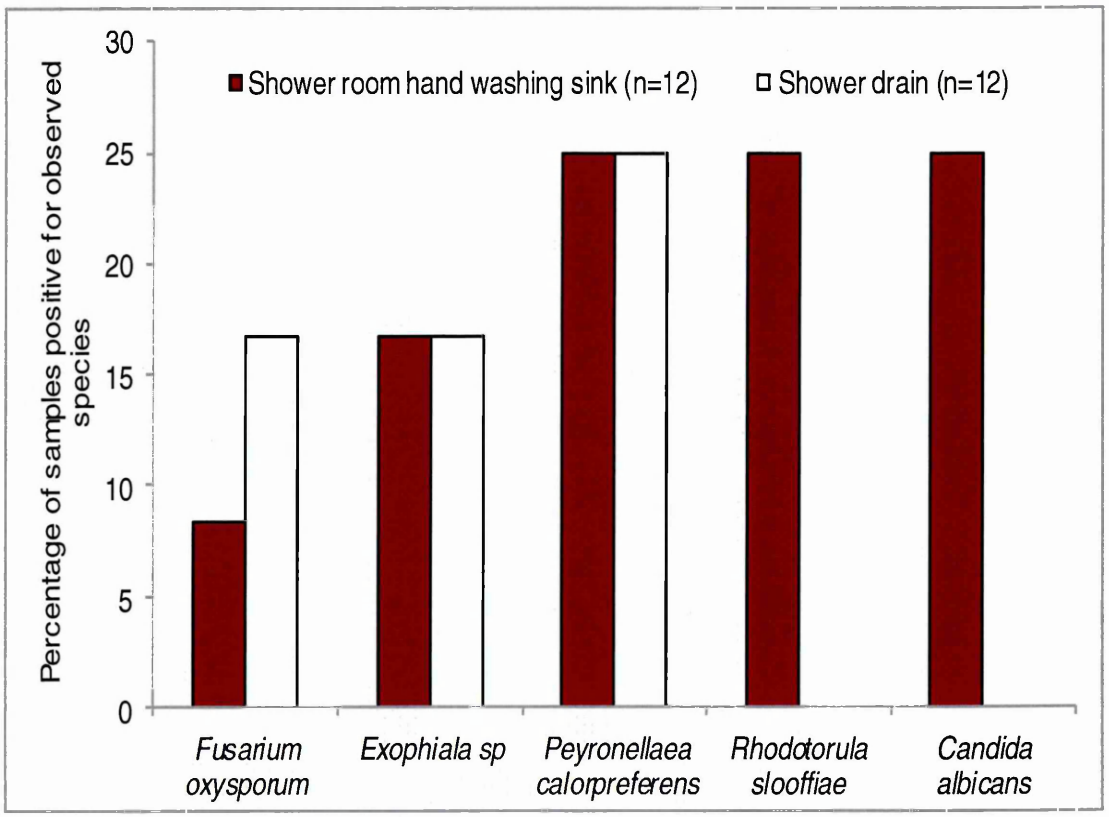

Figure 5.3 Comparison of fungal species identified in the orthopaedic ward shower drain and shower room sink using PCR-DGGE

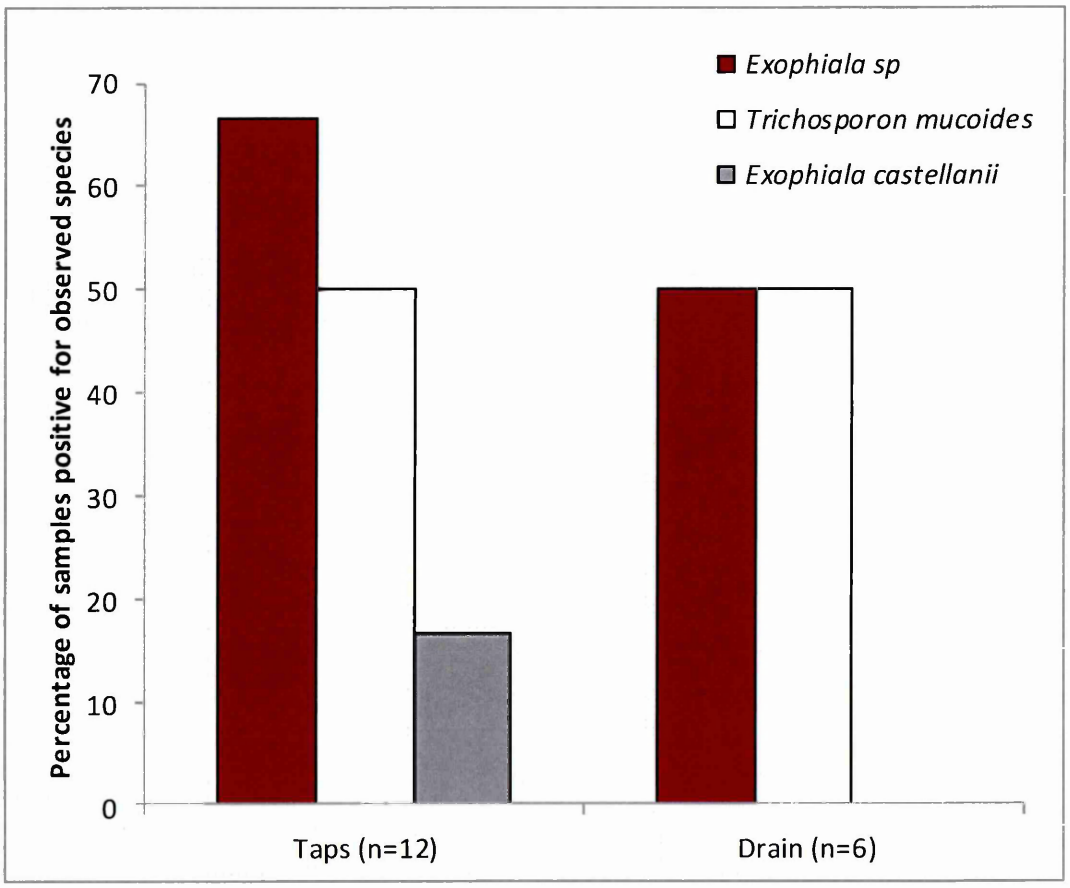

Figure 5.4 Comparison of fungal species identified in the orthopaedic ward taps and drain of the same sink unit using PCR-DGGE

5.4 Discussion

Previously, traditional cultural techniques have been used to study the distribution of airborne fungi in the hospital environment. The aim of this study was to characterise and compare reservoirs of fungi within the sink environment of an ICU and orthopaedic department.

The main difference between the hand washing sink drains of the departments was the dominance of non-albicans *Candida* species in the ICU sinks compared to sequences similar to predominantly *Exophiala* in the orthopaedic sinks. An association between the sequences retrieved from the taps and drain of the same sink was observed.

A dominance of non-*albicans Candida* in the ICU environment was previously reported by Kay *et al.* (2010) using cultural techniques. In the present study molecular methods investigating the same samples has also reported a dominance of non-*albicans Candida.* In addition PCR-DGGE has highlighted the presence of other potentially pathogenic yeast including *Trichosporon* and *Fusarium* that were not detected using culture. Here PCR-DGGE has shown the same results as culture, plus provided additional information not gathered using culture and therefore PCR-DGGE is an important tool for the investigation of the microbial ecology of the hospital sink environment.

Sequences similar to *Fusarium* sp were identified in the hand washing sinks of both the ICU and the orthopaedic department. Similarly a recent study by Short

et al (2011) highlighted the widespread distribution of *Fusarium* in drains in bathroom sinks. They suggested that its presence in these drains demonstrate an adaptation that allows them to survive in these environments (Short *et al.*,2011). Anaissie *et al* also identified *F. oxysporum* in taps and shower heads, and *F. solani* in sink drains and the hospital water tank (Anaissie *et al.*,2001).

Investigation into the genetic diversity of *F. oxysporum* in America indicated that isolates recovered from hospitalised patients genetically matched those isolated from the patients' hospital (O'Donnell *et al.*,2004). It has been suggested that *Fusarium* gain access through wounds or via indwelling medical devices. Water systems may promote the spread of *Fusarium* infection by 1) aerosol formation by running water leading to entry by respiratory apparatus, 2) access to wounds via exposure during washing and showering (Anaissie *et al.*,2001, Boutati and Anaissie,1997). Patients on both the ICU and orthopaedic wards may have surgical or trauma wounds and patients on the ICU may often require artificial ventilation, this may increase the risk of *Fusarium* infection to these groups of patients.

Sequences similar to *Exophiala* were retrieved in the orthopaedic department using PCR-DGGE. Previously *Exophiala* were frequently isolated from bathroom and kitchen sinks using cultural methods (Hamada and Abe,2010). Here PCR-DGGE has allowed detection of this genus in both drain and tap environments without the need for cultural enrichment.

The results of PCR-DGGE were compared to cultural analysis. Using PCR-DGGE sequences similar to *Trichosporon mucoides* were retrieved from the drains and taps of the hand washing sink in the orthopaedic department, culturable *T. mucoides* could also be isolated from the drain environment. Culturable *C. albicans* and *C. parapsilosis* were also isolated from the orthopaedic sink environment; however, these species were not identified in the orthopaedic department using PCR-DGGE. *Candida* species are typically fast growing and would dominate over other fungi in cultural studies. In comparison genera such as *Exophiala* (detected only using PCR-DGGE in the present study) are considered slow growing requiring 5-7 days of culture and are often outgrown by other faster growing species (Masoud-Landgraf *et al.*,2013). Molecular techniques such as PCR-DGGE are not biased by the ability of a particular species to grow under laboratory conditions.

It is recognised that PCR-DGGE enables detection of species that are numerically dominant in a population, however as culturable *Candida* strains were isolated in this environment, they should also be detectable using PCR-DGGE. Here the lack of retrieval of *Candida* sequences may be associated with co-migration of bands with other species preventing sequence identification. Alternatively this may be a result of the limited sequence information available for assigning sequence identity due to amplification of relatively short sequences (260 bp) with the selected primers.

PCR-DGGE analysis of the taps and drain of the same sink unit demonstrated that species of the genera *Exophiala* and *Trichosporon* could be detected in both environments, suggesting there is an association between the two environments. Where the water from the taps leads directly into the drain below, resulting in transfer of fungi from the taps to the drain.

PCR-DGGE analysis of the shower drain and hand washing sink located in the same room showed that sequences similar to the same genera could be retrieved from both environments (*Fusarium, Exophiala* and *Peyronellaea*). These results suggest that two different drains in close proximity are linked either by exposure to the same microorganisms or through aerosolisation of microbes in one environment spread to another nearby. Hota *et al.* (2009) previously reported that drain contents could be splashed up to one meter from a sink during handwashing (Hota *et al.*,2009).

Sequences similar to *Candida albicans* and *Rhodotorula slooffiae* were also retrieved from the hand washing sink drain but not the shower drain. The observed differences between the two drain environments may be a result of the different human activities undertaken at each, one used for hand washing of both patients and staff, the other for showering of patients.

Fungi and bacteria may form mixed species biofilms. It has been suggested that mixed species biofilms may exhibit increased resistance to antimicrobials due to complex extracellular matrix composition (Wargo and Hogan,2006).

Previous studies have shown that *Candida albicans* significantly increase the adhesion and biofilm formation on nonglycocalyx-producing bacteria (El-Azizi, Starks and Khardori,2004). In addition *Candida albicans* has been shown to enhance the resistance of bacteria to antimicrobials in mixed species biofilms (Swidergall, Ernst and Ernst,2013, Adam, Baillie and Douglas,2002). Therefore fungi in these environments may promote the survival and persistence of pathogenic bacteria. As such the study of fungi in the hospital environment is not only important to understanding the microbial ecology of fungi, but also to the ecology of bacteria.

In this study PCR-DGGE was used to investigate potential reservoirs of fungi in the sinks of an ICU and an orthopaedic department. PCR-DGGE has demonstrated a difference in the dominant genera observed on each ward. Sequences similar to non-*albicans Candida* were frequently retrieved from the ICU department, while sequences similar to *Exophiala* species were well distributed in the orthopaedic department.

6 Identification of resistance determinants in the orthopaedic ward environment

6.1 Background and aims

It has been suggested that the ward environment may act as a reservoir of antibiotic resistance genes and the link between antibiotic consumption on wards and resistance among environmental isolates has previously been reported (Dancer *et al.*,2006).

The extended spectrum beta-lactamase enzymes (ESBLs) are becoming increasingly common among members of the *Enterobacteriaceae* family including *Escherichia coli, Klebsiella pneumoniae* and other Gram negatives including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Falagas and Karageorgopoulos,2009). It has previously been shown that during outbreaks ESBL-carrying *Klebsiella* and *Enterobacter* contaminate the environment surrounding patients and that the environmental isolates and clinical isolates are often identical or closely related (Kac *et al.*,2004, Hobson, MacKenzie and Gould,1996). In addition multidrug-resistant Enterobacteriacea (MDE) have previously been shown to persist for extended periods in the hospital environment (Kramer, Schwebke and Kampf,2006). Therefore the environment may act as a reservoir for MDE, however currently little is know about the importance of the healthcare environment as a reservoir (Khan, Dancer and Humphreys,2012).

Bacterial infection following orthopaedic surgery is a serious complication that can increase the risk of morbidity and mortality and often necessitate revision of the joint. Gentamicin-loaded bone cement is widely used in orthopaedics as a prophylaxis to combat the risk of surgical site infection (Diefenbeck, Mückley and Hofmann,2006). The widespread use of gentamicin on the wards investigated in this study may promote the survival of gentamicin resistant bacteria.

Traditional sensitivity testing has previously been used to investigate resistance among clinical and environmental isolates. However it has been suggested that such methods can be problematic particularly for the detection of ESBL carrying isolates, therefore molecular methods that target the gene are considered more reliable (Pfaller and Segreti,2006). This study aimed to investigate the presence of antibiotic resistance genes in the environment of the orthopaedic department using a series of gene specific PCR reactions.

The objectives were to:

- Investigate distribution of antibiotic resistance genes in total DNA extractions from the orthopaedic ward environment (*mecA*, $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{TEM}, *aph(2")-Ia*, *aph(2")-Ic* and *aph(2")-Id*)
- Characterise phenotypically resistant bacteria (Gentamicin)

- Investigate the presence of aminoglycoside modifying genes in environmental *Staphylococci* isolated from wards where gentamicin is used as prophylaxis

6.2 Method Summary

Environmental samples were taken from ward A and B on 6 separate occasions from the ward floor, bed rails, patient chairs and underside and tops of over bed tables. Total DNA was extracted directly from microorganisms eluted from swabs. PCR was used to screen for bla_{CTX-M}, bla_{SHV}, bla_{TEM}, *mecA*, *aph(2")-Ia*, *aph(2")-Ic* and *aph(2")-Id* genes in total DNA extractions from the orthopaedic wards and the operating theatre.

Enumeration of environmental bacteria was performed on Iso Sensitest Agar with gentamicin 2mg/l. Representative colonies were sub-cultured from each plate and identified by sequencing of the 16S rRNA gene. Isolated *Staphylococci* were screened for aminoglycoside modifying enzyme genes using PCR.

6.3 Results

6.3.1 Detection of Extended spectrum beta-lactamase enzyme and *mecA* genes in total DNA extractions

None of the ESBL genes (bla_{CTX-M}, bla_{SHV}, bla_{TEM}) investigated nor the *mecA* gene were detected in total DNA extractions from swab samples taken in the orthopaedic wards or operating theatre environment (Figure 6.1 and 6.2). In order to exclude the possibility that the negative results were due to problems with the PCR reagents or amplification conditions; all PCR reactions were performed with appropriate controls (DNA template known to contain the target gene). Where no product was seen for environmental DNA extractions the PCR was repeated in order to confirm a negative result.

6.3.2 Detection of aminoglycoside modifying enzyme genes in total DNA extractions

PCR amplicons of the expected size for the *aph*(2")-Ia gene (644bp) and the *aph*(2")-Ic gene (527bp). Sequencing confirmed that the amplicons matched gene sequences in the GenBank database for the *aph(2")-Ia* gene (Table 6.i). Sequencing of product expected to be *aph(2")-Ic* based on size was unsuccessful, examination of the sequencing chromatogram suggested the product was possibly contaminated with other DNA.

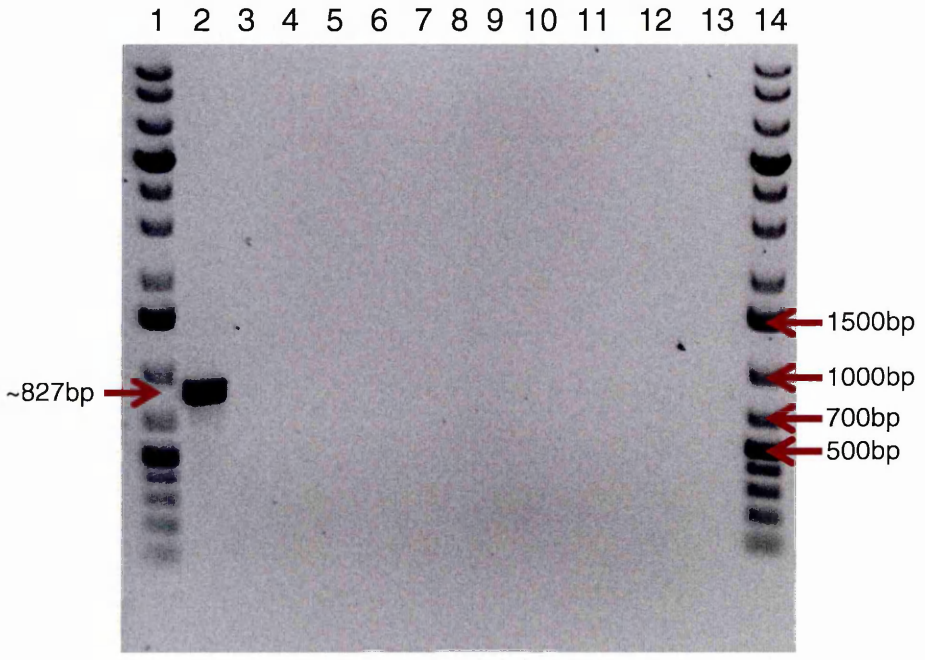

Figure 6.1 Detection of the *bla*$_{SHV}$ gene in total DNA extractions using PCR

PCR product is visible at the expected size for *bla*$_{SHV}$ gene (827bp) only for positive control

Lanes 1 & 14 1kb plus DNA ladder, lane 2 *Ecoli* SHV-2 positive control, lane 3 negative control (no DNA), lanes 4-13 total DNA extractions from environmental sampling sites; floor (4), bed rails (5-6), over bed tables (7-10), patient chairs (11-13)

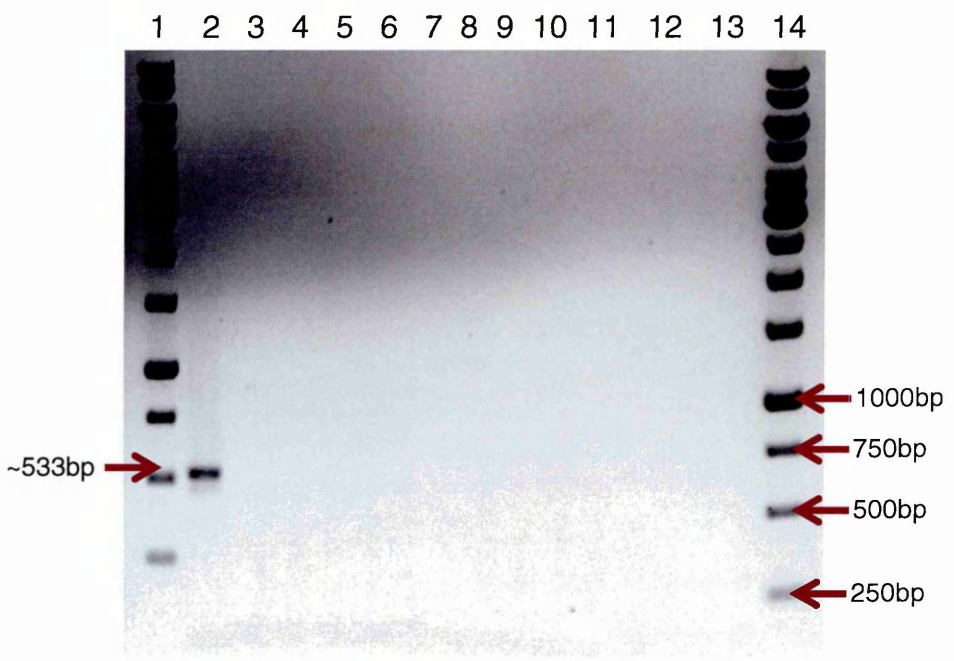

Figure 6.2 Detection of the *mecA* gene in total DNA extractions using PCR

PCR product is visible at the expected size for *mecA* gene (533bp) only for positive control.

Lanes 1 & 14 1kb DNA ladder, lane 2 MRSA positive control, lane 3 negative control (no DNA), lanes 4-13 total DNA extractions from environmental sampling sites; floors (4-7), bed rails (8-9), over bed tables (10-13)

Sequence number	Expected gene	Sequence similar to	Percentage match	Number of aligned residues
1	*aph(2")-Ia*	6'-aminoglycoside N-acetyltransferase (AAC(6')) / 2"-aminoglycoside phosphotransferase [*Staphylococcus epidermidis*]	99	583
2	*aph(2")-Ia*		99	581
3	*aph(2")-Ia*		99	582
5	*aph(2")-Ic*	No significant similarities	n/a	n/a

Table 6.i Results of BLAST search of sequences of PCR product amplified from total DNA extractions using AME gene PCR

Amplicons at the expected size of 644bp suggested that the *aph*(2")-Ia was the most frequently detected gene, detected in 54% (29/54) of total DNA extractions. Amplicons at the expected size for *aph*(2")-Ic were detected in 20% and *aph*(2")-Id was not detected in any of the total DNA extractions collected from the orthopaedic wards.

The gene *aph*(2")-Ia was widely distributed and could be detected at all sampling sites (Floors, bed rails, chairs and tables) on both wards. *aph*(2")-Ic was detected in total DNA extractions from the floors, tables and chairs (Figure 6.3). None of the AME genes investigated could be detected using PCR in total DNA extractions taken from the orthopaedic operating theatre.

AME genes were observed in a higher proportion of samples taken 23 hours after cleaning compared to 1 hour after cleaning. At 23 hours after cleaning 58% and 33% of total DNA extractions were shown to be positive for *aph*(2")-Ia and *aph*(2")-Ic repectively using PCR. At 1 hour after cleaning 42% and 25% were shown to be positive for *aph*(2")-Ia and *aph*(2")-Ic repectively (Fig 6.3).

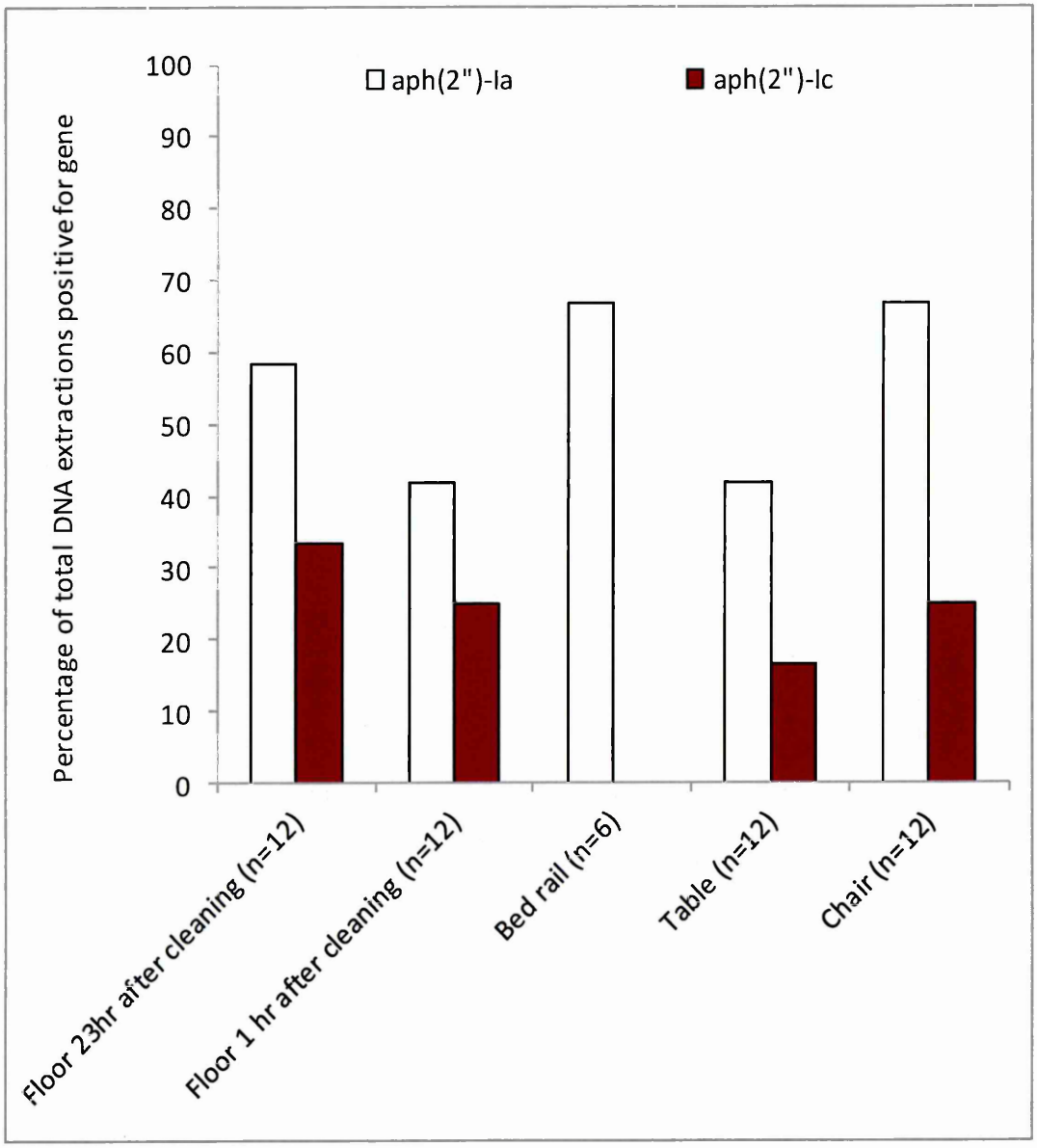

Figure 6.3 Frequency of aminoglycoside modifying enzyme genes *aph(2")-Ia* and *aph(2")-Ic* detected in total DNA extractions from the orthopaedic ward environment

6.3.3 Identification of gentamicin resistant environmental isolates

Using gentamicin (2mg/l) containing agar, resistant bacteria were isolated from all environments sampled on the orthopaedic wards, average aerobic colony counts (ACC) on gentamicin contaning agar ranged from 0.003 CFU/cm^2 (table underside) to 1.88 cfu/cm^2 (floor 23 hours after cleaning).

16S rRNA gene sequencing was used to determine which species were isolated on gentamicin containing agar. A dominance of Gram positive cocci was observed; *Staphylococci* were isolated from 72.2% (39/54) of samples. Three species of *Staphylococci* were isolated *S. haemolyticus* (43% of samples), *S. epidermidis* (39%) and *S. hominis* (11%). *Enterococcus* were the second most frequently isolated genus, present in 14.8% (8/54) of samples. Other bacteria identified included *Klebsiella pneumoniae* (9.3% of samples), *Chryseobacterium* sp. (1.9%) and *Aerococcus viridans* (1.9%) (Fig 6.4).

6.3.4 Identication of aminoglycoside modifying enzymes genes in gentamicin resistant environmental *Staphylococci*

Aminoglycoside modifiying enzyme genes were amplified from chromosomal DNA of the isolated environmental *Staphylococci* using a multiplex PCR; product was visible at the expected size for *acc(6')/aph(2")* (491bp), *aph(3')-IIIa* (242bp) and *ant(4')-Ia* (135bp) (Figure 6.5).

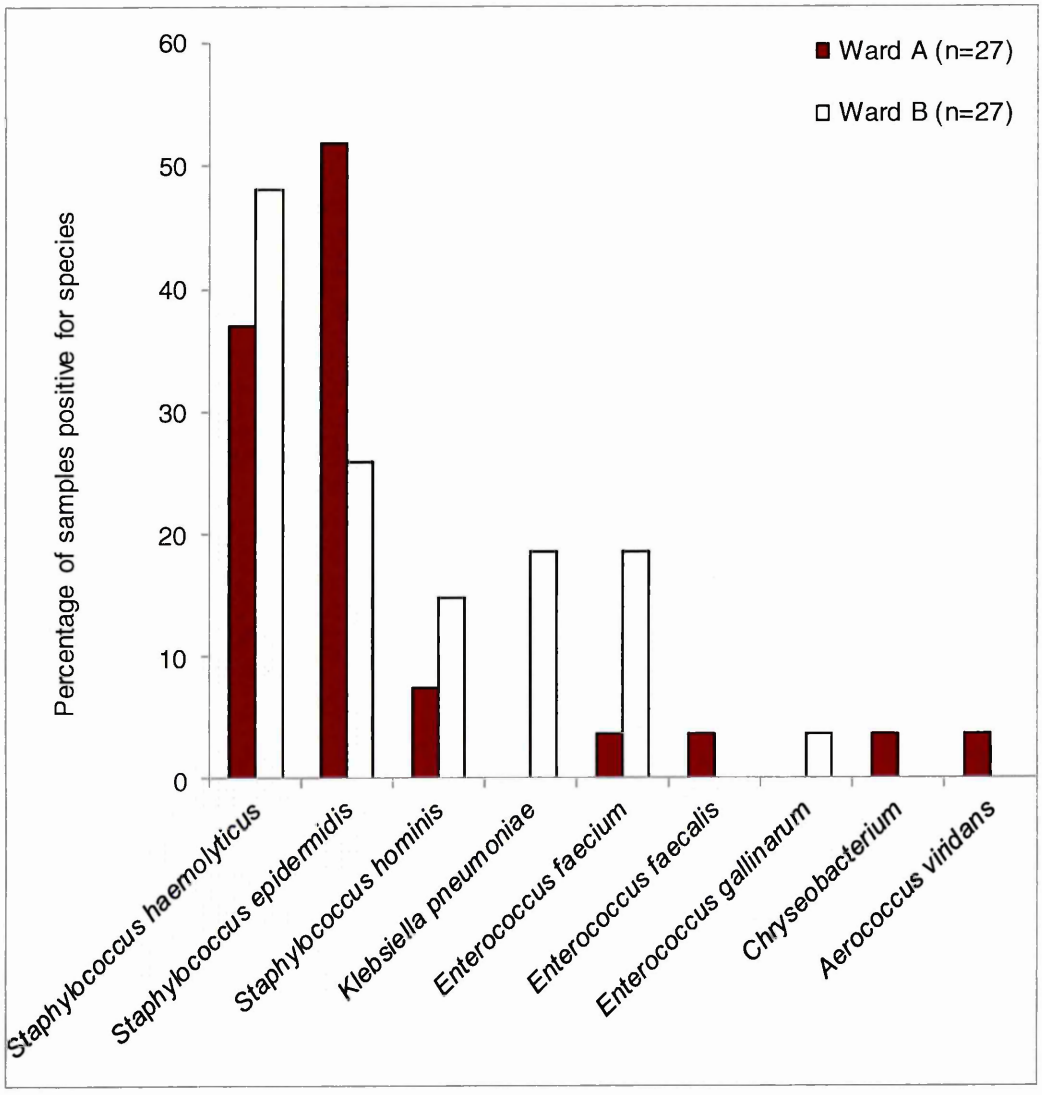

Figure 6.4 Percentage of environmental swab samples positive for each species of bacterium identified after culturing on gentamicin-containing agar

The *acc(6')/aph(2")* gene encoding the bifunctional AME was most frequently detected gene, identified in 83% of isolates using PCR. *aph(3')-IIIa* was detected in 17% and *ant(4')-Ia* in 10% of environmental isolates.

Multiplex PCR demonstrated that 15% of isolates had more than one AME gene. A single *S. epidermidis* isolate was positive for both *acc(6')/aph(2")* and *ant(4')-Ia* (Fig 6.5, lane 10). 29% of *S. haemolyticus* (7/24) isolates were positive for both *acc(6')/aph(2")* and *aph(3')-IIIa*.

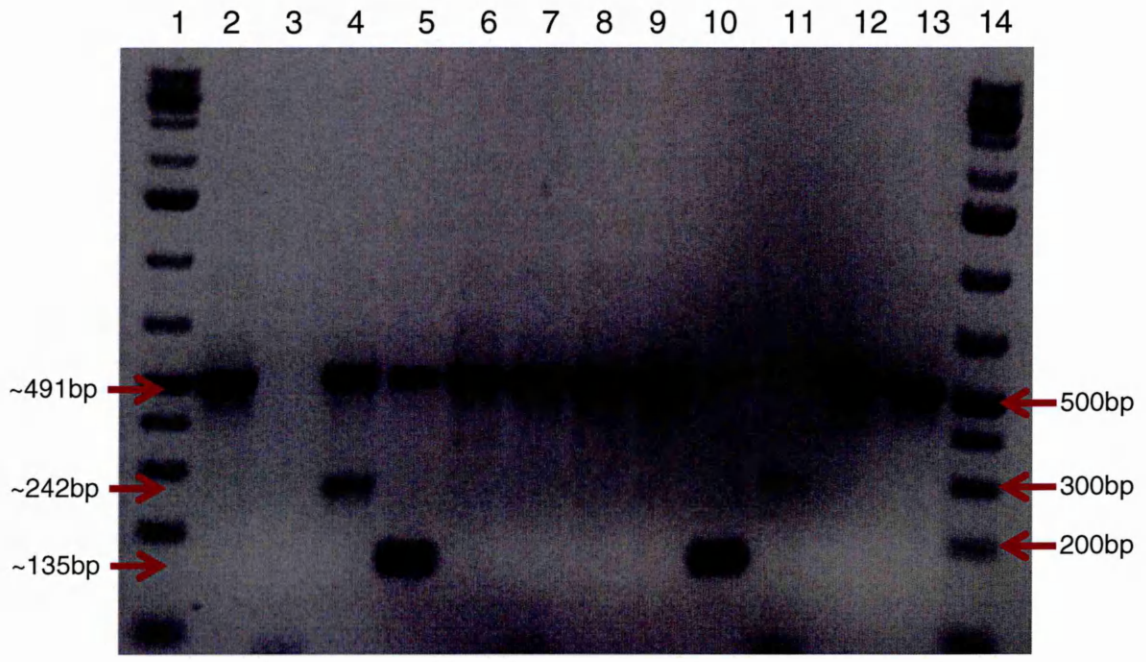

Figure 6.5 Detection of the aminoglycoside modifiying enzyme genes in isolated environmental *Staphylococci* using a multiplex PCR

PCR product is visible at the expected size for *acc(6')/aph(2")* (491bp), *aph(3')-IIIa* (242bp) and *ant(4')-Ia* (135bp).

Lanes 1 & 14 1kb DNA ladder, lane 2 *acc(6')/aph(2")* positive control, lane 3 negative control (no DNA), lanes 4-13 environmental *Staphylococcus* isolates

6.4 Discussion

Previous studies have isolated MRSA and ESBL-producing organisms in clinical samples and the environment during outbreaks using cultural techniques. In this study molecular analysis of total DNA extractions from environmental sites taken during non-outbreak conditions found no ESBL genes or the *mecA* gene to be present in detectable copy number.

Extended spectrum beta-lactamase enzymes are becoming increasingly prevalent in Enterobacteriaceae. However this study found none of the ESBL genes investigated could be detected in total DNA extractions from swab samples taken from the environment of two orthopaedic wards. Discussions with senior clinicians in the department have indicated that the incidence of ESBL producing Enterobacteriaceae is low among patients on these wards. In addition the present study demonstrated few Enterobacteriaceae could be detected in the environment using PCR-DGGE (Chapter 3). This study demonstrates that under normal operating conditions, in the absence of outbreaks, ESBLs are not readily detectable in the environment.

Kac *et al.* (2004) investigated an outbreak of ESBL-producing Enterobacteriaceae and found that moist surfaces such as taps and drains acted as a reservoir of these organisms (Kac *et al.*,2004). Similarly Guet-Revillet *et al.* reported that 19% of hospital surfaces were contaminated within rooms occupied by ESBL producing Enterobacteriaceae carrying or infected

patients. They also found that the environmental sites most frequently contaminated were baths and sinks (Guet-Revillet *et al.*,2012). In the present study ESBLs were not detected in swab samples taken from dry surfaces. The lack of ESBLs in this environment compared to previous reports may therefore be due to the preferential survival of the hosts (mainly Enterobacteriaceae) of these genes in wet or moist environments compared to dry surfaces (Gould and Chamberlain,1994). The sink drain and taps of the same orthopaedic ward are currently the subject of another PhD study.

In this study, on two orthopaedic wards where gentamicin is used regularly, gentamicin resistant bacteria were isolated from all sites sampled using cultural methods and aminoglycoside-modifying enzyme genes were readily detected in both total DNA extractions and cultured environmental *Staphylococci*. Loeffler *et al.* reported a positive correlation between gentamicin and other aminoglycoside usage and resistance among clinical CNS isolates (Loeffler *et al.*,2003). Similarly Lang *et al.* demonstrated higher levels of resistance to antibiotics including Gentamicin on a haematology ward compared to an ICU ward. This correlated with higher usage of aminoglycoside on the haematology ward (Lang *et al.*,2001).

The genus *Enterococcus* was isolated in 15% of environmental samples using gentamicin containing agar. *Enterococci* are known to have low level intrinsic resistance to aminoglycosides including gentamicin; in addition acquisition of

AME genes through horizontal gene transfer has also been described (Hollenbeck and Rice,2012, Mederski-Samoraj and Murray,1983).

Three AME genes were detected in cultured environmental *Staphylococci* using a multiplex PCR. The bifunctional enzyme encoded by *acc(6')/aph(2")* was the most frequently detected gene. Previous studies have shown this to be the most frequent AME in clinical *Staphylococci* (Ardic *et al.*,2006, Busch-Sorensen *et al.*,1996).

The present study has shown that, in the environment of a ward where patients have been treated with gentamicin-loaded bone cement, gentamicin-resistance bacteria namely *Staphylococci* and *Enterococci* can be isolated from the environment. PCR has shown that aminoglycoside phosphotransferase genes can be detected in total DNA extractions from this environment and the bifunctional *acc(6')/aph(2")* gene is the most frequently identified AME gene in cultivable *Staphylococci.* Extended spectrum beta-lactamase enzymes were not identified in total DNA extractions from the orthopaedic ward environment.

7 Identification of *Staphylococci* and their antibiotic resistance determinants in the orthopaedic ward environment

7.1 Background and aims

Although the *mecA* gene was not detectable in total DNA extractions using PCR, culturable bacteria were isolated using cefoxitin containing agar. Therefore these isolates were investigated further.

Methicillin resistant *Staphylococci* are important hospital acquired pathogens. The gene encoding methicillin resistance, *mec*A, is an important genetic marker. The environmental distribution and ecology of this gene is poorly understood. The *mecA* gene is carried on the Staphylococcal cassette chromosome (*SCCmec*), a mobile genetic element that may be transferred between *Staphylococci* (Ibrahem *et al.*,2009, Wielders *et al.*,2002). In addition to β-lactam resistance, *Staphylococci* may also be resistant to other antibiotics, for example aminoglycosides (Ardic *et al.*,2006), the genes for which may also be carried on the SCC*mec* (Turlej, Hryniewicz and Empel,2011).

Methicillin is now discontinued and no longer used to treat patients (Royal Pharmaceutical Society of Great Britain and British Medical Association 2012). Investigation of methicillin resistance in *Staphylococci* is currently performed

using either oxacillin or cefoxitin. It has been suggested that disk testing with cefoxitin is more reliable than oxacillin (Andrews *et al.*,2005, Skov *et al.*,2005, Skov *et al.*,2003, Felten *et al.*,2002) and the British society for antimicrobial chemotherapy (BSAC) now recommend the use of cefoxitin for sensitivity testing of *S. aureus* and coagulase negative *Staphylococci* (Andrews and BSAC Working Party on Susceptibility Testing,2008). Therefore in the present study cefoxitin containing agar (Colorex MRSA) was used to isolate resistant bacteria from the environment.

The present study aimed to investigate the ecology of the *mec*A gene in the orthopaedic department, in terms of frequency of the gene, host species and the genetic type. As *Staphyloccocci* may also be resistant to other antibiotics, this study also investigated the existence of resistance to aminoglycosides and quaternary ammonium compounds in cefoxitin-resistant isolates.

The objectives were to:

- Characterise cefoxitin resistant *Staphylococci* from the environment
- investigate the frequency of the *mecA* gene in different *Staphylococcus* species
- Assign SCC*mec* type to environmental *Staphylococci* according to the published guidelines

- Investigate the frequency of the *mecA* gene in clinical *Staphylococci* isolates

- Investigate the frequency of aminoglycoside modifying enzyme genes in cefoxitin resistant environmental *Staphylococci*

- Investigate the frequency of quaternary ammonium compound resistance genes in cefoxitin resistant environmental *Staphylococci*

- Compare the use of 16S rRNA and *tuf* gene sequencing for the identification of environmental *Staphylococci*

7.2 Method summary

Environmental samples were taken from ward A and B on 12 separate occasions from the ward floor, bed rails, patient chairs and underside and tops of over bed tables. Swab samples were taken from the floors of the operating theatres on a single occasion.

Enumeration of environmental bacteria was performed on Colorex MRSA selective (Cefoxitin containing) and non-selective (CLED, Blood) agars. Representative colonies were sub-cultured from each plate and identified by sequencing of the 16S rRNA and *tuf* gene.

Clinical isolates collected as part of routine diagnostics from patients on both wards were obtained from the hospital microbiology department for the period of environmental sampling and identified by sequencing of the 16S rRNA genes.

Isolated *Staphylococci* were screened for the *mecA* gene, aminoglycoside modifying enzyme genes and *qacA/B* genes using PCR. Where possible SCC*mec* type was assigned to environmental *Staphylococci* using PCR to type the mec and ccr elements of the chromosome.

7.3 Results

7.3.1 Enumeration of bacteria on Colorex MRSA agar

Using Colorex-MRSA agar, cefoxitin resistant staphylococci were isolated from all environments sampled, average aerobic colony counts (ACC) ranged from 0.001 CFU/cm^2 (table underside, ward A) to 0.783 CFU/cm^2 (bed rails, ward B) (Fig 7.1). PCR confirmed the presence of *mec*A in all representative isolates (n=149) from all sample sites; floors, bed rails, over bed tables and patient chairs. Analysis of variance (ANOVA) indicated no significant difference between the overall average ACC on Colorex MRSA agar of the two wards investigated (p=0.364).

7.3.2 Identification of *mecA* gene hosts

16S rRNA gene sequencing demonstrated that *S. haemolyticus* was the most frequent and widely distributed species in the ward environment, detected in

79% and 75% of samples from ward A (Elective 'MRSA-free' ward) and B (Trauma ward) respectively. A further nine non-*aureus* *mecA*-positive staphylococcal species were also detected on Colorex-MRSA agar (Fig 7.2).

MRSA was only detected in the environment of ward B in samples taken from the floor at 23 hours after cleaning (11% of samples), from a bed rail (17%) and chair arm (8%). Only two species were recovered from the orthopaedic operating theatre environment using Colorex MRSA agar. These were identified as *S. warneri* (8.3%) and *S. pasteuri* (8.3%) (Fig 7.2).

7.3.3 The effect of cleaning on environmental *mecA* positive *Staphylococci*

ANOVA demonstrated that the increase in microbial load between 1 and 23 h after cleaning on Colorex MRSA agar was statistically significant (p=0.002).

Samples taken just after cleaning yielded a lower diversity of *Staphylococcus* species on Colorex MRSA agar compared to 23 hours after cleaning. *Staphylococcus haemolyticus* and *Staphylococcus cohnii* were frequently isolated from samples taken 1 hour after cleaning, particularly on ward A where *S. haemolyticus* was detected in 100% of samples (n=9). Both species were also isolated from samples taken 23 hours after cleaning, however there was also an increase in diversity with a further six species detected across the two wards at 23 hours after cleaning.

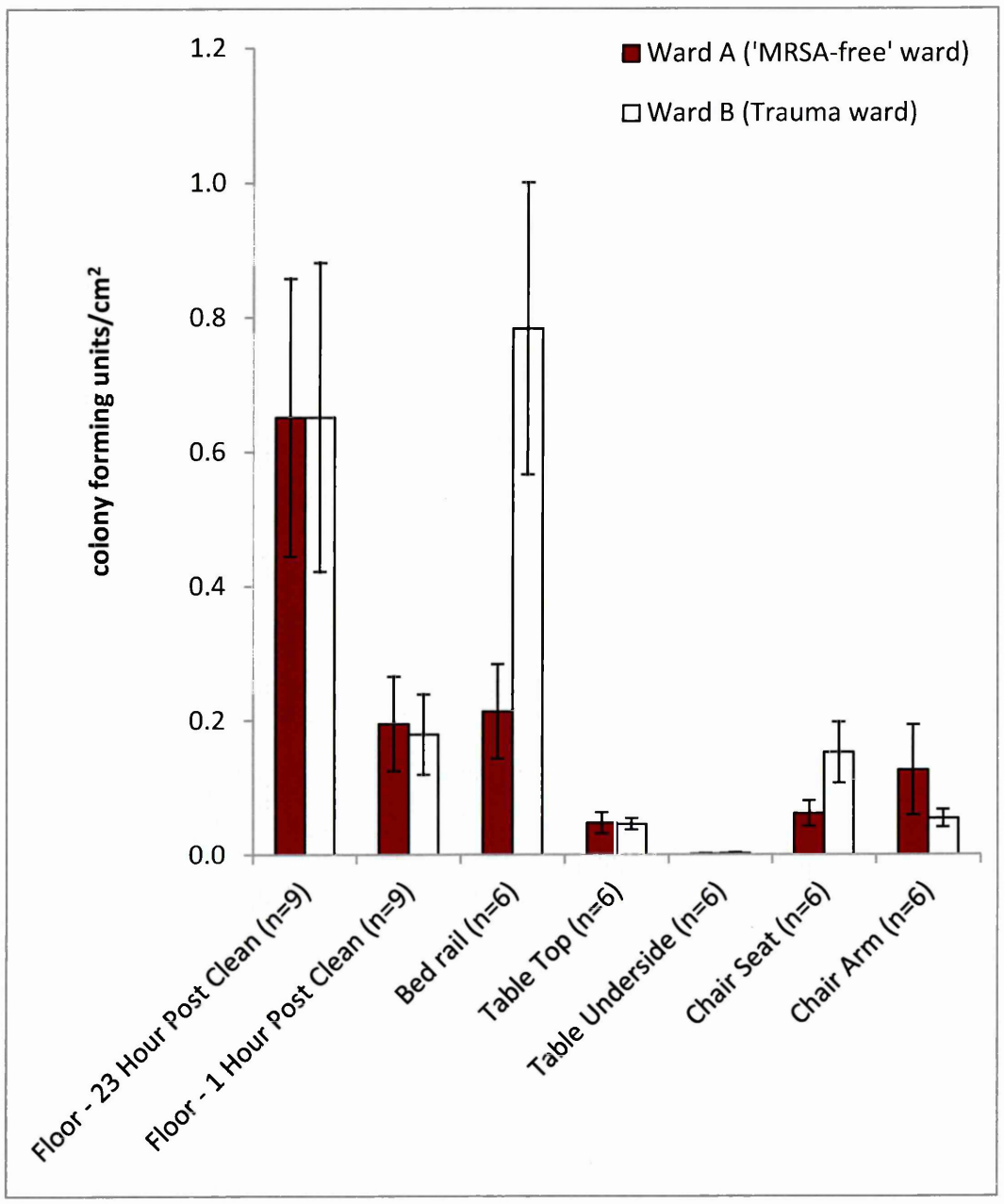

Figure 7.1 Average aerobic colony counts (ACC) of samples from orthopaedic wards on Colorex-MRSA agar

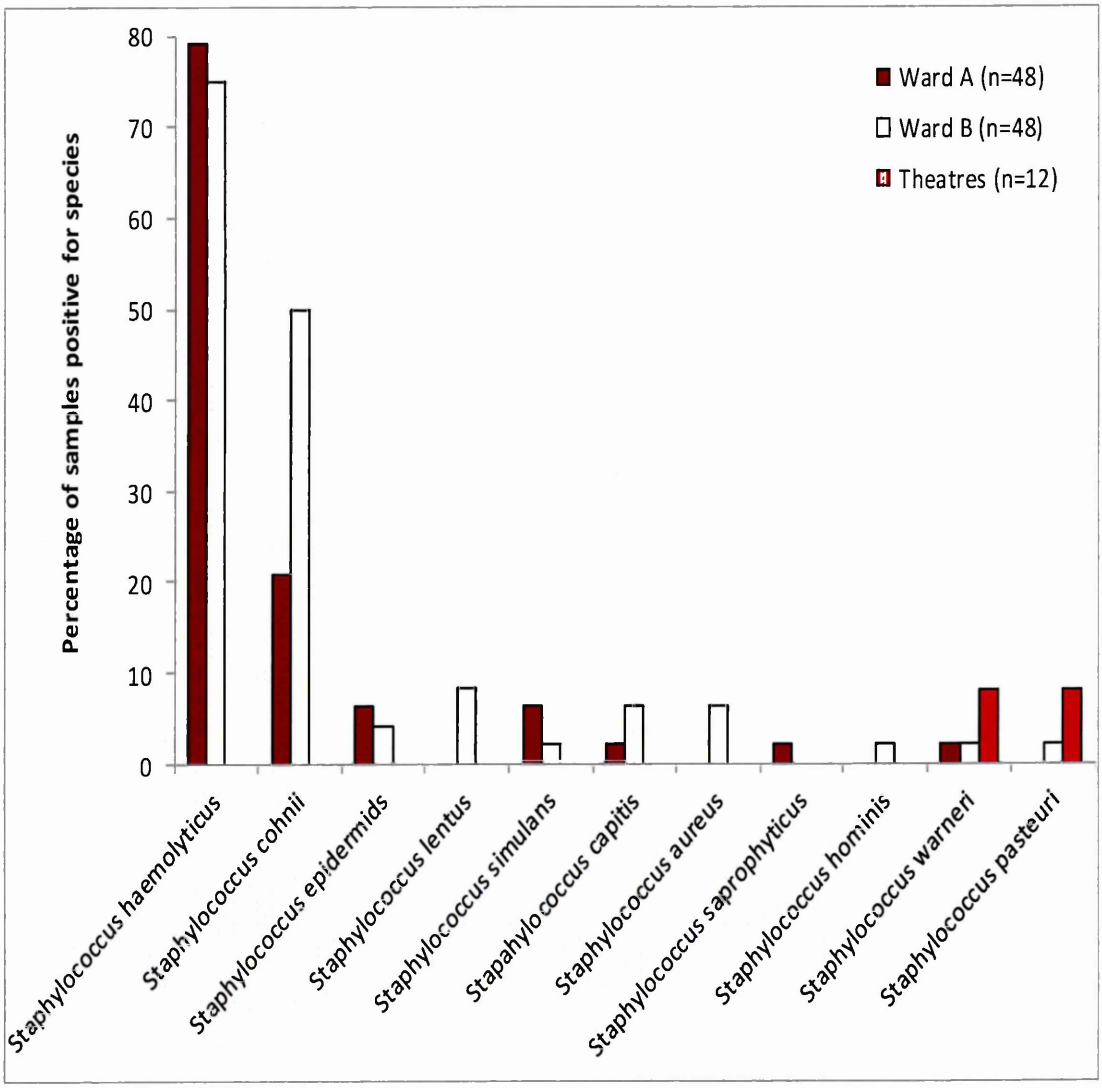

Figure 7.2 Percentage of environmental swab samples cultured on Colorex MRSA (Cefoxitin) agar yielding *mec*A positive *Staphylococcus* species

7.3.4 Estimate of the prevalence of the *mecA* gene in environmental *Staphylococcus* isolates isolated using non-selective agar

The presence of the *mecA* gene was investigated in *Staphylococci* isolated on blood and CLED agar in order to estimate the prevalence of the *mecA* gene in each species identified. Dry surfaces of the wards yielded thirteen staphylococcal species, including *S. aureus*. Isolates containing the *mecA* gene were found for only seven of these species *S. haemolyticus, S. hominis, S. cohnii, S. epidermidis, S. simulans, S. saprophyticus* and a single *S. aureus* isolate (Table 7.i). The overall proportion of *mec*A positive isolates detected using non-selective agar was 40% and 47% from wards A and B respectively. Isolates of *S. haemolyticus* most frequently carry the *mecA* gene; 71 and 82% of *S. haemolyticus* isolates from ward A and B.

In contrast, the floor of the operating theatre yielded eight staphylococcal species, only three of which were represented by isolates carrying the *mecA* gene; *S. pasteuri, S. hominis* and *S. warneri* (Table 7.i). The overall proportion of *mecA* positive isolates on non-selective agar isolated from the operating theatre environment was 19%.

Species identified by 16S rRNA gene sequencing	Percentage of isolates positive for *mecA* gene			
	Environmental isolates			Clinical isolates
	Ward A	Ward B	Theatres	
Staphylococcus haemolyticus	71 (n=7)	82 (n=11)	0 (n=1)	-
Staphylococcus cohnii	50 (n=4)	78 (n=9)	0 (n=2)	-
Staphylococcus saprophyticus	0 (n=1)	100 (n=1)	-	-
Staphylococcus epidermids	50 (n=24)	46 (n=13)	0 (n=8)	75 (n=4)
Staphylococcus hominis	50 (n=2)	25 (n=4)	67 (n=6)	0 (n=1)
Staphylococcus simulans	-	50 (n=2)	-	-
Staphylococcus aureus	14 (n=7)	0 (n=4)	-	0 (n=8)
Staphylococcus sciuri	0 (n=1)	0 (n=1)	-	-
Staphylococcus kloosii	0 (n=1)	-	-	-
Staphylococcus capitis	0 (n=1)	0 (n=3)	0 (n=3)	-
Staphylococcus lugdenensis	0 (n=2)	0 (n=2)	0 (n=4)	-
Staphylococcus warneri	0 (n=2)	0 (n=1)	17 (n=6)	-
Staphylococcus pasteuri	-	0 (n=2)	100 (n=1)	-
Total	**40 (n=52)**	**47 (n=53)**	**19 (n=31)**	**23 (n=13)**

Table 7.i Percentage of environmental and clinical isolates shown to be positive for the mecA gene using PCR

- Indicates that no isolates of a particular species were cultured from sampling sites

7.3.5 Prevalence and distribution of the *mecA* gene in clinical *Staphylococcus* isolates

Clinical isolates (n=13) were collected during the period of environmental sampling. Sequencing of the 16S rRNA gene confirmed that 62% (8/13) were *S. aureus,* 31% (4/13) were *S. epidermidis* and 7% (1/13) were *S. hominis.* No clinical isolates of *S. aureus* carried the *mec*A gene but 75% (3/4) of clinical *S. epidermidis* were *mecA* positive (Table 7.i).

7.3.6 Staphylococcal cassette chromosome typing of *mecA* positive environmental isolates

SCC*mec* type was investigated in the representative 149 environmental *Staphylococci* isolated on Colorex MRSA. SCC*mec* type was identified for 61 (41%) of these isolates using two multiplex PCRs. Among these, 97% had a single SCC*mec* type, including type II (20.3%), III (11.9%), IV (33.9%), V (28.8%) and VIII (5.1%), while 2 isolates (both *S. aureus*) had two types. Forty isolates could not be assigned a SCC*mec* type described by the current classification system and so were designated unnamed types (UT) (Table 7.ii).

A further 48 isolates could not be assigned a type; 10 isolates had two copies of the ccr complex and 4 isolates had two copies of the mec complex. The *ccr* genes could not be obtained from a total of 34 isolates despite repeated attempts.

Unnamed SCC*mec* Type	ccr complex	mec complex	Species
UT 1	1	A	*S. simulans* (n=2)
UT 2	1	C	*S. haemolyticus* (n=5)
UT 3	2	C	*S. haemolyticus* (n=23), *S. capitis* (n=1), *S. aureus* (n=1)
UT 4	4	C	*S. haemolyticus* (n=6)
UT 5	5	A	*S. cohnii* (n=1)
UT 6	5	B	*S. cohnii* (n=1)

Table 7.ii Unnamed SCC*mec* types identified in this study

7.3.7 Prevalence and distribution of aminoglycoside modifying enzyme genes in environmental *mecA* positive *Staphylococci*

At least one AME gene was detected in 62% (92/149) of the representative *mecA* positive environmental *Staphylococci* investigated. The bifunctional AME gene (*acc(6')/aph(2")*) was the most frequently detected AME gene, present in 46% of isolates using PCR. The *acc(6')/aph(2")* was detected most frequently in *S. haemolyticus* isolates (78% of *S. haemolyticus*) (Table 7.iii).

The two genes *aph(3')-IIIa* and *ant(4')-Ia* were carried least frequently by environmental *Staphylococci*, present in 13% and 5% of isolates respectively (Table 7.iii).

A single *S. haemolyticus* isolate was shown using multiplex PCR to carry more than one AME gene in combination with *mecA*. This isolate was positive for both *acc(6')/aph(2")* and *aph(3')-IIIa.*

Species Identity by 16S rRNA gene Sequencing	Number of isolates	Percentage of isolates of species with specified genotype	*MecA*	AME Multiplex		
				acc(6')/ aph(2")	*aph(3')-IIIa*	*ant(4')-Ia*
Staphylococcus haemolyticus	81	78%	+	+		
		14%	+			
		4%	+			+
		2%	+		+	
		2%	+	+	+	
Staphylococcus cohnii	42	64%	+			
		33%	+		+	
		2%	+			+
Staphylococcus epidermidis	5	60%	+			
		40%	+	+		
Staphylococcus aureus	3	100%	+			
Other CNS	18	78%	+			
		11%	+			+
		5%	+		+	
		5%	+	+		
Total (n=149)			**100%**	**46%**	**13%**	**5%**

Table 7.iii Frequency of aminoglycoside modifying enzyme genes in *mecA* positive *Staphylococci*

7.3.8 Prevalence and distribution of *qac* A/B genes in environmental *Staphylococci*

The presence of *qac* resistance genes was investigated in *Staphylococci* isolated from the floors of the two orthopaedic ward environments 1 and 23 hours after routine cleaning. Approximately 41% of *Staphylococci* from ward A (10/24) and ward B (9/22) isolated 23 hours after routine cleaning were shown using PCR to carry *qac* genes. At 1 hour after cleaning 86% (12/14) of ward A (Elective 'MRSA-free') isolates and 25% (4/16) of ward B (Trauma) isolates were found to carry *qac*. Overall isolates of *S. haemolyticus* was shown to most frequently harbour *qac* genes compared to other species (84% of *S. haemolyticus,* n=31).

The presence of *qac* resistance genes was also investigated in *Staphylococci* isolated from the theatre environment. The *qac* A/B genes were identified in 100% (10/10) of anaesthetic room isolates, 66% (8/12) of theatre prep room isolates, and 27% (3/11) of operating theatre isolates.

7.3.9 Identification of environmental *Staphylococci* using 16S rRNA and *tuf* gene sequencing

It has been suggested that 16S rRNA gene sequencing has low discriminatory power for some closely related *Staphylococci*. In the present study, 16S rRNA gene sequencing was used to determine the identity of the 149 representative *mecA* positive *Staphylococci* isolated on Colorex MRSA agar. Sequencing the

tuf gene of a small number of isolates (n=10) was used to validate the results of 16S rRNA gene sequencing. Both 16S rRNA and *tuf* gene sequencing assigned the same species identity to 9 out of 10 of the isolates analysed (Table 7.i). The remaining isolates were identified using 16S rRNA gene sequencing only.

Isolate	16S rRNA Gene		*tuf* Gene	
	Species	% Similarity	Species	% Similarity
1	*S. capitis*	99	*S capitis*	100
2	*S. cohnii*	99	*S cohnii*	99
3	*S. cohnii*	99	*S cohnii*	99
4	*S. epidermidis*	100	*S haemolyticus*	99
5	*S. epidermidis*	100	*S epidermidis*	99
6	*S. haemolyticus*	99	*S haemolyticus*	99
7	*S. haemolyticus*	99	*S haemolyticus*	99
8	*S. saprophyticus*	99	*S saprophyticus*	99
9	*S. simulans*	99	*S simulans*	100
10	*S. warneri*	100	*S warneri*	99

Table 7.iv Comparison of the use of 16S rRNA and *tuf* gene sequencing for the identification of environmental *Staphylococcus* isolates

7.4 Discussion

The aim of this study was to characterise the environmental and clinical *Staphylococci* of an orthopaedic department. Cefoxitin resistant *Staphylococci* were isolated on Colorex MRSA agar from all sites sampled on the wards. Coagulase negative *Staphylococci,* predominantly *S. haemolyticus* were shown to dominate the environment and regularly carry other resistance mechanisms as well as the *mecA* gene, including aminoglycoside modifying enzyme genes and quaternary ammonium compound resistance genes.

Previous studies have utilised cultural techniques and sensitivity testing for analysis of clinical and environmental *Staphylococci.* However it has been shown that methicillin resistance is often heterogeneous and phenotypic resistance is influenced by culture conditions (Hartman and Tomasz,1986, Sabath,1982). This complicates the detection of resistance, particularly for low level resistant strains (Murakami *et al.*,1991). Therefore molecular methods which detect the gene encoding PBP-2a are of use and are considered by the clinical laboratory the gold standard, like the PCR method used here to detect *mecA*.

This study demonstrates the frequent and wide environmental distribution of *mec*A in the non-*aureus Staphylococci* among the microflora in the 'low MRSA setting' of the orthopaedic department and in the environments around 'MRSA free' patients as well as in the trauma ward. Both of the wards investigated have

identical layout and cleaning specifications, the main difference between the two is that different ward admission criteria are used and therefore patient populations differ on the ward. Ward A is an elective 'MRSA-free' ward where patients are screened prior to admission and a strict admittance criterion is applied. Ward B is a trauma ward where patients are admitted without prior screening. Despite these differences, no statistically significant difference was observed in terms of total colony counts on Colorex MRSA agar.

These data highlight the significant distribution of *mec*A in non-*aureus Staphylococcus* species in the orthopaedic ward environment, particularly *S. haemolyticus*. Until recently non-*aureus Staphylococci* were considered to be commensals of normal human skin however their significance as pathogens is being increasingly recognised, often responsible for foreign body associated infections (e.g. catheters and joint prostheses) (Arciola *et al.*,2006, Kloos and Bannerman,1994).

Staphylococcus haemolyticus in the ward environment was found frequently to carry the *mecA* gene and other resistance determinants, including aminoglycoside modifying enzyme genes and quaternary ammonium compound resistance genes. Arciola *et al.* reported that *S. haemolyticus* was of great clinical and epidemiological relevance due to its broad antibiotic resistance (Arciola *et al.*,2006). It has been suggested that *S. haemolyticus* clusters resistance determinants with similar insertion sequence elements through homologous recombination, leading to a multi-resistant phenotype that may be

horizontally transferred to other *Staphylococci* (Hanssen and Ericson Sollid,2006, Kobayashi, Alam and Urasawa,2001a, Kobayashi, Alam and Urasawa,2001b). Therefore *S. haemolyticus* in the orthopaedic environment may act as a reservoir for acquired resistance mechanisms that are beneficial for survival in this environment.

These data are in accordance with Squeri *et al*, who recently reported that Methicillin resistant *Staphylococci* could be detected in 35.7% of samples taken from hospital surfaces. They also found higher levels of resistance in isolates of *S. epidermidis* and *S. haemolyticus* compared to *S. aureus* (Squeri, Grillo and La Fauci,2012). The current study demonstrated that 71% and 82% of *S. haemolyticus* from wards A and B, respectively, carried the *mecA* gene; similarly Ashimoto *et al* demonstrated that 80% of *S. haemolyticus* isolated from ward surfaces were Methicillin resistant (Ashimoto *et al.*,1995).

This study highlighted a dominance of *S. hominis* and *S. epidermidis* in the orthopaedic operating theatres in contrast to the dominance of *S. haemolyticus* on the orthopaedic wards. Previously Larson *et al.* reported that significantly more *S. haemolyticus* and significantly fewer *S. hominis* organisms were isolated from hospital patients compared to non-patient controls, suggesting that *S. haemolyticus* is nosocomially acquired or selected for in the hospital environment (Larson *et al.*,1986). In the present study the difference in dominant *Staphylococci* in the environment might therefore reflect difference in the skin flora of healthcare workers compared with patients. Healthcare

workers outnumber patients in the operating theatre and so the environment is likely to reflect predominantly healthcare workers skin flora. There are more patients on the ward compared to healthcare workers, therefore this environment is more likely to reflect flora from patient skin. In addition during an operation the patient is usually immobile and the healthcare workers are much more active, which may cause the healthcare workers to shed skin squams into the environment more rapidly. Mills *et al.* (2000) previously reported that surgical staff shed an increased number of microorganisms during physical activity (Mills, Holland and Hardy,2000).

The present study found that 75% of *S. epidermidis* isolated from clinical specimens carried the *mecA* gene. A high incidence of methicillin resistance in clinical CNS isolates (MR-CNS), particularly *S. epidermidis* (MRSE), has previously been reported (Zingg *et al.*,2009, Widerstrom *et al.*,2006).

The Staphylococcal cassette chromosome (SCC*mec*) is a mobile genetic element that contains the *mecA* gene. SCC*mec* is important as it contains the genes necessary for its movement (cassette chromosome recombinase genes) and may carry additional resistance determinants as well as the *mecA* gene. As such SCC*mec* in environmental *Staphylococci* may act as a reservoir of resistance mechanisms that may be transferred between *Staphylococci.* Various SCC*mec* types that differ in the genetic components have previously been reported (IWG-SCC,2009).

A range of previously described SCC*mec* types were identified in the environmental methicillin resistant coagulase negative *Staphylococci* (MR-CNS) isolated in this study. Where type could be assigned to the environmental isolates in this study, type IV and V were most common. Similar results have previously been reported in clinical isolates; type IV has been associated with clinical *S. epidermidis* and type V with clinical *S. haemolyticus* (Ruppe *et al.*,2009, Miragaia, Couto and de Lencastre,2005).

Various SCC*mec* types are known to be associated with other resistance determinants carried in the J regions of the chromosome. In this study over one third of the isolates typed where determined to be type IV. Type IV has previously been reported to carry transposon TN4001 encoding aminoglycoside resistance (Turlej, Hryniewicz and Empel,2011, Malachowa and DeLeo,2010, Ito *et al.*,2003). Therefore in this study the higher number of type IV SCC*mec* compared to other types in environmental *Staphylococci* may be due to the regular use of gentamicin in orthopaedic bone cement as prophylaxis to combat joint infection.

A large number of environmental isolates were not typeable using the previously published guidelines. Similarly Ibrahem *et al.* (2008) found that the majority of methicillin resistant *Staphylococcus epidermidis* (MRSE) isolated from bacteraemia patients had six non-typeable SCC*mec* elements according to

PCR results and hence the the MRSE isolates contained previously undescribed SCC*mec* types (Ibrahem *et al.*,2008). In addition Ruppe *et al.* found 44% of clinical *Staphylococci* had an un-typeable SCC*mec*, 34% contained multiple ccr elements (Ruppe *et al.*,2009).

The *ccr* complex was not typed for over one fifth of the isolates investigated. It has previously been suggested that the *ccr* gene of CNS with non-typeable SCC*mec* might contain uncharacterised types, deletions or mutations in the primer binding regions of the genes (Zong, Peng and Lu,2011, Hanssen and Ericson Sollid,2006). SCC*mec* could be assigned for less than half of the environmental *Staphylococci* using the previously described Multiplex PCR (Kondo *et al.*,2007). The large number of untypeable isolates in this and previous studies (Zong, Peng and Lu,2011); suggest that an extended classification system is required for MR-CNS in addition to the currently described system for MRSA (IWG-SCC,2009).

Staphylococci may carry other resistances in addition to the *mecA* gene, either integrated into the SCC*mec* or on separate plasmids. The presence of aminoglycoside modifying enzyme genes (AME) was investigated in isolates positive for the *mecA* gene. This study has detected AME genes in 62% of environmental *Staphylococci* isolated from the environment of two wards that regularly use gentamicin-loaded bone cement as prophylaxis.

S. haemolyticus was shown to frequently carry the *acc(6')/aph(2")* gene, while the incidence of this gene in other *Staphylococci* was much lower (Table 7.iii). The other AME genes investigated (*aph(3')-IIIa* and *ant(4')-Ia*) were less frequently detected in all species. Previous reports have shown that *acc(6')/aph(2")* is the most frequently detected AME gene in clinical isolates (Ardic *et al.*,2006, Choi *et al.*,2003, Schmitz *et al.*,1999).

Sampling immediately after cleaning demonstrated the persistence of *S. haemolyticus* in the environment following routine cleaning. Investigation of the presence of *qac* genes using PCR indicated that *S. haemolyticus* isolates frequently carried these genes, which may contribute to the species survival in the hospital environment. Ben Saida *et al* previously reported the survival of *S. haemolyticus* in disinfectant bottles and a high incidence of disinfectant resistance *qac* genes in both clinical and environmental isolates recovered from a neonatal ward (Ben Saida *et al.*,2009).

However it is possible that the presence of *Staphylococci* in the environment is due to recontamination following cleaning rather than persistance. Hardy *et al.* previously reported rapid recontamination with MRSA of a terminally disinfected environment following readmission of patients to the ward (Hardy *et al.*,2007). While Hardy *et al.* were able to demonstrate recontamination following terminal cleaning, which is a rare event; the present study was able to detect *Staphylococci* in the environment 1 hour after routine cleaning, which happens on a daily basis on the ward.

The presence of *qac* genes was also investigated in *Staphylococci* isolated from the floors of the orthopaedic operating theatre. The *qacA/B* genes were frequently isolated from the floors of the anaesthetic room and prep room, and less frequently from the operating theatre floor. The operating theatre environment is cleaned more frequently than the wards; cleaning with disinfectant takes place between each operation and at the end of the day. The results presented in this chapter show that *Staphylococci* are dispersed into the theatre environment, regular cleaning in this environment may promote the survival and dominance of strains positive for detergent resistance genes such as *qacA/B.*

Traditional phenotypic identification of CNS is often unreliable, irreproducible and commercially available systems are often unable to distinguish CNS to species level (Heikens *et al.*,2005). Methods of sequencing conserved bacterial genes have recently become more popular. The 16S rRNA gene encodes a functional RNA essential for the bacterial ribosome and so is highly conserved (Rajendhran and Gunasekaran,2011, Maidak *et al.*,1997). The *tuf* gene encodes the elongation factor Tu which is involved with peptide chain formation and so is an essential part of the ribosome also highly conserved in bacteria (Heikens *et al.*,2005). Here 90% of isolates were assigned the same identity using both 16S rRNA and *tuf* gene sequencing. Shin *et al* compared use of 16S rRNA and *tuf* for identification of CNS to species level. They found 16S rRNA had low discriminatory power for certain species, for example it was unable to

discriminate between *S. capitis* and *S. caprae*. They found that *tuf* gene sequencing had the best discriminatory power, however currently very few type strains are available in public databases for comparison. In contrast there is already a vast amount of sequence data for 16S rRNA genes in many bacterial species (Shin *et al.*,2011). In addition Heikens *et al.* (2005) suggested that misidentification of species by 16S rRNA and *tuf* gene sequencing may occur due to incorrectly assigned or poor quality sequences deposited in GenBank (Heikens *et al.*,2005). In the present study 16S rRNA gene sequencing was used to identify *Staphylococci* because of the larger number of sequences available in the database for comparison.

8 Overall discussion

The aim of this study was to utilise molecular techniques to characterise the microbial ecology of the orthopaedic department under normal operating conditions. This was achieved through the use of PCR-DGGE for the separation of fragments of the bacterial 16S rRNA gene amplified from total metagenomic DNA extracted directly from environmental swabs without prior enrichment. The microbial ecology was further characterised through the use of PCR to amplify antibiotic resistance genes from total environmental metagenomic DNA extractions and chromosomal DNA of isolates cultured from the environment using antibiotic containing agar.

PCR-DGGE analysis of the microbial ecology of the orthopaedic department environment indicated that surfaces sampled on the two wards had a higher microbial diversity than the operating theatres. The floors of the wards were associated with highest microbial diversity and samples taken at two time points after routine cleaning were able to demonstrate the increase in this diversity during the day.

Comparison of *Staphylococci* identified in the ward and theatre environments suggested that species found in the theatres reflect healthcare worker microflora and species found on the ward are more likely to originate from

patients' microflora. This is most likely due to the number of healthcare workers in an operating theatre and also the higher activity of healthcare workers compared to the immobilised patient during an operation.

PCR-DGGE has been used to identify bacterial species present on the floors, bed rails, chairs and tables of the two orthopaedic wards. Types of microorganism known to be associated with normal human skin, oral, and intestinal microbiota have been detected. A dominance of gram positive cocci, particularly *Staphylococcus* species was observed on both wards. Under non-outbreak conditions and despite differences in patient population and admission criteria no significant difference was observed between the environmental microbiology of the two wards.

The results of both PCR-DGGE and culture suggested that the orthopaedic ward environment may act as a reservoir for potentially pathogenic *Staphylococcus* species. *S. haemolyticus, S. epidermidis* and *S. hominis* in particular were readily identified using PCR-DGGE and 16S rRNA gene sequencing of culturable isolated species. The use of antibiotic resistance gene specific PCR reactions indicated that these isolates frequently carried the *mecA* gene and less frequently aminoglycoside modifying enzyme genes. In addition *qac* resistance genes were frequently detected in isolates, particularly *S. haemolyticus*, recovered from the floors. Carriage of these genes may aid the survival and persistence of these species in the environment.

In the orthopaedic wards both PCR-DGGE and culture suggested a dominance of gram positive cocci, particularly *Staphylococci*. PCR-DGGE analysis of the ward environment also revealed the presence of anaerobic Gram positive cocci; these were not detected using culture as the specialist techniques needed to grow anaerobes were not used in this study. Bone and joint infections due to anaerobic bacteria can be life threatening, however are frequently unrecognised due to the fastidious nature of the organisms that cause them, in addition polymicrobial mixed aerobe-anaerobe infections are common complicating diagnosis and treatment (Murphy and Frick,2013, Brook,2008). Gram positive bacteria, such as *Clostridium* species detected in the environment in this study, are anaerobes involved in polymicrobial bone and joint infections (Murphy and Frick,2013, Lazzarini *et al.*,2004). Previous studies have focused on the isolation of aerobic species from the environment and therefore future work could include the use of anaerobic culture conditions to isolate any viable anaerobic species from the environment for further investigation.

This study has shown the complementary nature of molecular techniques and conventional culture, however here culture was shown to be more sensitive for a number of species in two of the environments studied. In the orthopaedic operating theatres PCR-DGGE did not retrieve sequences similar to *Staphylococci*, despite the growth of culturable staphylococcal species on agar from samples taken from the same sites. Previous studies have reported that

the procedure using lysis buffer containing lysostaphin that was used in this study is sufficient to lyse staphylococcal cells. In further support of the effectiveness of this technique in detecting environmental *Staphylococci*,16S rRNA gene sequences identified as originating from *Staphylococci* were detected using PCR-DGGE in the orthopaedic ward environment where the same enzymatic lysis method was used. Similarly culturable *Candida* were isolated from the orthopaedic ward sinks using conventional culture, although *Candida* sequences were not retrieved using PCR-DGGE in the same environment. Sequences similar to *Candida* were retrieved, however, from the ICU sink environment using the same techniques.

Most previous studies of the indoor environment have focused on the detection of specific culturable pathogens (Kembel *et al.*,2012a). A few recent studies have aimed to utilise molecular techniques (e.g. high-throughput 16S rRNA gene sequencing). Kembel *et al.* (2012) recently conducted a comprehensive study of the use of such technology to characterise the total microbial population in the indoor environment of a hospital in the USA (Kembel *et al.*,2012a). Flores *et al.* also utilised high-throughput sequencing and reported that Gram positive species typically associated with the skin dominated the environmental surfaces of kitchens and bathrooms (Flores *et al.*,2013, Flores *et al.*,2011). Rintala *et al.* investigated seasonal changes in the microbial ecology of dust in indoor environments using molecular cloning techniques and observed a dominance of Gram positive bacteria, including *Corynebacterium* and *Staphylococcus* species (Rintala *et al.*,2008). Similarly Hewitt *et al.* (2012)

used pyrosequencing of amplified 16S rRNA sequences in the office environment and identified genera typically associated with human skin, oral and intestinal flora (Hewitt *et al.*,2012). In contrast Lee *et al.* (2007) reported that Gram negative bacteria, such as *Pseudomonas*, were the most frequently detected species using molecular techniques (Lee, Tin and Kelley,2007).

The high-throughput sequencing technique used in the study by Kembel *et al.* (2012) provided conclusive identification of a large number of sequences, providing greater detail than the present study where PCR-DGGE was used. However fingerprinting techniques, such as DGGE, have the advantage that they can be used to rapidly compare numerous samples on a single gel and are useful for the identification of the dominant species of a community (Hamady and Knight,2009, Anderson and Cairney,2004). In the present study PCR-DGGE was used to provide greater understanding of the hospital environment through comparison of specific environmental sampling sites (floors, bed rails, chairs and tables), rather than characterisation of airborne communities in Kembel's study. In addition the present study utilised parallel cultural analysis of environmental and clinical isolates.

The study by Rintala *et al.* (2008) used universal 16S rRNA gene primers to amplify the gene from total DNA extractions of collected dust samples and observed similar results to the present study. Both the Rintala *et al.* and the present study have reported that Gram positive cocci, including

Corynebacterium and *Staphyloccus* species dominated the indoor environment. In addition Rintala *et al.* retrieved sequences similar to genera typically associated with the human intestines, including *Clostridium* and *Ruminococcus* species, which were also identified in the present study. Rintala *et al.* also detected 16S rRNA gene sequences similar to species that were not detected during the present study including; *Propionibacterium, Lactococcus, Peptostreptococcus* and *Lactobacillus* species (Rintala *et al.*,2008).

This study characterised the microbial ecology of an orthopaedic department, including two wards and the operating theatre. Future work could include a more detailed study of the operating theatre environment, investigating more surface sampling sites over multiple sampling sessions to determine if the environmental microflora varies at different sites and whether it remains consistent over time. Other hospital environments worthy of investigation could include outpatient departments, NHS walk in service or community general practises, where a greater number of patients are seen each day that may have many different underlying conditions or illnesses.

Whilst the information that this study has given about the presence of pathogens, other microorganisms and the resistance in the orthopaedic environment is of interest, its primary aim was to evaluate molecular methods, characterise the overall microbial burden in this environment and provide baseline data. The data gathered have allowed characterisation of potential

reservoirs of pathogens in the environment and the dynamics of these microbiological communities during cleaning cycles.

The data will contribute to debates on the importance of hospital cleaning and the correlation between cleaning and microbiological risk. This study has demonstrated that there is a significant increase in microbial load over a 24 hour period following routine cleaning. Enumeration of viable microorganisms in swab samples taken from ward floors 1 hour after cleaning indicated that average aerobic colony counts were < 2.5 CFU/cm^2, the suggested microbiological surface hygiene standard for near-patient sites (White *et al.*,2008, Dancer,2004). This suggests that the cleaning regime used here is effective at reducing microbial contamination. In addition PCR-DGGE indicated that at 23 hours after cleaning a more diverse range of bacteria are detectable on the floors compared to 1 hour after cleaning. This suggested that routine cleaning is effective at removing a substantial proportion of the microbial population and that the environment is then recontaminated over the course of the day before the next clean. Futher research could evaluate the effect of decreasing the time between routine cleaning to determine if more frequent cleaning has an effect on microbial load.

It is hoped that the data presented in this study, in particular the characterisation of environmental *Staphylococcus* isolates, will inform debates on the role of the environment as a reservoir and source of pathogens.

Coagulase-negative *Staphylococci* carrying resistance genes including *mecA,* aminoglycoside modifying enzyme and quarternary ammonium compound resistance genes were readily isolated from the orthopaedic ward environment. Typing of the staphylococcal cassette chromosome (SCC*mec*), a mobile genetic element known to accumulate resistance genes, indicated that type IV was most common. Type IV is known to also encode aminoglycoside resistance (Turlej, Hryniewicz and Empel,2011, Malachowa and DeLeo,2010, Ito *et al.*,2003). This study shows that in an environment where gentamicin is used regularly, the environment may act as a reservoir for both this mobile genetic element and resistant *Staphylococci.*

Finally the present study will also contribute to the debate on methods used to assess microbial burden and cleaning effectiveness in the hospital environment. Investigation of the bacteria present in the orthopaedic ward and theatre surface environments and fungi present in ICU and orthopaedic sinks has demonstrated that the molecular and cultural techniques can provide complementary information and should therefore be used in combination. Future research could utilise high throughput sequencing, a technique that provides a greater amount of sequence information and as the price per base decreases each year the technique is becoming more attractive for the study of microbial populations (Logares *et al.*,2012). The present study used total RNA extraction to characterise the viable portion of the environmental microbial community. Further research could utilise extraction of RNA directly from the hospital environment to further characterise metabolically active microorganism and the

expression of genes under different conditions. The continued application of these methods to the hospital environment will aid our understanding of the role of the hospital environment in HAI, of the effectiveness of cleaning, and the interpretation of microbial environmental monitoring data.

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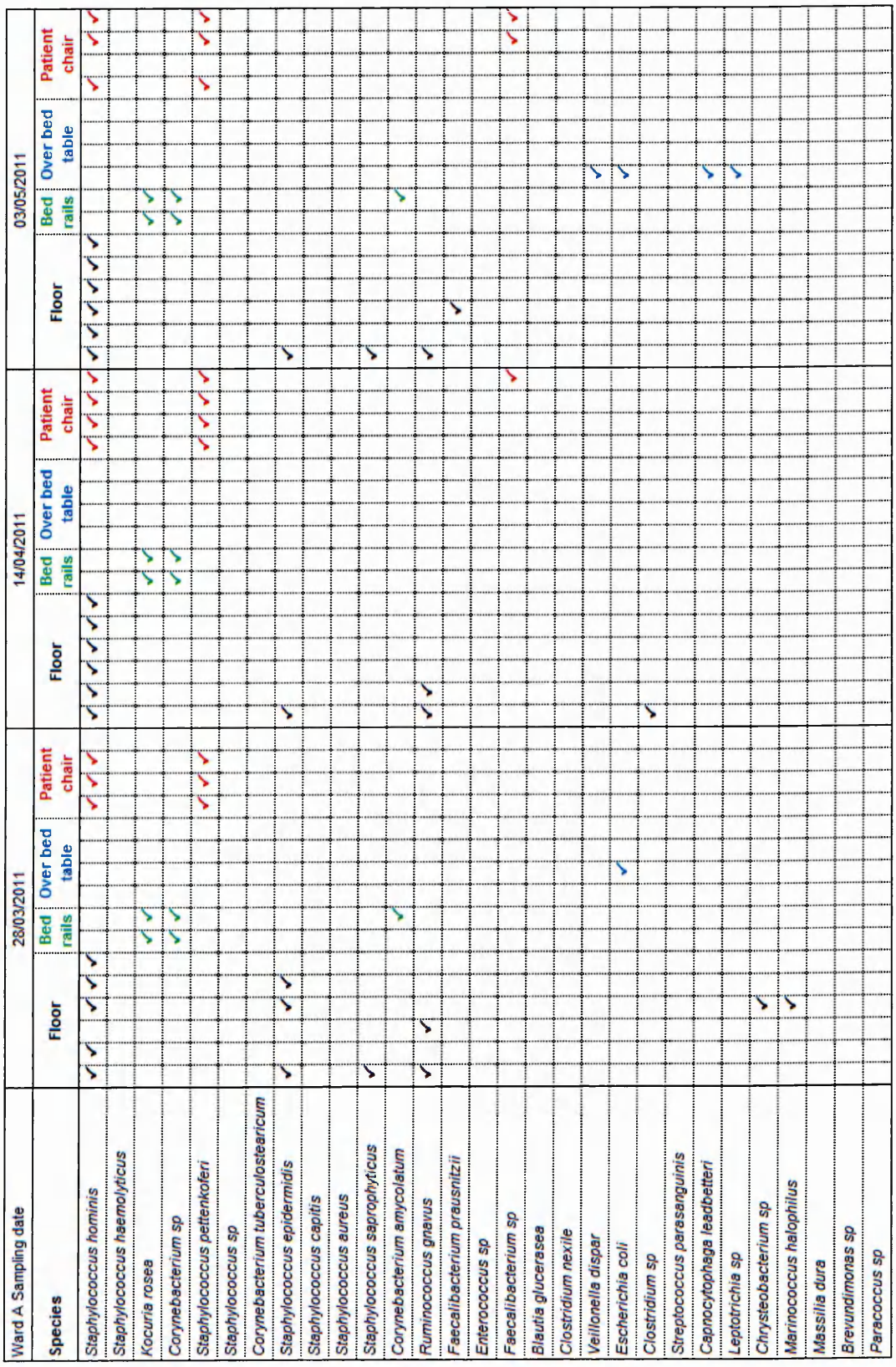
Appendix 1 – Species detected by sampling date



Ward A Sampling date	28/03/2011				14/04/2011				03/05/2011			
Species	Floor	Bed rails	Over bed table	Patient chair	Floor	Bed rails	Over bed table	Patient chair	Floor	Bed rails	Over bed table	Patient chair
Staphylococcus hominis	✓✓	✓✓		✓✓✓	✓✓✓✓			✓✓✓	✓✓✓✓	✓		✓✓
Staphylococcus haemolyticus												
Kocuria rosea		✓✓				✓✓				✓✓		
Corynebacterium sp		✓✓				✓✓				✓✓		
Staphylococcus pettenkoferi				✓✓✓				✓✓✓				✓✓
Staphylococcus sp												
Corynebacterium tuberculostearicum												
Staphylococcus epidermidis	✓	✓✓			✓							
Staphylococcus capitis												
Staphylococcus aureus												
Staphylococcus saprophyticus	✓								✓			
Corynebacterium amycolatum		✓								✓		
Ruminococcus gnavus	✓				✓✓				✓			
Faecalibacterium prausnitzii									✓			
Enterococcus sp												
Faecalibacterium sp												✓✓
Blautia glucerasea												
Clostridium nexile												
Veillonella dispar											✓	
Escherichia coli			✓								✓	
Clostridium sp					✓							
Streptococcus parasanguinis												
Capnocytophaga leadbetteri											✓	
Leptotrichia sp											✓	
Chrysteobacterium sp	✓											
Marinococcus halophilus	✓											
Massilia dura												
Brevundimonas sp												
Paracoccus sp												

Ward B Sampling date	04/04/2011														19/04/2011														09/05/2011													
Species	**Floor**						**Bed rails**		**Over bed table**			**Patient chair**			**Floor**						**Bed rails**		**Over bed table**			**Patient chair**			**Floor**						**Bed rails**		**Over bed table**			**Patient chair**		
Staphylococcus hominis	✓	✓	✓	✓	✓	✓									✓	✓	✓	✓	✓	✓									✓	✓	✓	✓	✓	✓								
Staphylococcus haemolyticus										✓		✓	✓	✓											✓	✓	✓								✓			✓		✓	✓
Kocuria rosea							✓	✓												✓	✓												✓	✓			✓				
Corynebacterium sp							✓	✓												✓	✓												✓	✓							
Staphylococcus pettenkoferi																																									
Staphylococcus sp																																									
Corynebacterium tuberculostearicum																																									
Staphylococcus epidermidis																																									
Staphylococcus capitis																																									
Staphylococcus aureus																																									
Staphylococcus saprophyticus																																									
Corynebacterium amycolatum																																									
Ruminococcus gnavus	✓	✓		✓												✓		✓																								
Faecalibacterium prausnitzii		✓			✓							✓				✓		✓		✓																						
Enterococcus sp																																										
Faecalibacterium sp																																										
Blautia glucerasea	✓	✓														✓			✓																								
Clostridium nexile													✓													✓															✓
Veillonella dispar										✓																										✓						
Escherichia coli																																										
Clostridium sp																																										
Streptococcus parasanguinis																																										
Capnocytophaga leadbetteri																																										
Leptotrichia sp																																										
Chrysteobacterium sp																																										
Marinococcus halophilus																																										
Massilia dura																														✓												
Brevundimonas sp																																										
Paracoccus sp																																										

Ward A Sampling date	28/09/2011									05/10/2011									24/10/2011								
Species	**Floor 23**		**Floor 1**		**Bed rails**	**Over bed table**		**Patient chair**		**Floor 23**		**Floor 1**		**Bed rails**	**Over bed table**		**Patient chair**		**Floor 23**		**Floor 1**		**Bed rails**	**Over bed table**		**Patient chair**	
Staphylococcus hominis																										✔	✔
Staphylococcus haemolyticus																											
Kocuria rosea																											
Corynebacterium sp																											
Staphylococcus pettenkoferi																											
Staphylococcus sp		✔									✔		✔							✔		✔					
Corynebacterium tuberculostearicum																	✔									✔	✔
Staphylococcus epidermidis																											
Staphylococcus capitis													✔										✔				
Staphylococcus aureus																											
Staphylococcus saprophyticus																											
Corynebacterium amycolatum																											
Ruminococcus gnavus																											
Faecalibacterium prausnitzii																											
Enterococcus sp		✔								✔	✔								✔	✔		✔					
Faecalibacterium sp																											
Blautia glucerasea																											
Clostridium nexile																											
Veillonella dispar																											
Escherichia coli																											
Clostridium sp																											
Streptococcus parasanguinis																											
Capnocytophaga leadbetteri																											
Leptotrichia sp																											
Chrysteobacterium sp																											
Marinococcus halophilus																											
Massilia dura																											
Brevundimonas sp																											
Paracoccus sp																											

Ward B Sampling date	03/10/2011									19/10/2011									26/10/2011								
Species	Floor 23		Floor 1		Bed rails		Over bed table	Patient chair		Floor 23		Floor 1		Bed rails		Over bed table	Patient chair		Floor 23		Floor 1		Bed rails		Over bed table	Patient chair	
Staphylococcus hominis	✔	✔	✔	✔				✔		✔	✔	✔					✔	✔	✔	✔	✔						✔	
Staphylococcus haemolyticus	✔	✔		✔						✔			✔						✔			✔					
Kocuria rosea																											
Corynebacterium sp																											
Staphylococcus pettenkoferi																											
Staphylococcus sp		✔	✔								✔									✔	✔						
Corynebacterium tuberculostearicum																	✔									✔	✔
Staphylococcus epidermidis																											
Staphylococcus capitis					✔																						
Staphylococcus aureus	✔									✔									✔								
Staphylococcus saprophyticus																											
Corynebacterium amycolatum																											
Ruminococcus gnavus																											
Faecalibacterium prausnitzii																					✔						
Enterococcus sp																											
Faecalibacterium sp		✔																	✔								
Blautia glucerasea																											
Clostridium nexile																											
Veillonella dispar																											
Escherichia coli																											
Clostridium sp																											
Streptococcus parasanguinis																											
Capnocytophaga leadbetteri																											
Leptotrichia sp																											
Chrysteobacterium sp																											
Marinococcus halophilus																											
Massilia dura																						✔					
Brevundimonas sp														✔									✔				
Paracoccus sp					✔																		✔				

Appendix 2 - Manufacturer's addresses

Bio-Rad Laboratories Ltd

Bio-Rad House

Maxted Road

Hemel Hempstead

Hertfordshire

HP2 7DX

E&O Laboratories

E&O Laboratories

Burnhouse

Bonnybridge

Scotland

FK4 2HH

Eurofins MWG Operon

Westway Estate

28-32 Brunel Road

Acton

London

W3 7XR

Life Technologies Ltd

3 Fountain Drive

Inchinnan Business Park

Paisley

PA4 9RF

Qiagen Ltd

Skelton House

Lloyd Street North

Manchester

M15 6SH

Sigma-Aldrich Company Ltd

The Old Brickyard

New Road

Gillingham

Dorset

SP8 4XT