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Microbial Ecology and Antibiotic Resistance of Microorganisms in Intensive Care Unit Environments

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Abstract

In the UK alone over 320,000 patients per annum acquire at least one nosocomial infection and one in four intensive care unit (ICU) patients worldwide acquire an infection during their hospital stay. Frequently the organisms that cause these infections are opportunistic and resistant to antibiotics. The costs to the NHS are six times higher if ICU patients acquire a nosocomial infection and the mortality rates are greater (30-60 % dependent on the infection).

This study investigated the ICU environment for bacterial reservoirs, fungal reservoirs and antibiotic resistance determinants. It was hoped that information about the microorganisms and antibiotic resistance determinants within the ICU may be useful in optimising infection control within the hospital. Samples were taken and analysed via PCR for the presence of bacterial 16S rRNA genes, antibiotic resistance determinants (including *mec*A and *tet*) and beta-lactamase genes. Parallel cultural analysis was used to assess the presence of fungi. Bacterial species, diversity and communities were identified using PCR-denaturing gradient gel electrophoresis (PCR-DGGE).

Using culture dependent and independent techniques, sequences similar to opportunistic pathogens were retrieved from a variety of ICU environmental sites (patient chair, floor and ward sink plughole). Clinically significant non-*albicans Candida* species were detected in the hospital environment where individual ICU patients were colonised, suggesting there is a reservoir in the ward environment. Despite the low detection frequency, resistance determinants of clinical relevance (*mec*A and bla_{TEM} genes) were observed in the ICU environment at sites that may have infection control significance.

Several sites used by hospital staff and patients (ward sink plughole, floor, patient chair, sluice room sink plughole, handwash bottles and curtains) within the ICU environment were shown to act as reservoirs for particular fungal (isolates of *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida guillermondii*) and bacterial (*Burkholderia* spp., *Stenotrophomonas maltophilia*, *Acinetobacter* spp. and isolates of *Micrococcus* spp., coaglulase negative *Staphylococci*) opportunistic pathogens. Routine ICU ward cleaning was largely effective on hard surfaces (floors and patient chairs). Opportunistic pathogens (*Stenotrophomonas maltophilia*, *Burkholderia* spp.) could be retrieved via PCR-DGGE after cleaning from ICU ward sink plugholes. There was a wide distribution of bla_{TEM} genes in the ICU environment and detection in clinical isolates is of significance. The results of this study indicate that changes in routine ICU ward cleaning of ward sink plugholes may be beneficial in removing opportunistic pathogens and antibiotic resistance determinants from within these sites.

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Abbreviations

CLED	Cysteine Lactose Electrolyte Deficient
CVC	Central Venous Catheter
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribo Nucleic Acid
ESBL	Extended Spectrum Beta-Lactamase
HAI	Hospital Acquired Infection
HDU	High Dependency Unit
HIV	Human Immunodeficiency Virus
ICU	Intensive Care Unit
MDR	Multi Drug Resistant
MIC	Minimum Inhibitory Concentration
MRSA	Meticillin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NaDCC	Sodium DiChloroisoCyanurate
NICU	Neonatal Intensive Care Unit
NGH	Northern General Hospital
PBP	Penicillin Binding Protein
PCR	Polymerase Chain reaction
PDG	Potato Dextrose Glucose
PFGE	Pulse Field Gel Electrophoresis
RHH	Royal Hallamshire Hospital
RSV	Respiratory Syncytial Virus
RT	Room Temperature
TTC	Triphenyl Tetrazolium Chloride
UTI	Urinary Tract Infection
VZV	Varicella Zoster Virus
YPD	Yeast Peptone Dextrose

1. Introduction

1.1 Overview

Extensive work has been carried out on the transmission of nosocomial (hospital-acquired) infections based upon clinical isolates, however, the role of the ward environment as a potential source or reservoir for these organisms is much less well understood. The impact of nosocomial infections caused by antibiotic resistant organisms has been high on the political and public agenda. There is an awareness based on the role of the ward environment in the transmission of nosocomial infections and the importance of effective infection control measures. Intensive care unit (ICU) patients are particularly vulnerable to infections which can lead to an increase in hospitalisation, antibiotic usage, mortality and costs. This study uses molecular and cultural methods to identify the microbial (bacterial and fungal) ecology and associated antibiotic resistance determinants in the ICU environment, to locate, identify, characterise and describe the distribution and classification of potential reservoirs of nosocomial infection-causing organisms.

1.2 An overview of hospital acquired infections

Hospital acquired infections (HAIs) are also referred to as nosocomial infections. By definition these infections are either not incubating or present at the time the patient is admitted to hospital (or other health-care facility) **(WHO, 2002; Vincent, 2003)**. Urli *et al.* (2002) defined ICU-acquired infections as infections occurring more than 48 hours after admission to the ICU.

HAIs are a major concern, because among 9.6 million admissions to publicly-funded hospitals in the UK during 2002-2003, 0.5-1 million patients acquired a hospital infection. Added to this, 5-10% of inpatients acquired an infection during their stay **(Wilcox, 2003)**.

Although all hospitalised patients are at a risk of HAIs, patients in the ICU are especially vulnerable. ICU patients are often severely immuno-compromised, meaning they have a weakened immune system as it is impaired by disease and/or treatment. Many organisms which cause HAIs are also opportunistic pathogens, meaning they generally do not cause disease/infection in healthy individuals with intact defence mechanisms. However, such organisms can cause infection in immuno-compromised patients and when introduced during invasive procedures **(Mims *et al.*, 1998a)**.

HAIs are often caused by antibiotic resistant micro-organisms (some are multi-drug resistant). The high frequency of antibiotic resistant infections among ICU patients is due to the extensive use of broad-spectrum antibiotics as well as the increased use of invasive medical techniques **(Mims *et al.*, 1998b)**. Examples include MRSA (meticillin- and multi- resistant *Staphylococcus aureus*), VRE (vancomycin resistant enterococci) and multi-resistant Gram-negative bacilli (i.e. *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas aeruginosa* and *Escherichia coli*) **(Crowe *et al.*, 1998; WHO, 2002; French, 2005)**.

The most frequent pathogens to cause HAIs include *S. aureus*, *P. aeruginosa*, Gram-negative aerobes and the *Candida* spp. (e.g. *Candida albicans*, *Candida glabrata*, *Candida krusei*) **(Urli *et al.*, 2002)**. HAIs are not only caused by bacteria and fungi, they can be viral. Some examples of hospital acquired viruses are; respiratory viruses (including influenza and respiratory syncytial virus - RSV), herpes virus (including varicella zoster virus - VZV), hepatitis viruses, rotavirus as well as viruses acquired via the respiratory route (including rubella and measles) **(Mims *et al.*, 1998a)**.

Hospital infections can be acquired from two sources: -

1. an endogenous source, for example from another site within the patient (self/auto infection) or by the patients commensal flora
2. an exogenous source, for example from another patient or the environment **(Mims *et al.*, 1998a)**.

HAIs are rarely transmitted via the airborne route; the main transmission route (cross-infection) is by hospital workers (either directly or indirectly) **(Wilcox, 2003)**. The most common HAIs are pneumonia, urinary tract infections (UTIs), central venous catheter (CVC) infections and CVC-related sepsis **(Urli *et al.*, 2002)**. Bacteraemia can arise from multiple sources and can be primary i.e. by the direct inoculation of organisms into the patient's blood from contaminated intravenous fluids. Or it can arise secondary to a focus of infection already present, for example UTIs **(Mims *et al.*, 1998a)**. Each of these infections carries significant mortality rates for ICU patients. UTIs (which may include cases of urosepsis) have an extremely high mortality rate of 25-60 % among ICU patients **(Leone *et al.*, 2001)**. For those with pneumonia or bacteraemias mortality is approximately 34 % **(Blot *et al.*, 2002; Rello *et al.*, 2003)**.

The host response to any infection is dependent upon a number of factors. For example very young individuals are highly susceptible due to the immaturity of their immune system. Similarly, the elderly are at a greater risk due to pre-disposing underlying disease, immobility, impaired blood supply and general decline in immune function **(Mims *et al.*, 1998a; Urli *et al.*, 2002)**. However, in all age groups underlying disease and treatment can pre-dispose to infection, resulting in all ICU patients being at risk.

1.3 Intensive care units

Patients in ICUs are extremely vulnerable to infections, largely because ICU patients are extremely immuno-compromised. According to prevailing medical practice in UK hospitals there are four major priorities which determine whether a patient is considered for admission to the ICU: -

1. Patients who are critically ill and are in a medically unstable state which requires monitoring and treatment (i.e. they require an intensive level of care)
2. Those who require intensive monitoring but may also need emergency interventions
3. Patients who are either medically unstable or critically ill but have a chance of recovery (due to the severity of their illness or trauma)
4. Patients who are not eligible for admission to the ICU as they are not expected to survive. If a patient falls into this category the director of the ICU needs to give their approval before admission **(Gulli *et al.*, 2006)**.

There are several types of intensive care unit: -

General ICU – for the general treatment of critically ill patients

Coronary care unit (CCU)

Paediatric ICU (PICU) - for the treatment of critically ill children

Newborn ICU (NICU) - for the care of premature and critically ill neonates

Surgical ICU (SICU) - for the treatment of postoperative patients **(Gulli *et al.*, 2006)**

The ICUs studied were classed as general ICUs, for the treatment of critically ill patients after trauma or underlying diseases.

The cost to the NHS is greater for ICU patients compared with patients on other wards because of the need for specialist care **(Dean *et al.*, 2002)**. 0.13 % of the annual budget for the NHS is spent on ICU services. The total annual budget for the NHS was £90.7 billion in 2007-2008 which resulted in £1700 per patient in England **(HM Treasury, 2008)**. The average financial cost of treating a patient in the ICU increases six-fold if the patient has or develops sepsis (characterised by a whole body inflammatory ststae which can be due to the presence of bacteria) **(Edbrooke *et al.*, 1999)**. Hence, the costs to the NHS is far higher if ICU patients gain HAIs and there is a great need to identify if the ward environment is acting as a source or reservoir for the organisms that cause HAIs.

1.4 Bacterial HAIs

1.4.1 Bacterial HAIs and risk factors

The majority of HAIs are caused by bacteria which are often members of our normal flora **(WHO, 2002)**. Between 1985 and 1995 the number of bacteraemia and fungaemia HAI cases increased dramatically from 17.7 to 80.3 per 1000 admissions **(Crowe *et al.*, 1998)**. In the early 1990's Gram-negative bacteria were the cause of high mortality but Gram-positive organisms were becoming increasingly important **(Neu *et al.*, 1993)**. MRSA, *Stenotrophomonas maltophilia, P. aeruginosa* and enterococci also became common pathogens among ICU patients **(Neu *et al.*, 1993)**.

Frequently reported in the literature now are bacterial nosocomial outbreaks, and common pathogens include *S. aureus*, *E. coli, Enterobacter cloacae, Klebsiella* spp., *P. aeruginosa*, *S. maltophilia*, *Acinetobacter baumannii* and other commensal organisms including *Staphylococcus epidermidis* which can act as opportunistic pathogens **(Crowe *et al.*, 1998)**.

To monitor the severity of HAIs, in 2001 the Department of Health made the surveillance of MRSA mandatory; this was extended in 2003 to include glycopeptide-resistant enterococci and in 2004 *Clostridium difficile* **(HPA, 2006a)**.

Risk factors for HAIs are often similar and the same organism can present as numerous infections. For example *Acinetobacter* spp. can cause ventilator-associated pneumonia, skin and soft tissue infections, UTIs, bacteriaemia and wound infections in critically ill patients **(Dijkshoorn *et al.*, 2007)**. The majority of risk factors for these infections are common in critically ill patients on ICUs. These include: -

- Mechanical ventilation
- Invasive procedures
- Indwelling devices
- Previous stay and length on an ICU
- Exposure to contaminated medical equipment **(Dijkshoorn *et al.*, 2007; Markogiannakis *et al.*, 2009)**

1.4.2 Prevention of bacterial HAIs

The Health Act 2006 introduced strict measures to help reduce and combat HAIs. One area of focus was upon the need to provide and maintain a clean and appropriate environment for healthcare **(Department of Health, 2008)**. Eight guidelines were laid out in the health act: -

1 – Effective communication to be made between infection control staff and facilities management

2 – Designate a lead manager for cleaning and decontamination of medical equipment used for treatment

3 – All areas of the ward involved in healthcare are kept clean and maintained in good physical condition and repair

4 – The cleaning arrangements meet the standard of cleanliness required and the cleaning regimes are available to the public

5 – Sufficient availability of handwash facilities and anti-bacterial handrubs

6 – Arrangements for effective decontamination of medical equipment

7 – Linen and laundry supplies comply with the Health Service Guidance (95) 18

8 – Ensure the staff uniform is clean and appropriate for the duties being carried out **(Health Act, 2006)**.

Since these guidelines have been introduced there have been reductions in some HAIs, in particular MRSA. However, there is still extensive research into the effectiveness of routine cleaning **(Danforth *et al.*, 1987; Dharan *et al.*, 1999; Wilcox *et al.*, 2003; Bhalla *et al.*, 2004; Dancer, 2004; Denton *et al.*, 2004; Kramer *et al.*, 2006; Dancer, 2009; Whittington *et al.*, 2009)**.

Some organisms including *A. baumannii* are able to survive in the ward environment due to multi-drug resistance and tolerance of drying **(Denton *et al.*, 2004)**. Additional cleaning was introduced after an outbreak of *A. baumannii* on a neurosurgical ICU and initially the number of *A. baumannii* positive environmental sites increased. When the outbreak occurred, several cleaning practices were revised on the ICU; one was to make the ward cleaning staff responsible for the cleaning of the ICU environment and the nursing staff responsible for cleaning patient equipment. Denton *et al.* (2004) demonstrated that when strict cleaning protocols were not followed the levels of *A. baumannii* in the environment correlated with patient infection and colonisation.

Dancer *et al.* (2009) investigated the impact an additional cleaner had on the presence of *S. aureus* (and MRSA). Overall a reduction was observed on the microbial contamination of hand-touch sites. The areas of greatest contamination were areas

closest to the patient (bedside locker and bed frame) as opposed to infusion pumps and computer keyboards. Molecular epidemiological evidence supported the conclusion that patients acquired MRSA from these particular environmental sites **(Dancer *et al.*, 2009)**.

Not only were the number of MRSA infections reduced but this also resulted in lowered hospital costs. Each MRSA infection costs on average £9000 and by introducing the enhanced cleaning the hospital saved between £30,000-70,000 **(Dancer *et al.*, 2009)**. Although this study showed the clear benefits of enhanced cleaning others have previously shown evidence that certain cleaning methods are not effective.

Comparison of detergent (cleaning aid which facilitates physical removal) and disinfectant (to kill micro-organisms) used for floor cleaning showed no difference in the levels of microbial floor contamination and the nosocomial infection rate did not alter **(Danforth *et al.*, 1987)**. Other studies to reduce infection rates by increasing cleaning have also failed **(Dancer, 1999; Wilcox *et al.*, 2003)**. Dharan *et al.* (1999) demonstrated the nosocomial infection rate did not alter over a four month period using various cleaning agents.

Organisms such as MRSA and *C. difficile* have been shown to persist in the environment even after general disinfection so other methods have been explored. The most effective cleaning reagent for the removal of persistent organisms such as *C. difficle* (which produces highly resistant endospores), MRSA and *A. baumanni* is hypochlorite on a large scale to disinfect the whole ward environment **(Wilcox *et al.*, 2003)**, however, this reagent does require the evacuation of patients and ward shut down.

1.4.3 Presence of bacteria in the ICU environment

The effectiveness of ward cleaning has been based upon controlling outbreak situations and little is known of the general microbial ecology of the ICU or any ward environment where these organisms may be surviving. Rather than trying to control an outbreak situation prevention would be better. It is acknowledged that the majority of infections are caused by patients own flora, however by investigating multiple environmental sites within the ICU for bacterial organisms more information and evidence would be provided on reservoirs of organisms.

The use of 16S rDNA sequences have become increasingly important in studying bacterial communities from environmental samples **(Muyzer *et al.*, 1993)**. Culture-independent methods are generally more reliable and rapid as they overcome the limitations of culture-dependent methods **(Ercolini, 2004)**. There is limited knowledge of the exact conditions which bacteria require to grow in any given environment which can result in no isolation **(Barer, 1997; Ercolini, 2004)**. This can however, lead to the question: are the bacterial cells viable?

A study based on microbial ecology in the environment can not solely rely on either method (culture dependent or independent). It is possible that bacteria could be detected via molecular methods but not be cultured.

This could be due to a number of factors: -

1. Bacterial cells are not viable
2. Bacterial cells are viable but non-culturable
3. Bacterial cells are in a dormant state **(Barer, 1997)**

It could also be the case that bacteria are not detected by molecular methods but can be cultured, however, this would generally occur as molecular techniques cannot detect a single bacterial cell but under favourable growth conditions could form a single colony forming unit **(Ware and Hawker, 1979)**. However, there are advantages to using culture techniques particularly in the clinical setting due to low cost, non-specialist training/equipment, rapid growth of organisms and detection. Therefore for studies observing microbial ecology both culture-dependent and independent methods should be used to gain the widest knowledge of that environment.

Computer keyboards and taps are environmental sites common to all hospital wards and are in constant use and contact with the hands of hospital staff. Bures *et al.* (2000) using a culture-dependent method sampled the taps and computer keyboards of an ICU. The computer keyboards presented as the most contaminated site (24 %), with taps being 11 % and it was noted that the keyboards were contaminated regardless of patient proximity. Although not all the bacteria cultured were identified, MRSA was isolated from the tap and keyboard in a room with an MRSA positive patient **(Bures *et al.*, 2000)**. This study had significant limitations, in particular the samples were only taken on one sampling session and no repeats were carried out at another time.

Several studies showed similar findings; *A. baumannii* was identified on computer keyboard covers but not from other environmental sites within the patients' room **(Neely *et al.*, 1999)**. In a surgical ICU the computer keyboards were shown to have the highest levels of pathogenic organism colonisation compared to other environmental sites **(Hartmann *et al.*, 2004)**. A hospital which had high MRSA infection rates was shown to have high MRSA colonisation on computer keyboards **(Devine *et al.*, 2001)**.

Conversely, a study based on computers in an ICU showed that the central unit of computers did not act as a reservoir for nosocomial pathogens **(Quinzio *et al.*, 2005)**.

Again this was culture-dependent which inevitably provides limitations to the study. No pathogenic bacteria or fungi were cultured from numerous sites around the computer (fan, ventilator and base) **(Quinzio *et al.*, 2005)**.

All these studies show the potential for computer keyboards and taps to be a reservoir of pathogenic bacteria in the ICU setting. However, with only using culture-dependent methods it is possible that some organisms were missed if they could not be cultured on the pathogenic specific media used or were out competed by other organisms (on non-specific media).

Some of the most important pathogenic organisms able to survive in the environment are *C. difficile*, MRSA and VRE. Despite these pathogenic organisms and others identified in the ICU (and other ward) environments **(Devine *et al.*, 2001; Naas *et al.*, 2002; Shiomori *et al.*, 2002; Wilcox *et al.*, 2003; French *et al.*, 2004; Lemmen *et al.*, 2004; HPA, 2006b; Mammina *et al.*, 2007; HPA, 2008)** it is unclear whether they are a source of patient colonisation/infection or a reservoir of these organisms (either from the environment or patients) **(Hota, 2004)**. For example, by following VRE-colonised ICU patients, the environment was positive for VRE from 25 % of patient cases **(Drees *et al.*, 2008)**. The environment was not concluded to be acting as a reservoir but contaminated from the patient **(Drees *et al.*, 2008)**.

A previous study has also shown that nosocomial infection rates were unchanged when movement of a department to a new hospital led to a decrease in environmental contamination **(Maki *et al.*, 1982)**.

Conclusions can be drawn from the current data available: -

1 – Environmental surfaces can become contaminated from colonised patients

2 – Non-colonised patients can be at risk of developing HAIs from a contaminated room

3 – Specific pathogenic micro-organisms may be dominant in the environment but are not colonising or infecting patients **(Hota, 2004)**

At present there is no conclusive evidence of the extent to which the ward environment is the source for HAIs. Available data supports the possibility that specific environmental sites may act as reservoirs for nosocomial pathogenic organisms **(Neely *et al.*, 1999; Devine *et al.*, 2001; Naas *et al.*, 2002; Hartmann *et al.*, 2004; Hota *et al.*, 2009; Whittington *et al.*, 2009)**. However, there are numerous environmental sites within an ICU ward including floors, bedside, ward sink plughole, computer keyboards, patient/staff chairs, curtains, trolleys, fans, sluice room sink plughole, pictures, door handles, ward sink taps and switches, but limited information is available to indicate whether any of these sites are reservoirs of nosocomial pathogens. Environmental sites are often tested if an outbreak situation has occurred (e.g. MRSA or *A. baumannii* infections) or one environmental site is tested just for bacterial contamination or to demonstrate effective cleaning methods.

The ward environment (particularly the ICU) needs to be sampled for a prolonged period of time to identify whether reservoirs of nosocomial pathogens are present and whether they persist in the environment.

1.5 Fungal nosocomial infections

1.5.1 Fungal organisms that cause HAIs

Approximately 10% of the known 200 *Candida* species are able to cause human infections. *Candida* spp. are the most common cause of fungal nosocomial infections

and over the past 20 years the incidence of yeast infections has risen dramatically **(Verduyn-Lunel *et al.*, 1999; Leone *et al.*, 2003; Hobson, 2003; Bassetti *et al.*, 2006)**. *C. albicans* had been the most common cause of fungal hospital infections for many years but non-*albicans Candida* species are increasing in prevalence **(Verduyn-Lunel *et al.*, 1999; Hobson, 2003; Shelenz and Grandsen, 2003; Bassetti *et al.*, 2006)**. *Candida parapsiolosis* and *Candida tropicalis* have been isolated more frequently than *C. albicans* in some European and Latin American countries **(Bassetti *et al.*, 2006)**. It has been reported that *Candida* infections can result in an increased hospital stay of approximately 30 days and a 60 % crude mortality rate **(Abi-Said *et al.*, 1997)**. *Candida glabrata* is the fourth most common *Candida* species isolated from blood samples and yet has a similar mortality rate to infections caused by *C. albicans* **(Rentz *et al.*, 1998; Leone *et al.*, 2003)**.

1.5.2 Risk factors for fungal HAIs

The two main factors which predispose to invasive *Candida* species infections are immunosuppression and treatment on an ICU **(Blumberg *et al.*, 2001; Hobson, 2003)**. The majority of fungal nosocomial infections are blood-related and referred to as candidaemia **(Hobson, 2003)**. The *Candida* spp. can cause a wide range of infections from superficial to invasive candidiasis **(Rentz *et al.*, 1998; Verduyn-Lunel *et al.*, 1999; Blot and Vandewoude, 2004; Laverdiere *et al.*, 2007)**, with the more extreme infections often seen in ICU patients.

The most common risk factors are mechanical ventilation, presence of CVCs and the use of antibiotics **(Laverdiere *et al.*, 2007)**. Up to 80 % of hospitalised patients have mucosal surfaces colonised with *C. albicans* **(Verduyn-Lunel *et al.*, 1999)**. This acts as a high pre-disposing factor for critically ill patients' developing disseminated infections **(Laverdiere *et al.*, 2007)**. Fungal colonisation and infections have been high

among ICU patients, with 71 % being colonised with yeasts and the remaining 29 % being infected **(Verduyn-Lunel *et al.*, 1999)**.

1.5.3 Prevention of fungal HAIs

Due to the high numbers of patients colonised with *C. albicans*, prevention of infections can be difficult especially in patients with numerous risk factors. The main focus has been on hand hygiene since it was reported that 80 % of general nosocomial infections are transmitted by the hands of hospital staff **(Gniadek and Macura, 2007)**. One study showed 57-61 % of ICU staff to be hand carriers of *Candida* species **(Brunetti *et al.*, 2008)**. They concluded that appropriate use of gloves could reduce the presence and spread of pathogenic yeasts from hospital staff hands to patients.

It has been suggested that patient screening may be of some benefit for certain patient groups (e.g. ICU patients) **(Schelenz, 2008)**. The majority of ICUs already follow good operative techniques, selective antibiotic usage and good line care policies but there is also the possibility of anti-fungal prophylaxis **(Schelenz, 2008)**. Using prophylaxis may lead to the increased risk of antifungal resistance development, but for those patients at the greatest risk of developing Candidiasis this preventative measure may be beneficial **(Bolt and Vandewoude, 2004)**.

1.5.4 Presence of fungi and infected patients on ICUs

Limited research has been carried out on fungi in the hospital or ICU environment. However, Gniadek and Macura (2007) identified *Candida* spp. from indoor air and walls of an ICU in a Polish hospital. A number of infections were shown to be non-*albicans* species, with the most common being *C. parapsilosis*, *C. tropicalis* and *C. glabrata*.

The majority of research is based on patient infections, particularly reporting a shift from *C. albicans* infections to non-*albicans* infections **(Nguyen *et al.*, 1996; Hobson, 2003; Bassetti *et al.*, 2006)**. In Italy, a five year study showed a decrease in *C. albicans* infections but an increase in those caused by *C. parapsilosis*, *C. tropicalis* and *C. glabrata* **(Bassetti *et al.*, 2006)**. Generally *C. tropicalis* was the second most frequently isolated *Candida* spp. after *C. albicans*, followed by *C. glabrata* from patients **(Kornshian *et al.*, 1989; Nguyen *et al.*, 1996)** but some studies show *C. parapsislosis* to be the most common non-*albicans* *Candida* spp. isolated from ICU patients **(Bassetti *et al.*, 2006)**.

Although differences in the distribution of *Candida* spp. in patient infections has been observed between different ICUs, no difference has been seen between the ICU and conventional wards within the same hospital **(Leone *et al.*, 2003)**. The majority of patients were colonised with *C. albicans* (68 %) but a number were colonised with *C. glabrata* (9.4 %) **(Leone *et al.*, 2003)**. Similar results were seen from a Canadian ICU with 60.2 % of all patients being colonised with a fungal species; among which 72 % were *C. albicans* and 5 % *C. glabrata* **(Laverdiere *et al.*, 2007)**.

It is possible (although rare) for patients to be colonised with multiple *Candida* species. 4.2 % of patients from a Texas cancer unit were colonised with $\geq$2 *Candida* species. The majority of these patients (90 %) were colonised with *C. albicans* and either *C. glabrata, C. parapsilosis* or *C. tropicalis* but two patients were colonised with two non-albicans species (*C. tropicalis* with *C. glabrata* or *C. parapsilosis*) exemplifying the shift towards non-*albicans* species **(Abi-Said *et al.*, 1997)**.

Not only is Candidemia a life-threatening yeast infection but there are significant additional costs to the NHS as a direct result of such infections **(Rentz *et al.*, 1998)**. The cost of a candidemia case in an ICU has been estimated at €16,000 **(Schelenz, 2008)**. With the majority of risk factors common in ICU patients, the high mortality risk

and the additional costs related to these infections, there is a need to observe the ICU environment for any potential reservoirs of fungal organisms to further reduce the risk of infection in this vulnerable group.

1.6 Antibiotic resistance

Antibiotic resistance is the term used when a bacterium or other micro-organism is permanently non-susceptible to a specific antibiotic **(Walsh *et al.*, 2004)**. There are several mechanisms which have evolved among bacteria to enable antibiotic resistance, for example, efflux pumps, antimicrobial target alteration, membrane permeability alteration, enzymatic modification and metabolic bypass **(Smith & Jarvis, 1999)**.

Bacteria can either acquire antimicrobial resistance or they are naturally resistant possessing the necessary resistance mechanism. Resistance can be acquired by horizontal gene transfer, from transmissible plasmids or transposons **(Mims *et al.*, 1998b)**. Transposons which possess antibiotic resistance genes have the ability to integrate into plasmids or chromosomes. They also possess the ability to "jump" between plasmids i.e. from a non-transmissible plasmid to a transmissible one **(Mims *et al.*, 1998b)**.

Bacteria can acquire antimicrobial resistance in any of four ways: -

1. Mutation - resistance can arise from a mutation i.e. a single chromosomal mutation may result in the synthesis of an altered protein. Or a series of mutations can result in multiple changes i.e. in penicillin-binding proteins **(Mims *et al.*, 1998b)**

2. Conjugation - Gram-negative bacteria transfer plasmids containing resistance genes to adjacent bacteria via the pilus. With Gram-positive bacteria conjugation is initiated by the production of sex pheromones by the mating pair **(Tenover, 2006)**
3. Transduction - resistance genes are transferred between bacteria via bacteriophage **(Tenover, 2006)**
4. Transformation - bacteria are able to take up naked DNA. Bacteria can release DNA segments into the environment via cell lysis, and other bacteria are able to acquire and incorporate these DNA segments **(Tenover, 2006)**

The study described in this thesis focused on detecting the antibiotic resistance determinants *mec*A, $bla_{CTX\text{-}M,\ SHV,\ TEM}$ and *tet*(M, O, W) in the ICU and HDU ward environments. The following sections not only describe the mechanisms of resistance caused by these genes but also the associated organisms (including MRSA and common ESBL-producing organisms). Risk factors, infections, treatment and the presence of these organisms and genes in the ICU environment are also discussed.

1.7 Extended spectrum beta-lactamases

1.7.1 Overview of ESBLs

One of the most clinically significant resistance mechanisms to have emerged is the production of extended spectrum beta-lactamases (ESBLs). ESBLs have become a predominant feature in Gram-negative hospital infections over the last 25 years **(Sturenburg and Mack, 2003)**, since the identification of the first ICU ESBL characterised back in 1985 from *K. pneumoniae* isolated from patients in France **(Sirot *et al.*, 1987)**. Since then there has been extensive research into the detection of ESBL-producing bacteria and prevention and treatment of the diseases they cause.

ESBLs are plasmid-mediated bacterial enzymes that are able to hydrolyse a wide variety of β-lactam antibiotics **(Naiemi *et al.*, 2005)**. They have evolved from native serine active-site, Ambler class A β-lactamases i.e. TEM-1, TEM-2 and SHV-1 by genetic mutation **(Pfaller & Segreti, 2006)**. The TEM-1 β-lactamase is most commonly seen in ampicillin-resistant Gram-negative enteric organisms. However, the most common β-lactamase among the *Klebsiella* spp. is SHV-1 **(Philippon *et al.*, 1989)**. TEM-1, -2 and SHV-1 are able to hydrolyse ampicillin at a greater rate compared with oxacillin, cephalothin and carbenicillin. However, they show little or no activity against antibiotics such as cefotaxime or ceftazidime **(Philippon *et al.*, 1989)**.

ESBLs are more frequently found in *P. aeruginosa*, *Serratia marcescens* and *Salmonella enterica* **(Pfaller & Segreti, 2006)**. Not only do these enzymes confer resistance to penicillins and first and second-generation cephalosporins, but also to the newer classes e.g. oxyimino cephalosporins (including cefotaxime and ceftazidime) and monobactams (including aztreonam) **(Sturenburg & Mack, 2003)**.

UTIs are the main clinical manifestations of ESBL-producing organisms but bloodstream infections are also seen **(Pessoa-Silva *et al.*, 2003; Sturenburg and Mack, 2003; Tumbarello *et al.*, 2007; Zahar *et al.*, 2009)**. Risk factors include increased age, previous UTIs, catheterisation, female sex, previous antibiotic usage and diabetes mellitus **(Pessoa-Silva *et al.*, 2003; Livermore and Woodford, 2006; Livermore *et al.*, 2007; Falagas and Karageorgopoulos, 2009)**.

Patients with infections caused by ESBL-producing organisms have a high mortality rate and for those with blood-stream infections the mortality rate is further increased due to treatment failures. One study showed that after 21 days from infection onset the mortality rate was 38 % **(Tumbarello *et al.*, 2007)**. These risk factors apply to all hospitalised patients but ICU patients are more prone to these factors (particularly with regard to catheterisation and antibiotic usage).

Serious infections caused by ESBL-producing organisms are treated with carbapenems **(Rodriquez-Bano and Pascual, 2008)**. Inadequate initial treatment of these infections has been found as a predictor for mortality, with many ESBL-producing organisms being multi-drug resistant, treatment failures are high **(Tumbarello *et al.*, 2007)**.

In some cases initial treatment is delayed because of a lack of rapid identification (up to 72 hours after infection onset) **(Song *et al.*, 2009; Trecarichi *et al.*, 2009)**. Therefore there is a need for a quick and accurate identification technique for these organisms; the main method of identification is by culture and disk diffusion. Data from this present study contributed to the development of a simple disk diffusion overnight method to identify ESBL and AmpC-producing organisms **(Derbyshire *et al.*, 2009)**.

A variety of β-lactamases have been classified into classes A, B, C and D according to their amino acid sequences. Class A enzymes are serine hydrolases with a serine active site which is acylated by the β-lactam antibiotic **(Fernandez-Varon *et al.*, 2005)**. There are three pre-dominant groups of ESBLs bla_{SHV} (class A), bla_{TEM} (class A) and $bla_{CTX\text{-}M}$ (class A).

CTX-M-type ESBLs are a new group of class A ESBLs **(Brinas *et al.*, 2005)**. The CTX-M β-lactamases have a potent hydrolytic activity against cefotaxime and some CTX-M-type ESBLs are able to hydrolyse ceftazidime **(Paterson & Bonomo, 2005)**. These enzymes are not closely related to either TEM or SHV ESBLs, but they do share a high amino acid identity with chromosomal β-lactamases from *Kluyvera georgiana*, *Kluyvera cryocrescens* and *Kluyvera ascorba*. **(Pitout *et al.*, 2004)**.

SHV-type ESBLs are more commonly found in clinical isolates compared with other ESBLs **(Paterson & Bonomo, 2005)**. SHV stands for sulfhydryl variable, this is because it was believed that inhibition of SHV activity was by p-chloromercuribenzoate

and was substrate-related but was variable according to the substrate **(Paterson & Bonomo, 2005)**. However, this has since never been confirmed but the name has remained. SHV-1 is a β-lactamase but not an ESBL. This is because it can only hydrolyse penicillin and cephalosporins but not extended-spectrum antibiotics (i.e. oxyimino cephalosporins) **(Hammond *et al.*, 2005)**. However, conversion to an ESBL can be caused by the single amino acid substitution G238S. The additional substitution E240K leads to further spectrum extension and increased enzyme activity **(Hammond *et al.*, 2005)**. This enzyme has been denoted as SHV-2 and since its discovery within 15 years it has been identified across the world. SHV-type ESBLs have been detected in a wide range of *Enterobacteriaceae* and outbreaks of SHV-producing *P. aeruginosa* and *Acinetobacter* spp. are reported **(Paterson & Bonomo, 2005)**.

TEM-type ESBLs are derived from TEM-1 and TEM-2. TEM-1 was initially reported in 1965 from an *E. coli* isolate of a patient named Temoniera (hence TEM) **(Paterson & Bonomo, 2005)**. Beginning in the early 1980s ESBLs derived from TEM-1 began appearing in the clinical setting **(Livermore, 1995)**. TEM-1 possesses the ability to hydrolyse ampicillin at a higher rate than it hydrolyses carbenicillin, oxacillin or cephalothrin and has a very low activity against extended spectrum cephalosporins **(Paterson & Bonomo, 2005)**. TEM-1, -2 and -13 are not classed as ESBLs due to this low activity. However, certain *K. pneumoniae* isolates in 1987 possessed a novel plasmid-mediated β-lactamase which is now referred to as TEM-3 **(Paterson & Bonomo, 2005)**. TEM-3 differs only in two amino acid substitutions compared with TEM-2 and has an enhanced activity against the antibiotic cefotaxime **(Paterson & Bonomo, 2005)**. There have now been greater than one hundred TEM-type beta-lactamases identified with the majority being ESBLs. Some TEM derivatives have been identified which possess a lowered affinity for β-lactamase inhibitors (such as clavulanic acid, which inhibits TEM-1 and many other beta-lactamases). However, these enzymes tend to have no hydrolytic activity against the extended cephalosporins

(Wu *et al.*, 1994). Although recently there has been the discovery of mutant TEM β-lactamases that have inhibitor resistance but also maintain the ability to hydrolyse third generation cephalosporins **(Paterson & Bonomo, 2005)**.

1.7.2 Presence of ESBL-producing organisms in the environment and transmission in ICUs

There is limited research into distribution and transmission of ESBL-producing organisms in the hospital environment; the majority of work focuses on clinical isolates and their spread between patients **(Pena *et al.*, 1998; Shannon *et al.*, 1998; Coque *et al.*, 2002; Naas *et al.*, 2002; Naiemi *et al.*, 2005; Zarnayova *et al.*, 2005; Brinzio *et al.*, 2006; Damjanova *et al.*, 2007; Manzur *et al.*, 2007)** rather than identifying the source or reservoir of these infections from the environment. There have been numerous outbreaks of nosocomial infections caused by ESBL-producing organisms **(Livermore, 1995; Naiemi *et al.*, 2005; Brinzio *et al.*, 2006; Damjanova *et al.*, 2007; Manzur *et al.*, 2007)**, particularly in ICUs and evidence shows that these organisms can persist in the hospital environment, particularly in sinks **(Naas *et al.*, 2002)** so research is required to identify these potential reservoirs.

ESBL-producing organisms have been found to persist in the ICU environment after routine cleaning. An ESBL-producing *Citrobacter freundii* strain was identified on an ICU and a clonal outbreak was linked from the ICU to other medical wards. This organism produced the ESBL TEM-21 and was identified from the sinks in the ICU; after decontamination of this area the organism still remained **(Naas *et al.*, 2002)**. Only after the sinks were replaced was the spread of this ESBL-producing organism stopped, indicating that these organisms can persist in the ICU environment and the sink was able to act as a reservoir despite cleaning.

Naiemi *et al.* (2005) showed two outbreaks in an ICU of multi-drug resistant *Enterobacter cloacae* and *A. baumannii* to possess the SHV-12 ESBL gene as well as the TEM-116 β-lactamase gene. Between June and November 2000 aminoglycoside-resistant and ESBL producing *E. cloacae* were isolated from ten patients. After the *E. cloacae* outbreak, infection control measures were taken including extra vigilance with hand hygiene, wearing gowns and gloves during patient care and isolating patients with multi-drug resistant (MDR) *E. cloacae*. However, these control measures did not prevent the multi-drug resistant *A. baumannii* outbreak in the ICU. These events suggest that by preventing the spread of resistant *E. cloacae* it was possible for other strains to become prevalent. Naiemi *et al.* (2005) showed that by further increasing the infection control measures it was possible to halt the outbreak of *A. baumannii.*

In 2004 a Spanish hospital saw only sporadic and non-related cases of ESBL-producing *E. cloacae*, however, an outbreak was observed in 2005 in the cardiothoracic ICU (CT-ICU) due to an epidemic strain (which caused infection in the majority of ICU patients) **(Manzur *et al.*, 2007)**. Emergence of this strain was attributed to the application of an antibiotic cycling strategy, which led to an increased use of cefepime and quinolones **(Manzur *et al.*, 2007)**. As the infections were device-associated it was assumed that cross-transmission was the cause of spread throughout the CT-ICU.

As previously mentioned ESBLs are mainly associated with *E. coli* and *K. pneumoniae* and outbreaks are generally associated with high rates of intestinal carriage, however, this is not often seen with *E. cloacae* **(Pena *et al.*, 1998)**. Of the *E. cloacae* strains identified with this outbreak one was identified by gene sequencing to possess two ESBLs (SHV and CTX-M-9) and the other possessed one (a TEM enzyme) **(Manzur *et al.*, 2007)**. The initial outbreak was therefore believed to have started from a transferable plasmid encoding ESBL production, isolated from other ESBL-producing organisms from three patients **(Manzur *et al.*, 2007)**. This hospital had only

experienced a handful of ESBL cases during the year before the outbreak. *E. cloacae* are not common ESBL producers but with ESBL genes found on transferable plasmids this suggests that any Gram-negative organism could potentially acquire a plasmid and produce an ESBL.

The emergence of ESBL-producing organisms on neonatal-ICUs (NICU) is usually due to antibiotic usage because of the restricted spectrum available. However, Mammina *et al.* (2007) showed a high percentage of ESBL isolates when observing the levels of multi-drug resistant Gram-negative (MDRGN) bacilli to be due to poor infection control compliance. It was shown that from one year surveillance, over 50% of patients were colonised with MDRGN and that a very high cross-transmission rate was observed **(Mammina *et al.*, 2007)**. Through molecular tracing using pulse-field gel electrophoresis (PFGE) they observed large cluster-related multi-drug resistant (MDR) organisms and identified the possibility of a common environmental reservoir interacting with healthcare workers. This study showed that not only can the use of antibiotics lead to the colonisation by MDR Gram-negatives but a lack of infection control measures can increase the risk of emergence of ESBLs enormously. Where Naiemi *et al.* (2005) had to quickly implement altered infection control measures (to halt outbreaks) Mammina *et al.* (2007) observed how easily cross-transmission can occur in NICUs if these measures are not strictly followed.

However, other researchers have shown alternative hypotheses for ESBL transmission within and between ICUs. Studies in Spain and Slovakia suggest that SHV-2a ESBL distribution is not caused by plasmid dissemination and suggest independent evolution of variants from geographically wide spread broad-spectrum non-ESBL enzymes may be the reason for the identification of different ESBL variants found in various locations **(Coque *et al.*, 2002; Zarnayova *et al.*, 2005)**.

Where many studies have shown that ESBL-producing organisms are the cause of epidemics **(Naiemi *et al.*, 2005; Manzur *et al.*, 2007; Mammina *et al.*, 2007)** Coque *et al.* (2002) observed very little evidence of an epidemic (produced by a single bacterial clone) or even an endemic situation (a single clone maintained over a length of time). It is unknown why there is high clonal turnover however it is possible for clonal competition and those organisms possessing an ESBL maybe selected. With these ESBL genes being present in various bacterial populations, via antibiotic selective pressure these genes could enter a variety of *K. pneumoniae* and other bacterial clones **(Coque *et al.*, 2002)**.

1.8 Meticillin resistant *Staphylococcus aureus*

1.8.1 Overview of MRSA

S. aureus is a Gram-positive coccus which can often be found as part of the normal flora of the human skin and many individuals are nasal carriers **(Berkeley, 1979)**. MRSA is a *S. aureus* organism which confers resistance to the widely used antibiotic meticillin.

MRSA has been identified in the hospital environment and is a frequent cause of infections seen on ICUs **(French *et al.*, 2004, Oztoprak *et al.*, 2007)**. In 1999, 57% of all ICU-acquired *S. aureus* infections were meticillin resistant in Europe **(Oztoprak *et al.*, 2006)**. ICU patients who acquire an MRSA bacteraemia infection have a 22 % increased risk of death. This results in an additional 0.3 % to the overall mortality rate on the general ICU **(Thompson *et al.*, 2008)**. Vancomycin was initially the drug of choice to treat MRSA infections but resistance has been reported in America and Japan with reduced susceptibility observed in the UK, and so a combination of glycopeptide antibiotics is now used **(Corey, 2009)**. However, the most effective

treatment for decolonisation of the nasal cavity is the use of the antibiotic mupirocin which is prescribed as a nasal ointment **(Crossley, 2009)**.

There is a need for a rapid detection method of MRSA and many researchers have developed a polymerase chain reaction (PCR) method **(Murkami *et al.*, 1991; Unal *et al.*, 1992; Geha *et al.*, 1994)**. However, with costs and technical ability/training required the methods used in the clinical microbiology laboratories are currently still culture based techniques on MRSA selective media **(HPA, 2008)**.

1.8.2 Risk factors for MRSA infections

MRSA infections can result in high morbidity and mortality and some strains are classed as endemic in several American and European hospitals **(Oztoprak *et al.*, 2006)**. *S. aureus* most commonly causes skin infections which result in abscesses and boils. In patients who have undergone invasive medical techniques, both *S. aureus* and MRSA can cause bloodstream infections (bacteraemia/sepsis) which is the majority of MRSA nosocomial infections, especially among ICU patients. **(Gordon and Lowy, 2008)**.

Oztoprak *et al.* (2006) identified the following risk factors for ICU-acquired MRSA: -

- Long period of ICU hospitalisation
- Presence of >1 MRSA positive patient on the ICU
- CVC insertion
- Previous antibiotic usage

They also showed the need to study the ICU environment for MRSA, as some uncolonised patients acquired MRSA infections from another source, which could be hospital staff or the environment.

Weist *et al.* (2002) showed that where multiple patients were present in the same room, 11.1 % saw all the patients to have an MRSA infection or colonisation. This suggested the presence of mobile reservoirs, either hospital staff or medical devices. This indicates that there is a need to identify these mobile reservoirs and if they are present in the ICU environment.

1.8.3 Resistance mechanism of MRSA

MRSA organisms possess a *mecA* gene that encodes an altered penicillin binding protein (PBP2a), 78kDa in size **(McKeegan *et al.*, 2002)**. PBP2a is a cell wall enzyme which unlike other PBPs has a lower affinity for β-lactam antibiotics and enables cell wall formation despite the presence of drug concentrations which render other PBPs inactive. **(Geha *et al.*, 1994)**. PBPs include transpeptidases that are essential for cross-linking peptidoglycan (which is the essential strength-conferring component of the bacterial cell wall), and beta-lactam antibiotics inhibit these enzymes (as they are analogues of the natural peptide substrates of the enzymes). Inhibition occurs by acylating the active site serine of the PBPs and blocks the active site. The plasmid encoding the altered PBP often possesses other antibiotic resistance genes, rendering MRSA a multi-drug resistant organism. Other such resistance genes include those for rifampicin, erythromycin, tetracycline, aminoglycosides and clindamycin **(Smith & Jarvis, 1999)**.

1.8.4 Prevention of MRSA infections

Many patients (approximately 40 %) are colonised with *S. aureus* and approximately 1 % are colonised with MRSA making prevention difficult. In 2008/2009 the HPA introduced MRSA screening for all elective admissions and before 2011 this will include emergency admissions (however the logistics of 2011 MRSA screening is unclear). Routine screening was introduced to monitor community-acquired MRSA (CA-MRSA) so that patients could be isolated and decolonised to prevent spread of MRSA between patients. Infection control is required to prevent the spread of MRSA among patients and the ward, for example isolation of colonised patients, effective hand hygiene and the use of gloves and gowns **(Furuno *et al.*, 2008)**.

In ward areas of previously colonised or infected patients, MRSA has been detected on multiple environmental surfaces **(French *et al.*, 2004)**. This suggests that beside staff to patient transmission, the ward environment is able to act as a reservoir for MRSA organisms (as described in Sections 1.4.2 and 1.4.3).

All hospital wards have routine cleaning carried out daily (often more than once per day) but questions have arisen as to whether this regime is sufficient to remove MRSA **(Dancer, 1999; Dharan *et al.*, 1999; Wilcox *et al.*, 2003; Dancer, 2004; French *et al.*, 2004)**. In some studies cleaning did not significantly reduce the number of MRSA organisms in the environment. Before cleaning 74 % of environmental swabs were MRSA positive and routine cleaning only reduced this to 66 % **(French *et al.*, 2004)**. After cleaning, Blyth *et al.* (1998) showed that 41 % of the isolation rooms (from a total of 41) were still contaminated with MRSA. Hydrogen peroxide vapour decontamination showed a high reduction in MRSA from 72 % down to 1.2 % **(French *et al.*, 2004)**. However, for this method of cleaning the rooms/wards need to be evacuated first

before decontamination can begin due to the high toxicity of hydrogen peroxide vapour, therefore more information is required on the environment and cleaning regimes.

1.8.5 Transmission of MRSA on the ICU

Reports of MRSA infections on ICUs have been frequent and outbreaks have been observed around the world **(Devine *et al.*, 2001; Wilcox *et al.*, 2003; HPA, 2008; Thompson *et al.*, 2008; Khan *et al.*, 2009)**. One Canadian surgical ICU reported an outbreak when two MRSA cases were identified within a four week period **(Khan *et al.*, 2009)**. Routine screening was not in action and after all patients were screened an additional two patients were found to be infected. Despite implementing specific infection control measures (education, increased surveillance and enhanced environmental cleaning) a further three cases appeared. Patients were placed into contact isolation and the hospital staff were screened. After a two week period another two cases appeared and decolonisation of all positive patients was implemented **(Khan *et al.*, 2009)**. This report shows the ability of MRSA to spread rapidly among patients despite initiating infection control measures and increasing environmental cleaning.

Bed occupancy has been shown to affect the incidence of MRSA infections on ICUs **(Howie and Ridley, 2008)**. One study from Hope hospital (in the UK) showed 7.8 % of ICU admissions to be colonised with MRSA and a further 10.3 % acquired MRSA infections during their hospitalisation. When bed occupancy was high, particularly in cramped ICUs (where airborne and environmental MRSA contamination is more likely) the number of MRSA cases increased **(Howie and Ridley, 2008)**. Physical factors including environmental contamination and patient proximity affect airborne transmission **(Shiomori *et al.*, 2002)** while MRSA contaminates the air and environment around known MRSA positive patients **(NHS Estates, 2003)**. It can be

concluded therefore that the ward environment plays an important source in hospital cross-infection.

1.9 Tetracycline resistance

1.9.1 Overview of tetracycline

Tetracycline is a broad-spectrum antibiotic which has been used for many years to treat bacterial infections in both humans and animals **(Villedieu *et al.*, 2003)**. It can also be used as a growth promoter in animals and in humans as an immunosuppressor. Tetracycline is used to treat a number of Gram-positive and negative infections as well as those caused by intracellular pathogens and protozoa **(Roberts, 2005)**.

The tetracyclines are a family of antibiotics with a common four-ring structure to which a variety of side chains are attached **(Prescott *et al.*, 2002)**. There are five members of this antibiotic family, tetracycline, chlortetracycline, oxytetracycline, doxycycline and minocycline.

The mode of action of the tetracyclines is to inhibit protein synthesis by stopping the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site **(Chopra & Roberts, 2001)**. Since their action is bacteriostatic the success/effectiveness of the treatment is also dependent upon the host defence mechanisms.

Due to extensive use of tetracyclines since their discovery in the 1940's resistance mechanisms have been identified **(Chopra & Roberts, 2001)**. As mentioned tetracyclines can be used as a growth promoter in animal feeds (this mainly occurs in the USA), but this is a rather controversial issue. Many believe that this process may

be significantly contributing to the emergence of resistance in human pathogens **(Chee-Sanford *et al.*, 2001; Chopra & Roberts, 2001; Roberts, 2005)**.

1.9.2 Tet genes in the environment

With tetracycline antibiotics having been used to treat humans and animals and in agriculture, tetracycline resistance genes are commonly found in the environment **(Bryan *et al.*, 2004)**. Several studies show the detection of tetracycline resistance genes from different environmental sources.

These genes have entered the environment as a direct result of agriculture and have been identified from groundwater samples, providing a possible source of antibiotic resistance in the human and animal food chain **(Chee-Sanford *et al.*, 2001)**. The majority of organisms associated with this resistance are of gastrointestinal origin, but by identifying these genes in organisms inhabiting soil this suggests that the vector is not limited and can spread between different bacterial classes **(Chee-Sanford *et al.*, 2001)**.

The tetracycline genes can act as a common antibiotic resistance determinant known to be present in the environment **(Anderson and Sandaa, 1994; Aminov *et al.*, 2001; Roberts, 2005)**.

1.9.3 Mechanism of tetracycline resistance

Bacteria have developed a number of resistance mechanisms against the tetracycline antibiotic class. At present there are 29 different known tetracycline resistance (*tet*) genes and 3 oxytetracycline resistance (*otr*) genes. There are three resistance

mechanisms against tetracyclines; efflux system, ribosome protection and tetracycline modification.

This present study focused on the detection of ribosome protection resistance *tet* genes. There are nine ribosomal protection proteins, named *tet* M, O, S, W, Q, T, *otr*(A), *tet*P(B) and one unnamed *tet*. These are cytoplasmic proteins and confer a wider spectrum of tetracycline resistance compared with the efflux pumps. This method also enables resistance to doxycycline and minocycline **(Chopra & Roberts, 2001)**.

The action is to enable the aminoacyl-tRNA to bind to the acceptor site of the ribosome. These ribosomal protection proteins (RPP's) have sequence similarity to the elongation factors Tu and G. It is the N-terminal regions of these proteins that share the greatest similarity. The tet proteins compete with the elongation factors to bind to the ribosomes. The RPP's bind to the ribosome and alter the ribosomal conformation, the energy for this alteration is provided by GTP hydrolysis **(Chopra and Roberts, 2001; Connell *et al.*, 2003)**. So in the presence of GTP and either Tet(M) or Tet(O) protein the ability of tetracycline to bind to the ribosome is reduced. But also, by altering the conformation the antibiotics can not bind when the protein is released. Tet(M) has a greater affinity than the elongation factors for the ribosome binding site. Tet(M) binds to the ribosome and causes a conformational change. Elongation factor Tu can form the amino acid-tRNA-GTP-EF-Tu complex. The protein dissociates allowing the elongation factor complex to bind and enable protein synthesis to continue **(Connell *et al.*, 2003)**.

1.10 Aims and objectives of the present study

Extensive research has been carried out into the acquisition and transmission of HAIs (particularly in outbreak situations) however; the role of the ward environment in HAIs

has not been fully investigated. The overall aim of the study was to characterise the reservoir of microorganisms (bacterial and fungal) and resistance determinants in the ICU and HDU ward environment under a non-outbreak situation.

Before hospital sampling began an effective sampling regime was required and several methods were optimised to meet the aim of this present study (detailed in Chapter 3). Two hospitals were investigated throughout this study, the Royal Hallamshire Hospital (RHH) and Northern General Hospital (NGH) ICU/HDU departments. Samples were taken from both hospitals however the aim was not to compare the two hospitals; therefore the results have been separated into RHH and NGH. Similarities or contrasts in the results have however been highlighted where appropriate.

The objectives were to: -

- detect bacteria and antibiotic resistance determinants in the ICU and HDU environment using a sensitive molecular method
- isolate bacteria and fungi in the ICU and HDU environment
- identify sites where bacteria and fungi were most frequently detected
- identify bacterial and fungal species
- compare bacterial and fungal species from environmental sites with clinical isolates
- identify bacterial species carrying target genes ($bla_{CTX\text{-}M,\ SHV,\ TEM}$, *mec*A, *tet*M, O, W)
- assess the effect of routine ward cleaning

2. Materials and methods

2.1 Ethical approval

Full ethical approval was granted by the National Research Ethics Service (REC reference number 08/H1310/2). Sheffield teaching hospitals provided the letter of authority for the study period 9th March 2007 to 21st December 2009, along with project authorisation granted on 20th February 2008. This approval enabled entry into the ICU environment at both the RHH and the NGH to collect samples from a variety of sites within the wards (Appendix 1).

2.2 Hospital sampling regime

The ICUs and HDUs of the Sheffield Teaching Hospitals Trust were sampled. At the start of the project (September 2006) there was one general ICU and HDU (six beds on each unit) located at the Royal Hallamshire Hospital (RHH), Glossop Road, Sheffield, S10 2JF. During the project several changes occurred, in March 2008 a new ICU/HDU (two four-bed wards and ten isolation rooms for each unit) was commissioned at the Northern General Hospital (NGH), Herries Road, Sheffield, S5 7AU. When sampling began in March 2008 both ICU and HDUs from the RHH and NGH were included. In July 2008 the RHH HDU moved floors within the hospital and included a larger HDU department (ten beds).

Hard surfaces (bedside, computer keyboard, fan, floor, computer stand, patient chair, picture, staff chair, ward sink plughole, ward sink taps, trolley, sluice room sink plughole, window ledge, door handle and machine handle) were sampled with a neutralising solution (1 ml - 1.5 M NaCl and 1 M Tris base) moistened DACRON swab

(FB57833 Thermo Fisher Scientific Inc). Swabs were taken of the same sample sites for each sampling session. Larger areas (including floors) were sampled using a 20 cm x 15 cm disposable plastic grid, details of each sample site are provided in Table 2.1a/b. Each sample was given a unique code number that specified the type of environment sampled, the hospital and unit from which the sample was taken and the date of the sampling (Appendix 2).

The DNA was extracted from each swab and stored at -20 °C in the 703/-20/2 freezer in room 703 BMRC labelled with the codes from Appendix 2. Microbial enumeration was performed by culturing on solid media (Section 2.4.1 Bacterial growth conditions and 2.4.2 Fungal growth conditions) (Figure 2.1).

Textured surfaces (chairs and curtains) were sampled (Appendix 2) by firmly placing a nutrient, blood (blood agar no2 and horse blood) and potato dextrose agar contact plate (Cherwell Laboratories cat no 101060, 101050 and 101280) onto the sample area.

Nutrient agar dipslides (Cherwell Laboratories cat no CLO500) containing triphenyl tetrazolium chloride (TTC) were used to sample small hard surfaces (hand wash bottles, intercom buttons and switches) by being placed firmly on the sample area (Appendix 2). TTC is a dye used to enable easy enumeration of microorganisms. In the oxidised form TTC appears colourless but upon reduction by microorganisms appears red, this is due to the formation of formazan dye from tetrazolium salts (by dehydrogenase or reductase enzymes).

The results of DNA extraction and the detection of prokaryotic cells and antibiotic resistance determinants are described in Chapters 4 and 5. Microbial enumeration from culturing of swab samples, dipslides and contact plates are reported in Chapter 4. The results of fungal detection after culture from swab samples are described in

Table 2.1a RHH sample sites and description

Sample site	Number of sites sampled per session	Description
Bedside (ICU)	1	One side of the bed (plastic)
Computer keyboard (ICU)	1	The areas between the keys and the base of the keyboard
Fan (ICU)	1	Each plastic slat in the small fan located on the far wall of the ICU
Floor (ICU)	2	A 20 cm x 15 cm area immediately adjacent to the machine nearest the patient bed
HDU computer keyboard	1	The areas between the keys and the base of the keyboard
HDU computer stand	1	The base of the stand
HDU patient chair	3	A 20 cm x 15 cm area from the seat and back of the chair and the arm
HDU picture	1	Horizontal upward-facing surface of the frame
HDU staff chair	1	A 20 cm x 15 cm area from the back of the chair
Picture (ICU)	1	Horizontal upward-facing surface of the frame
Plughole (ICU ward sink)	2	Swab was inserted and turned 3 to 4 times in the plughole
Taps (ICU ward sink)	2	Left and right tap handles of the ICU ward sink located on the far wall of the ICU
Trolley (ICU)	1	A 20 cm x 15 cm area of a supplies trolley surface
Sluice room sink plughole	2	Swab was inserted and turned 3 to 4 times in the plughole
Window ledge (ICU)	1	Horizontal ledge at the joint of the glass with plastic frame

Table 2.1b NGH sample sites and description

Sample site	Number of sites sampled per session	Description
Computer keyboard (ICU)	1	The areas between the keys and the base of the keyboard
Door handle (ICU)	1	The whole of the sluice room door handle (metal)
Floor – isolation room (ICU)	2	A 20 cm x 15 cm area immediately adjacent to the machine nearest the patient bed
Floor – ward (ICU)	2	A 20 cm x 15 cm area immediately adjacent to the machine nearest the patient bed
HDU computer keyboard	1	The areas between the keys and the base of the keyboard
HDU door switch	1	A round electrical switch that is pressed to unlock the door of the HDU on the wall (plastic)
HDU floor	1	A 20 cm x 15 cm area immediately adjacent to the machine nearest the patient bed
HDU ward sink plughole	1	Swab was inserted and turned 3 to 4 times in the plughole
Machine handles (ICU)	2	Left and right handles of the machine nearest the patient bed (patient monitoring equipment)
Patient chair (ICU)	4	A 20 cm x 15 cm area from the seat and back of the chair and the arms
Plughole (ICU ward sink)	2	Swab was inserted and turned 3 to 4 times in the plughole
Sluice room sink plughole	2	Swab was inserted and turned 3 to 4 times in the plughole
Window ledge (ICU)	1	Horizontal ledge at the joint of the glass with plastic frame between two rooms

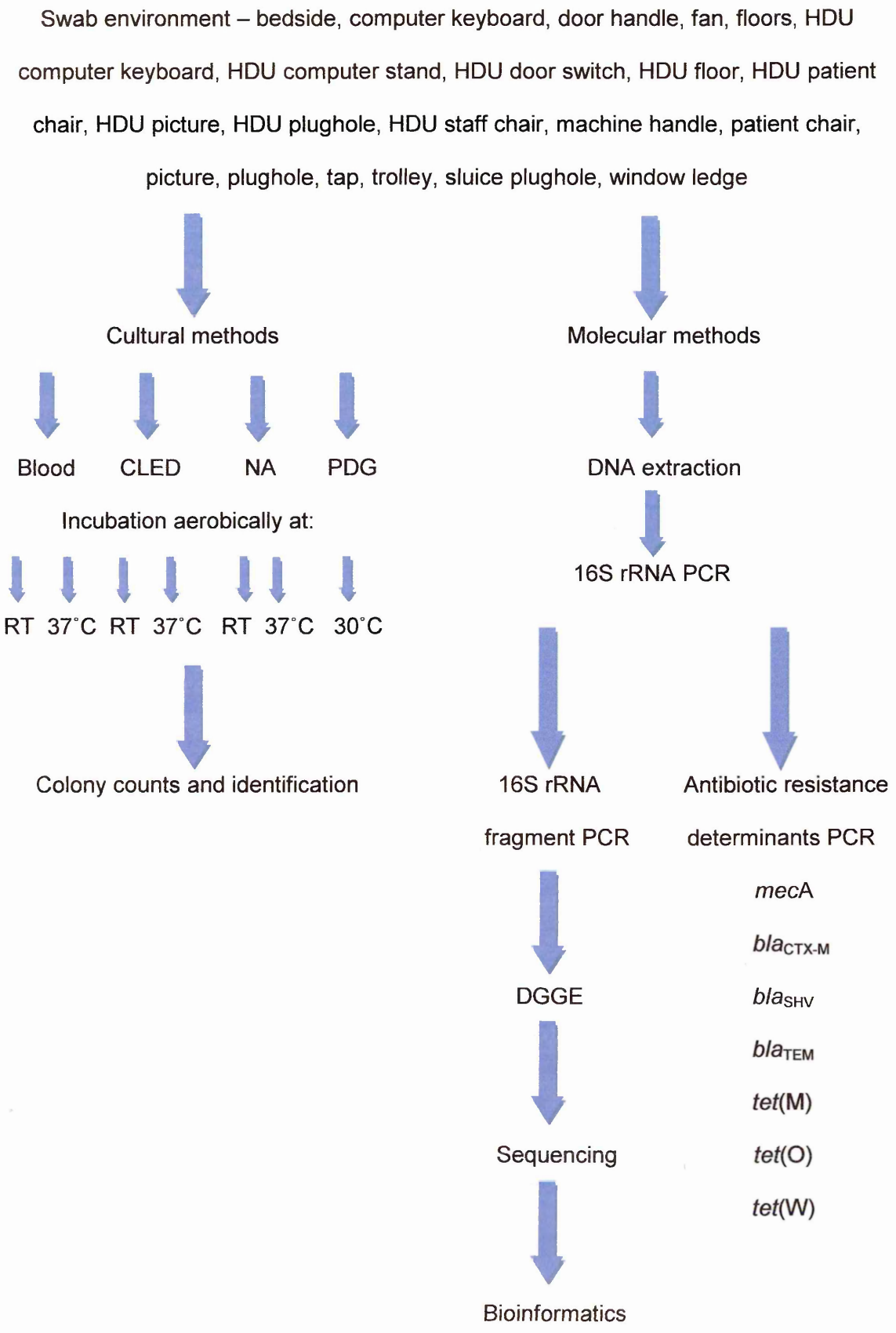


Figure 2.1 Methods for each environmental swab sample

Chapter 6. A period of intensive sampling was carried out to report on the effect of routine ward cleaning and details of the extra sampling regime are laid out in Chapter 7.

2.3 Identification and subculturing of clinical isolates from ICU patients

RHH and NGH microbiology departments have an intercalated database (providing access to clinical isolate information from both ICU sites) of all clinical isolates. A database search was established to locate all clinical isolates from both RHH and NGH ICU and HDU. Clinical samples were screened routinely by the hospital staff, ICU and HDU clinical isolates were collected weekly from storage on nutrient agar (NA) plates.

The bacteria were streaked out to yield single colonies and grown on nutrient agar (NA) (cat no 70148 Sigma) plates overnight at 37 °C. Pure cultures were sub-cultured overnight in nutrient broth (cat no 70123 Sigma) at 37 °C with shaking at 200 rpm.

Glycerol stocks were made from 900 µl bacterial suspension and 100 µl 80 % glycerol (cat no G5516), then gently mixed and stored at -80 °C.

2.4 Microbiological growth conditions

2.4.1 Bacterial growth conditions

Dipslides and contact plates were incubated aerobically at room temperature 20-25 °C (RT) (in a laminar flow hood – Heraeus) for 48 h and also at 37 °C (in a static incubator – Heraeus). All contact plates and dipslides were stored at 4 °C after incubation for

further analysis and any colonies grown in liquid culture (nutrient broth) were stored at -80 °C as glycerol stocks (including antibiotic gene-carrying organisms).

All ICU/HDU clinical isolates throughout this study were grown aerobically in 5 ml nutrient broth at 37 °C with shaking at 200 rpm in an orbital incubator S1 50 (Stuart Scientific).

All environmental swab samples were plated out in duplicate (NA, blood and cysteine lactose electrolyte deficient agar) and incubated aerobically at RT (48 hours) and 37 °C (overnight) (Table 2.2).

2.4.2 Fungal growth conditions

Throughout this study all fungal cultures were grown using the following standard conditions; aerobic incubation at 30 °C with shaking at 200 rpm in an orbital incubator (Gallenkamp) in 9 ml YPD broth (1 L 20 g peptone, 20 g dextrose and 10 g yeast extract - Sigma) for up to 5 days.

Throughout this study environmental fungal contact plates were grown aerobically at 30 °C in a static incubator (Heraeus) on potato dextrose glucose (PDG) (cat no 70139 Sigma) agar plates for up to 5 days.

All environmental swab samples were tested for fungal species by inoculating on PDG agar plates and incubated aerobically at 30 °C in a static incubator (Heraeus) for up to 5 days.

Table 2.2: Growth conditions for cultivation of bacteria from swab samples

Agar	Volume spread (µl)	Temperature
Blood	50	RT
Blood	50	37˚C
CLED	50	RT
CLED	50	37˚C
Nutrient agar	50	RT
Nutrient agar	50	37˚C

2.5 DNA extraction

All DNA extractions from swabs and cultures were performed to isolate genomic bacterial DNA or fungal DNA via a Qiagen spin column method (QIAamp® DNA mini kit cat no 51304 Qiagen Ltd).

Whilst chemical treatment alone can release DNA from Gram-negative bacteria, treatment of whole cells with a peptidoglycan-digesting enzyme is necessary for effective release of DNA from Gram-positive bacterial cells. This may be because the Gram-positive cell wall contains substantially more of the strength-conferring peptidoglycan, compared with the outer membrane of Gram-negative cells. Therefore the enzymes lysozyme and lysostaphin are required to enable better lysis of Gram-positives. Lysozyme hydrolyses 1,4 β-linkages between N-acetylmuramic acid and N-acetyl D-glucosamine residues in peptidoglycan. However, lysozyme is inactive against certain Gram-positive organisms, notably *Staphylococci* including *S. aureus*. To enable lysis of staphylococcal cells, lysostaphin was added to the cell pellet or to the tip of the swab that was used for taking the sample. Like lysozyme, lysostaphin disrupts the peptidoglycan, but differs from lysozyme in that it cleaves the polyglycine cross-links. Lysozyme and lysostaphin were used in combination because lysostaphin is inactive against the majority of micro-organisms where the peptidoglycan lacks the pentaglycine bridge between the peptide moieties.

Yeast cell walls can form capsules or resistant spores so lyticase was added to aid in fungal DNA extraction from cultures. Lyticase is a lysing enzyme that hydrolyses poly-β(1-3)-glucose which is present in the cell wall compound glucan. This enzyme enables the partial formation of spheroplasts which can then be easily lysed to release DNA.

2.5.1 DNA extraction from swab samples

DNA was extracted from swab samples following the Qiagen QIAamp DNA mini kit, buccal swab spin protocol adapted by Lee *et al.* (2007). After swabbing, the DACRON swab tip was placed into a 2 ml Eppendorf tube and 200 µl of lysozyme extraction mix (20 M Tris, 2 mM EDTA pH 8.0, 1.2 % [vol/vol] P40 detergent [which causes cellular membrane breakdown], 20 mg/ml lysozyme, 20 µg/ml lysostaphin) was added and incubated for 30 min at 37 °C. Proteinase K (20 µl – cat no 19131 Qiagen) was added (in order to digest protein cellular components) and 400 µl of buffer AL (lysis buffer) was added and incubated for 10 min at 70 °C. Samples were vortexed with 400 µl of 100 % ethanol and 700 µl of mixture was applied to the spin column and centrifuged (micro centrifuge 5415D – Eppendorf UK Limited) at 8000 rpm for 1 min, and the flow-through was discarded. The DACRON swab tip was removed from the mixture and the remaining solution was applied to the spin column, and re-centrifuged at 8000 rpm for 1 min. The flow-through was discarded and 500 µl of buffer AW1 was applied and centrifuged at 8000 rpm for 1 min. The flow-through was discarded, and 500 µl of buffer AW2 was added and the column re-centrifuged at 13000 rpm for 3 min. The flow-through was discarded and the column re-centrifuged for 1 min. The spin column was placed in a clean 2 ml Eppendorf and 150 µl of buffer AE was added to the column and incubated at RT for 1 min then the column was re-centrifuged at 8000 rpm for 1 min. The spin column was discarded and extracted DNA was stored at -20 °C.

2.5.2 DNA extraction from bacterial cultures

DNA was purified from Gram-positive cultures using the QIAamp® DNA mini kit (cat no 51304 Qiagen Ltd). The manufacturer's instructions were followed from protocol D - isolation of genomic DNA from Gram-positive bacteria. Briefly, 1 ml of bacterial culture was pelleted and 200 µg/ml of lysostaphin (cat no L0761 Sigma) was added to the

pellet of *S. aureus* cultures and 20 mg/ml of lysozyme (cat no L6876 Sigma) was added to other Gram-positive species. The tissue protocol was followed from step 4 as instucted, and 200 µl of buffer AE was added to the column and incubated at RT for 5 min, and the eluted DNA was stored at -20 °C.

DNA was purified from Gram-negative cultures using the QIAamp® DNA mini kit (cat no 51304 Qiagen Ltd). The manufacturer's instructions were followed from protocol Cb - isolation of genomic DNA from bacterial cultures. Briefly, 1 ml of bacterial culture was pelleted and 180 µl of lysis buffer ATL was added to the pellet. The tissue protocol was followed from step 4 as instructed, 200 µl of buffer AE was added to the column and incubated at RT for 5 min, and the eluted DNA was stored at -20 °C.

2.5.3 DNA extraction from fungal cultures

Fungal genomic DNA was extracted using the Qiagen QIAamp DNA (Qiagen Ltd) mini kit, by following the manufacturer's protocol for yeast followed by the tissue protocol from step 2. Briefly, the yeast culture was grown in YPD media to an $OD_{600} = 10$. 3 ml of fungal culture was centrifuged for 10 min at 7500 rpm. The pellet was resuspended in 600 µl of sorbital buffer with 200 U of lyticase (cat no G5516 Sigma) and incubated for 30 min at 30 °C. The sample was centrifuged at 7500 rpm for 5 min (to pellet spheroplasts). The spheroplasts were resuspended in 180 µl of buffer ATL. Proteinase K (20 µl – cat no 19131 Qiagen) was added (to digest protein cellular components), and incubated at 56 °C until the cells were completely lysed (approximately 2 hours). Buffer AL (200 µl) was added (lysis buffer) and incubated at 70 °C for 10 min, 200 µl of 100 % ethanol was added to the sample, then applied to the QIAamp spin column and centrifuged at 8000 rpm for 1 min. The flow-through was discarded and 500 µl of buffer AW1 was added and the column re-centrifuged at 8000 rpm for 1 min. The flow-through was discarded and 500 µl of buffer AW2 was

added and the column re-centrifuged at 13000 rpm for 3 min. The flow-through was discarded and the column re-centrifuged at 13000 rpm for 1 min. Buffer AE (200 µl) was added to the column and incubated at RT for 5 min. The column was centrifuged at 8000 rpm for 1 min and extracted DNA was stored at -20 °C.

2.6 Polymerase chain reaction

Polymerase chain reaction (PCR) was used frequently throughout this project in order to detect the presence of specific genes e.g. bacterial 16S rRNA genes and antibiotic resistance determinants. The technique of PCR involves three main steps: -

1. Denaturation - DNA was heated to separate double stranded DNA molecules to single strands
2. Annealing - the temperature was reduced to the optimum for annealing of the oligonucleotide primers to the complementary sequence of the DNA template
3. Amplification - Thermoprime *Taq* DNA polymerase amplified the primed DNA sequence using the dNTPs provided to create a copy of the original DNA

This process was cycled to enable multiple strand amplification which could subsequently be visualised using agarose gel electrophoresis (Section 2.8 Agarose Gel Electrophoresis).

All PCRs were carried out using 2x master mix (cat no AB-0575-DC ABgene Ltd), which in a 25 µl total reaction volume consisted of the following final concentrations: -

0.625 U ThermoPrime Taq DNA polymerase

75 mM Tris-HCl (pH 8.8 at 25 °C)

20 mM $(NH_4)_2SO_4$

1.5 mM $MgCl_2$

0.01 %(v/v) Tween 20

0.2 mM each of dATP, dCTP, dGTP, dTTP

The constituents of the master mix have a specific role in the PCR reaction. $MgCl_2$ was a source of magnesium required for the activity of DNA polymerases (a lack of free magnesium results in the inactivity of *Taq* and other DNA polymerase). Tween 20 is a non-ionic detergent which also stabilises *Taq* polymerase and can suppress the formation of unwanted DNA secondary structures. Tris-HCl maintains the pH of the reaction and ammonium sulphate precipitates DNA resulting in a reduction in non-specific binding.

Reagents were added to the tube in the following order: -

7.5 µl sterile water

12.5 µl master mix (x2)

1 µl 30 pmol forward primer

1 µl 30 pmol reverse primer

3 µl template (>6 ng/µl)

Negative control - the template was replaced with 3 µl sterile water.

Positive control - the template was replaced with DNA known to possess the target gene.

PCR cycle:

[95 °C 5 min, annealing temperature (AT) °C 30 s, 68 °C 1 min] x 1;

[95 °C 30 s, AT °C 30 s, 68 °C 1 min] x 25;

[95 °C 30 s, AT °C 30 s, 68 °C 10 min] x 1

All primers were custom synthesised by Invitrogen.

2.6.1 Antibiotic resistance determinant PCR

Primers were identified from the literature to amplify highly conserved regions of the *mec*A, $bla_{CTX\text{-}M,\ SHV,\ TEM}$ and *tet*(M, O and W) genes (Table 2.3). All primers were tested against known positive and negative controls to optimise the amplification parameters (detailed in Chapter 3).

2.6.2 Bacterial 16S rRNA gene PCR

Primers were identified from the literature to enable the amplification of the highly conserved 16S rRNA gene for the detection of prokaryotic cells (all known eubacteria, Gram-positive and Gram-negative bacteria) (Table 2.4). The primers were optimised using *S. aureus* SH1000 and *E. coli* XL1 to determine the optimum amplification parameters (detailed in Chapter 3).

2.6.3 PCR-DGGE

Universal primers were identified from the literature to enable the amplification of the variable nt 341-926 16S rDNA fragments for the detection and identification of bacterial species (Table 2.5), and optimum amplification was established using CNS and *Klebsiella* spp (detailed in Chapter 3).

Table 2.3: Primers for antibiotic resistance determinant detection

Gene	Primer sequence (5'-3')	Annealing temp (AT) (°C)	Expected amplicon size (bp)	Reference
bla$_{TEM}$	**TEM-F1** ATGAGTATTCAACATTTCCG **TEM-R1** GACAGTTACCAATGCTTAATCA	50	862	Naiemi *et al.*, 2005
tet(o)	**TETO-F** ACGGARAGTTTATTGTATACC **TETO-R** TGGCGTATCTATAATGTTGAC	52	171	Aminov *et al.*, 2001
bla$_{SHV}$	**SHV-F1** CTTTACTCGCCTTTATCG **SHV-R1** TCCCGCAGATAAATCACC	55	827	Naiemi *et al.*, 2005
bla$_{CTX-M}$	**CTX-M F1** GCGTGATACCACTTCACCTC **CTX-M R1** TGAAGTAAGTGACCAGAATC	55	540	Weill *et al.*, 2004
tet(w)	**TETW-F** GAGAGCCTGCTATATGCCAGC **TETW-R** GGGCGTATCCACAATGTTAAC	60	168	Aminov *et al.*, 2001
*mec*A	**MECA-1** AAAATCGATGGTAAAGGTTGGC **MECA-2** AGTTCTGCAGTACCGGATTT	60	533	Geha *et al.*, 1994

Table 2.4: Primers for bacterial 16S rRNA gene detection

Gene	Primer sequence (5'-3')	Annealing temp (AT) (°C)	Expected amplicon size (bp)	Reference
16S rRNA	**16S1** AGAGTTTGATCMTGGCTCAG **16S2** TACGGYTACCTTGTTACGACTT	50	1500	Bodrossy *et al.*, 1999

Table 2.5: Primers for amplification of 16S rDNA fragments for DGGE

Gene	Primer sequence (5'-3')	Annealing temp (AT) (°C)	Expected amplicon size (bp)	Reference
16S rDNA fragments	**926R(907R)** CCGTCAATTCMTTTGAGTTT **341F-GC** CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCGCCT ACGGGAGGCAGCAG	55	600	Brinkhoff *et al.*, 1998 Muyzer *et al.*, 1993

2.6.4 Fungal PCR

Primers were identified from the literature to enable the amplification of the 18S-25/28S rDNA fragments for the detection of medically relevant yeasts (Table 2.6). These primers target a sequence which contains an intergenic region that varies in length between fungal species. The length of the PCR product provides information about the species identity of the yeast.

2.7 PCR-Restriction Fragment Length Polymorphism

Fungal PCR products were digested with *Mwo*I (cat no R0573S New England Biolabs) at 60 °C for 2 hours.

Reaction: -

1 µl *Mwo*I enzyme

5 µl buffer 3

10 µl PCR product

34 µl sterile water

50 µl total reaction volume

To enable the differentiation of *C. tropicalis* and *C. parapsilosis* and *C. guillermondii* and *C. membranaefaciens* a second digestion of the PCR product was required with *Bsl*I (cat no R0555S New England Biolabs) at 55 °C for 2 hours (Table 2.7).

Table 2.6: Primers for amplification of fungal 18S-25/28S rDNA fragments for species identification

Gene	Primer sequence (5'-3')	Annealing temp (AT) (°C)	Expected amplicon size (bp)	Reference
18S-25/28S rDNA fragments	**Primer 1** GTCAAACTTGGTCATTTA **Primer 3** TTCTTTTCCTCCGCTTATTGA	50	*C. albicans* 586 *C. dubliniensis* 589 *C. glabrata* 925 *C. krusei* 560 *C. tropicalis* 576 *C. parapsilosis* 570 *C. guillermondii* 657 *C. membranaefaciens* 686 *S. cerevisiae* 891	Trost *et al.*, 2004

Table 2.7: Fragment sizes for fungal species identification after restriction digestion

Species	Fragment sizes after *Mwo*I digestion (bp)	Fragment sizes after *Bsl*I digestion (bp)
C. albicans	261, 184, 141	
C. dubliniensis	325, 264	
C. glabrata	414, 174, 171, 86, 80	
C. krusei	289, 134, 83, 49, 5	
C. tropicalis	325, 154, 97	326, 187, 63
C. parapsilosis	336, 146, 88	413, 94, 63
C. guillermondii	355, 302	356, 238, 63
C. membranaefaciens	387, 299	623, 63
S. cerevisiae	343, 207, 173, 168	

2.8 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments by size using the fact that DNA is negatively charged (due to phosphate moieties present in the DNA backbone) and migrates towards the positively charged electrode upon application of an electric field across the agarose gel (polysaccharide matrix). The agarose provides a cross-linked matrix which enables smaller DNA molecules to migrate further through the gel at a higher rate compared with larger DNA molecules. PCR products were run on agarose gels to enable approximate size quantification.

In order to visualise the products, the gel requires staining and during this project two stains were used for various samples; ethidium bromide (cat no E-8751 Sigma) and Sybr Green 1 (cat no 86205 Sigma). Both of these stains intercalate with the DNA to enable visualisation under UV light. Ethidium bromide was used to stain all gels and provides a 25-fold increase in fluorescence when bound to ds-DNA and generally does not require de-staining and is added directly to the gel prior to setting. SybrGreen 1 was used as an ultra sensitive stain compared to ethidium bromide, in order to visualise smaller quantities of ds-DNA (as low as 1-2 ng). For all PCR products run from environmental samples the gel was initially stained with EtBr followed by staining with SybrGreen 1 solution (4 µl SybrGreen 1 in 40 ml TE buffer).

When analysing amplicons (DNA fragments <1 kb), 0.8 % agarose (cat no 15510-019 Invitrogen) gels were used, when analysing fungal restriction digestion products 2 % agarose gels were used. When pouring the agarose gel (50 ml) 2 µl of 10 mg/ml ethidium bromide was added. A total of 10 µl of sample and 2 µl of 6x loading dye (cat no R0611 Fermentas), were vortexed to mix and centrifuged briefly to collect the full volume. A total of 12 µl was loaded onto the gel along with the corresponding DNA ladder. When identifying amplicons >500 bp a 1 kb DNA ladder (cat no SM0311

Fermentas) was used, to identify fungal restriction digestion products and amplicons <500 bp a 100bp DNA ladder (cat no N3231L New England BioLabs) was used. DNA was visualised using a light box or UVP camera (Epi Chemi 11 darkroom – UVP).

2.9 Denaturing gradient gel electrophoresis

Denaturing gel electrophoresis (DGGE) is a technique for separating PCR fragments of the same size but different sequences. During DGGE the DNA product is subjected to increasing concentrations of a chemical denaturant (urea and formamide) as they migrate through a polyacrylamide gel, resulting in separation based on their differential denaturation (melting) profile defined by the DNA sequence. DGGE is a very sensitive technique to the sequence composition and is able to resolve even single nucleotide differences. DNA is run through the gel of increasing denaturant concentration driven by an electric field, resulting in DNA denaturation.

The PCR primer is designed to include a GC-clamp at the 5' end in order to alter the denaturation pattern of the DNA fragments (as detailed in Section 2.6.3 PCR-DGGE). The GC clamp regions remain annealed at a particular formamide concentration which prevents further migration through the polyacrylamide gel. The PCR was carried out as detailed in Section 2.6 Polymerase chain reaction.

To set up the parallel gradient gel sandwich, the manufacturer's instructions were followed from BIO-RAD the DCode universal mutation detection system (cat no 170/9080-9104), as follows. The glass plates were cleaned thoroughly using soap (dish washing liquid) and water, followed by 100 % ethanol (cat no M/4450/17 Thermo Fisher Scientific), then acetone (cat no A/0560/17 Thermo Fisher Scientific). The edges of the spacers were greased with petroleum jelly and placed on the larger glass plate; the small glass plate was placed on top. The clamps were attached onto the gel

sandwich and tightened. The sandwich assembly was placed in the alignment slot of the casting stand, and the alignment card placed between the glass plates and the plates were aligned (ensuring the glass plates were sat flush). The sponge was placed onto the front casting slot and the gel sandwich assembly was attached. Two 30 ml syringes were labelled low and high: -

High density solution (8% polyacrylamide gel)

15 ml 70% denaturant solution (see below)

300 µl 6x loading dye (cat no R0611 Fermentas)

120 µl 10 % (w/v) APS (cat no A3678 Sigma)

12 µl TEMED (cat no T9281 Sigma)

Low density solution (8% polyacrylamide gel)

15 ml 30% denaturant solution (see below)

120 µl 10 % (w/v) APS

12 µl TEMED

70% denaturing solution

20 ml 40% N,N'-methylene bis-acrylamide (37:1 wt/wt) (cat no 154563 Sigma)

2 ml 50x TAE buffer

28 ml formamide (cat no F9037 Sigma)

29.4 g urea (cat no U6504 Sigma)

50 ml distilled water

30% denaturing solution

20 ml 40% N,N'-methylene bis-acrylamide (37:1 wt/wt)

2 ml 50x TAE buffer

12 ml formamide

12.6 g urea

66 ml distilled water

The solutions were drawn into the syringes and the high density solution filled syringe was attached to the bottom filling side of the gradient delivery system and the low density solution filled syringe to the top filling side. The cam wheel was rotated slowly and steadily to deliver the gel solution. The comb was inserted and the polyacrylamide gel left to set (Figure 2.2). Water was run back through the delivery system to remove any traces of unpolymerised acrylamide. 7 L of 1x TAE was prepared and heated to 60 °C using the electrophoresis tank and temperature control module (power turned on). To load the gel 10 µl of PCR product and 2 µl of 6x loading dye was used. The power was turned off and left for 15 s, then the temperature control module was removed and samples loaded. The temperature control module was replaced and power restored, when the temperature reached 60 °C the gel was run at 20 V for 15 min to run the samples into the gel. The gel was run overnight at 60 V for 16 hours (960 min). The power was turned off and left for 15 s, then the gel sandwich was removed and the polyacrylamide gel was stained with SybrGold for 40 min in the dark then visualised using the UVP imager (Epi Chemi 11 darkroom – UVP). The amplicons were visualised during this project using SybrGold, which is a highly sensitive nucleic acid stain and provides a >1000 fold increase in fluorescence when bound to ds- or ss-DNA.

2.10 DIG-labelled probe and colony blotting

This method was used to enable easy detection of antibiotic resistance determinants from colonies blotted onto hybridisation membranes. Digoxigenin-11-dUTP was incorporated into the PCR products for the detection of tetracycline resistance genes *tet* O, W and M using *Taq* DNA polymerase to create a digoxigenin-labelled oligonucleotide probe. All chemicals were purchased from Roche Products Limited unless otherwise stated.

In brief PCR reactions were carried out using the cycles stipulated in Section 2.6.1 and were amplified using the following reaction: -

4 µl 30 pmol forward primer

4 µl 30 pmol reverse primer

1 µl Taq DNA polymerase (5 U/µl)

5.2 µl $MgCl_2$ (50 mM)

10 µl PCR buffer (x10)

10 µl PCR DIGmix (2 mM dNTP, 1.3 mM dTTP, 0.7 mM digdUTP)

53.8 µl sterile water

The reaction was mixed and aliquoted into 4 x 22 µl and 3 µl DNA template (>6 ng/µl) was added.

Solutions were prepared fresh on the day of use and included (details provided by Roche Products Limited): -

Denaturation solution (0.5 M NaOH, 1.5 M NaCl, 0.1% SDS)

Neutralisation solution (1.0 M Tris-HCl, 1.5 M NaCl)

20x SSC stock solution (3 M NaCl, 0.3 M sodium citrate)

Washing buffer x1

Blocking solution

Detection buffer

Antibody solution

BCIP/NBT colorimetric detection tablets

The nylon membrane was placed over the whole contact plate (avoiding any air bubbles) and left for 1 min. Filter paper was soaked in denaturation, neutralisation and 2x SSC solutions. The filter paper soaked in denaturation solution was placed on the

membrane and incubated at RT for 15 min. The same was carried out using the neutralisation then the 2x SSC soaked filter papers (for 15 min and 10 min respectively). The membrane was placed on a UV transilluminator for 1 min in order to cross-link the DNA. To digest any potential interfering proteins the membranes were incubated at 37 °C for 1 hour with 2 mg/ml Proteinase K.

To prepare the hyb-probe solution, the dig-easy hyb solution was incubated at 42 °C for 1 hour and the probe denatured at 100 °C for 5 min. The hyb-probe was added to the pre-warmed dig-easy hyb solution and poured over the membrane and re-incubated at 42 °C for 2 hours. The dig-probe solution was poured off and stored at -20 °C for up to 1 year. The membrane was washed twice with 2x SSC and 0.1 % SDS for 5 min at RT, followed by two washes with 0.5x SSC and 0.1 % SDS for 15 min at 68 °C. In order for colorimetric detection, the membrane was equilibrated in washing buffer for 1 min, then blocked with blocking buffer for 1 hour at RT with gentle agitation. The antibody solution (anti-digoxigenin-AP Fab fragments) was added and incubated for 30 min at RT. Membranes were washed twice with washing buffer for 15 min, followed by the detection buffer for 2 min. The BCIP/NBT colour substrate solution was added and the samples were placed in the dark overnight and the reaction stopped by washing the membrane in water. Positive reactions were initially seen by purple colouration after approximately 30-60 min.

2.11 PCR purification

PCR purification was used to purify ds-DNA products via a spin column method similar to that used for DNA extraction for sequencing. Buffer PBI enables efficient binding of ds- and ss- PCR products (>100 bp) onto the silica spin column. PBI also acts as a pH indicator to ensure the maintenance of an optimum pH $\leq$7.5 for DNA binding. Impurities and unwanted primers in the PCR reaction do not bind to the column during

DNA adsorption and salts are removed by the addition of buffer PE containing ethanol. Elution buffer EB (10 mM Tris-Cal pH8.5) was used to elute the DNA from the column at optimum salt concentration and pH conditions and to enable storage at -20 °C with minimal DNA degradation.

PCR products were purified following the manufacturers instructions from QIAquick spin handbook (cat no 28104 Qiagen Ltd), QIAquick PCR purification kit protocol, as follows; 5 volumes of PBI buffer was added to 1 volume of PCR sample and mixed. The sample was applied to the column and centrifuged at 13000 rpm for 1 min, and the flow-through discarded, 0.75 ml of buffer PE was added to the column and re-centrifuged at 13000 rpm for 1 min, and the flow-through discarded. The column was placed in a sterile 1.5 ml eppendorf and 30 µl of elution buffer was added, and the column incubated at RT for 1 min, and re-centrifuged at 13000 rpm for 1 min. The purified PCR products were stored at -20 °C.

2.12 DNA sequencing

PCR products were purified following the methods laid out in Section 2.11 PCR purification. 20 µl of the PCR products (≥5 ng/µl) were sent to Euro Fins Genetic Services Ltd for DNA sequencing.

After DNA sequencing of purified DNA from isolated bacteria, organisms were identified from GenBank database searches. Retrieved sequences from PCR-DGGE were sequenced and GenBank database searches used to generate percentage identity/similarity to bacterial sequences. Chromatograms were studied to check sample purity and unresolved residues.

For manufacturer details please refer to Appendix 3

3. Method optimisation

3.1 Background and aims

The aim of the work described in this chapter was to create a robust sampling regime and down-stream sample processing for molecular and cultural analysis of environmental samples. Before sampling began in the hospital environment, the sampling methodology was developed by using samples from the environment of the research laboratory and by sampling pieces of flooring material, some of which were deliberately contaminated with microbial cultures.

The objectives were to: -

- identify a variety of solid media to enable the isolation of a wide range of bacteria and fungi from environmental swab samples
- establish a DNA extraction method for the isolation of Gram-negative and Gram-positive bacterial DNA from environmental sites
- identify a method to detect and characterise fungi of clinical significance
- optimise the use of DGGE to identify bacterial diversity and enable the resolution and retrieval of bacterial sequences
- optimise PCR conditions for the detection of eubacterial DNA and antibiotic resistance determinants (*mec*A, $bla_{CTX\text{-}M,\ SHV,\ TEM}$ and *tet*O, M, W genes)

3.2 Culturing on solid media

Culturing techniques were used to enable the isolation of most clinically relevant and commensal bacteria and fungi. The solid media selected was blood, CLED and NA for culturing bacteria and potato dextrose agar for fungi. Blood agar was used to select for

fastidious organisms and show haemolytic activity (i.e. *Staphylococcus* species). CLED was used to select for urinary tract bacteria (e.g. *E. coli*). NA was used to enable the growth of most bacteria and some fungal species. PDG agar was used to enable the growth of fungi.

3.3 Bacterial identification

3.3.1 DNA extraction directly from swab samples

Initially the swab DNA extraction method was followed directly from the Qiagen QIAamp DNA extraction kit. After carrying out the extraction from a dry swab of *S. aureus* SH1000 and a separate PBS soaked swab from a molecular laboratory bench, eubacterial DNA was amplified from the *S. aureus* SH1000 sample but no product was amplified from the laboratory bench sample (data not shown). This was perhaps due to the low yield of the extraction as the DNA concentration in environmental sites could have been very low or the swab was too dry to pick up sufficient prokaryotic cells.

In order to improve the removal of cells from the surfaces sampled and to promote their adhesion to the swab, it was decided to investigate pre-wetting of the swab by adding sterile neutralising solution (see Section 2.2 hospital sampling regime) to the swab holder to wet the swab before use, as described by Lee *et al.* (2007). Extraction of DNA from an unused swab dipped in neutralising solution confirmed neither were sources of prokaryotic contamination (Figure 3.1).

To test the sensitivity of the DNA extraction method a small piece (10.5 cm x 8 cm) of plastic cushioned flooring was spiked with 100 µl and 500 µl of *S. aureus* SH1000 overnight broth cultures (approximately 10^7 cells). Positive amplification of 16S rRNA

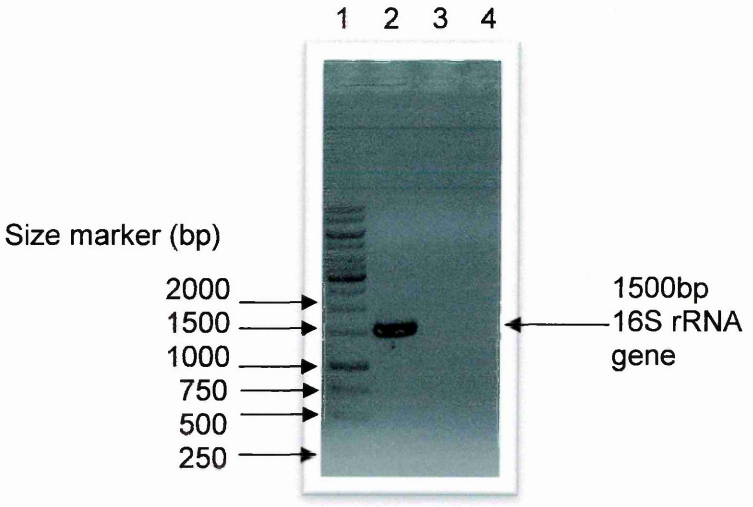


Figure 3.1 Evaluation of the swab wetting method for contamination from the neutralising solution

Lane 1 marker, lane 2 positive control, lane 3 no DNA template control, lane 4 blank swab extraction

(Using the eubacterial 16S rRNA gene specific primers detailed in the materials and methods)

gene was seen from both samples (Figure 3.2). This demonstrated that the method was sufficient to detect pure cultures from a floor piece.

In order to perform a more relevant test, a piece of used ICU flooring was obtained during replacement of the ICU floor at the RHH. After swabbing, DNA extraction and PCR (directly from the floor piece with no bacterial spiking), no DNA or 16S rRNA gene product could be visualised, indicating the levels of bacteria were extremely low or absent. This was confirmed as only ten colonies were cultured from 100 µl of swab solution on NA incubated aerobically at 37 °C overnight.

According to a modification proposed by Lee *et al.* (2007), recovery of bacterial DNA can be improved by incubation of the swab at 37 °C for 30 min with a lysozyme reaction mixture to improve extraction of bacterial DNA by digesting the bacterial cell wall.

To test this method, an area of the molecular biology laboratory floor was swabbed and DNA was purified by using the altered version of the kit extraction method described by Lee *et al.* (2007) (Section 2.5.1 in Materials and Methods). Subsequent PCR and gel analysis showed a visible 16S rRNA gene product (Figure 3.3). The kit was sensitive enough to detect prokaryotic cells from relevant environmental sites such as linoleum-covered floors. A Gram-specific PCR reaction was used to determine whether both Gram-positive and Gram-negative DNA could be extracted using this method (Figure 3.4). However, when the first ICU sampling began some sites yielded high colony numbers of *Staphylococci* species on blood agar, but no visible product following 16S rRNA gene amplification. To rectify this, lysostaphin was also added to the lysozyme reaction mix to ensure the extraction of *S. aureus* DNA from the swab samples.

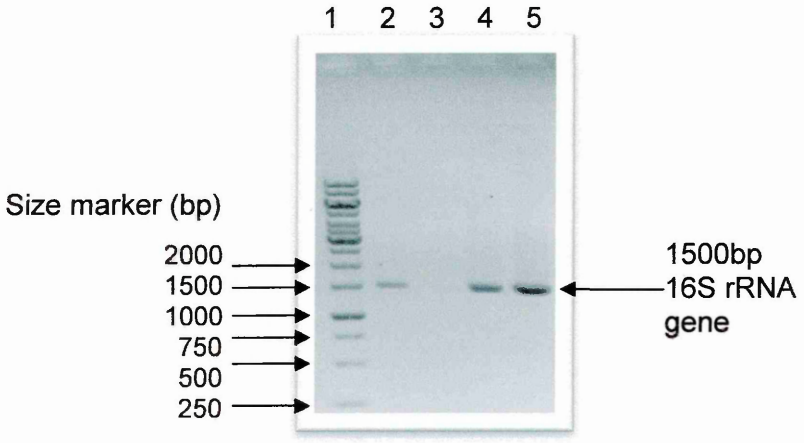


Figure 3.2 Amplification of 16S rRNA genes from a floor piece inoculated with *S. aureus*

Lane 1 marker, lane 2 positive control, lane 3 no DNA template control, lane 4 100 µl *S. aureus* culture, lane 5 500 µl *S. aureus* culture

(Using the eubacterial 16S rRNA gene specific primers detailed in the materials and methods)

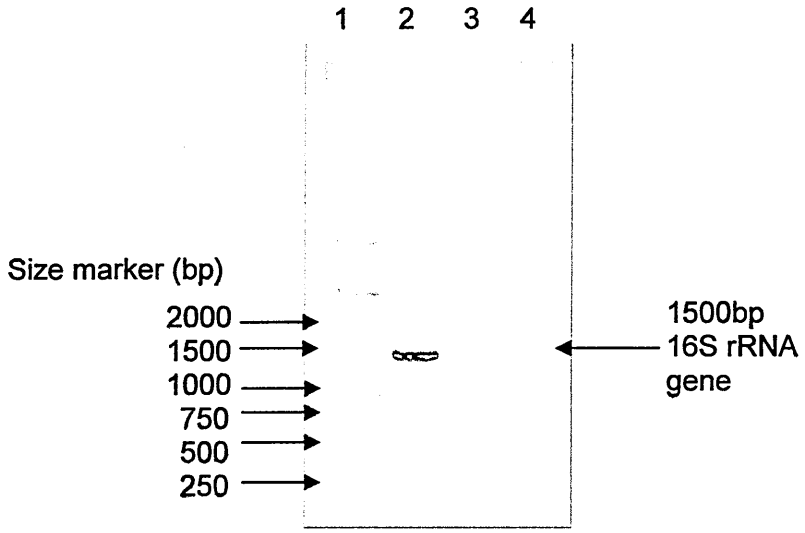


Figure 3.3 PCR detection of bacterial 16S rRNA genes from molecular biology laboratory floor samples

Lane 1 marker, lane 2 positive control, lane 3 no DNA template control, lane 4 molecular laboratory floor swab sample

(Using the eubacterial 16S rRNA gene specific primers detailed in the materials and methods)

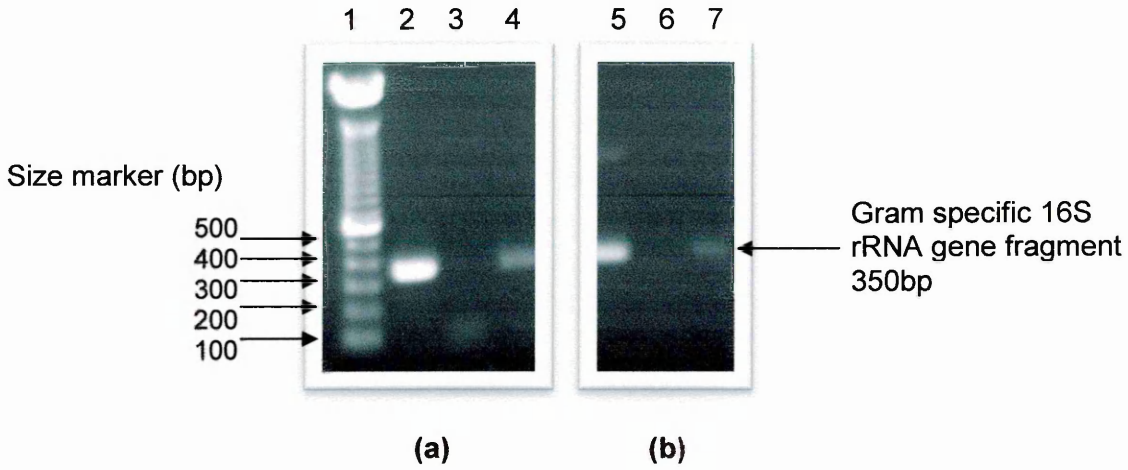


Figure 3.4 (a) Amplification of Gram-positive specific DNA

Lane 1 marker, lane 2 *S. aureus* SH1000 Gram-positive control, lane 3 no DNA template control, lane 4 positive Gram +ve PCR product from template DNA purified from environmental tap swab samples

Figure 3.4 (b) Amplification of Gram-negative specific DNA

Lane 5 no DNA template control, lane 6 *E. coli* XL1 Gram-negative control, lane 7 no DNA template control, lane 8 positive Gram –ve PCR product from template DNA purified from environmental tap swab samples

3.3.2 Selection of staining technique

With most of the methods requiring agarose gel electrophoresis, a staining technique was required to enable the visualisation of faint PCR products. Ethidium bromide had been used widely for many years to visualise DNA on agarose gels. However, recently new stains have been identified which are more sensitive. As it was possible there could be extremely low DNA yields from the ICU environment, a stain was required to enable the visualisation of DNA and PCR products at low concentrations. Ethidium bromide enabled visualisation down to 0.6 ng of plasmid DNA (Figure 3.5); however SybrGreen enabled visualisation down to 0.07 ng (Figure 3.6). Throughout this study a sequential staining with ethidium bromide and then SybrGreen was established for the study.

3.3.3 Use of PCR for the detection of prokaryotic cells

The 16S rRNA gene sequence can be used to detect and identify bacteria to the species level **(Woese *et al.*, 1983; Bodrossy *et al.*, 1999)**, and it was decided to use 16S rRNA gene primers 16S1 and 16S2 **(Bodrossy *et al.*, 1999)** to amplify the whole 1500bp gene fragment which is specific to prokaryotic cells and is highly conserved.

Eubacterial 16S rRNA gene PCR was optimised using pure cultures followed by environmental samples to show a single sized amplicon that could be used to identify the presence of prokaryotic cells in a given sample. Positive amplification was achieved for *S. aureus* SH1000, *E. coli* XL1 and an environmental DNA sample (from sink slime in the trap in the waste pipe) at the expected size of 1500 bp (Figure 3.7).

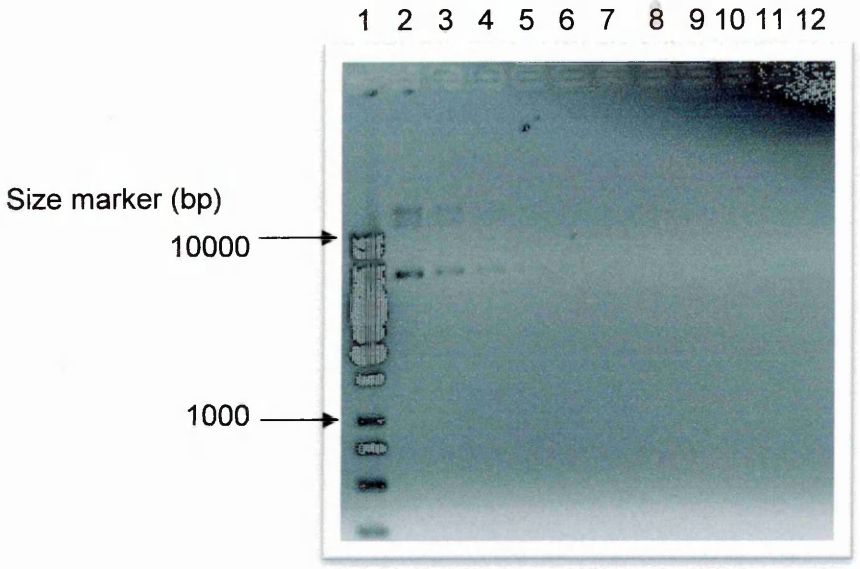


Figure 3.5 Visualisation of plasmid DNA using ethidium bromide staining

Lane 1 marker, lane 2 4.6 ng DNA, lane 3 2.3 ng DNA, lane 4 1.15 ng DNA, lane 5 0.575 ng DNA, lane 6 0.288 ng DNA, lane 7 0.144 ng DNA, lane 8 0.07 ng DNA, lane 9 0.04 ng DNA, lane 10 0.02 ng DNA, lane 11 0.01 ng DNA, lane 12 0.005 ng DNA

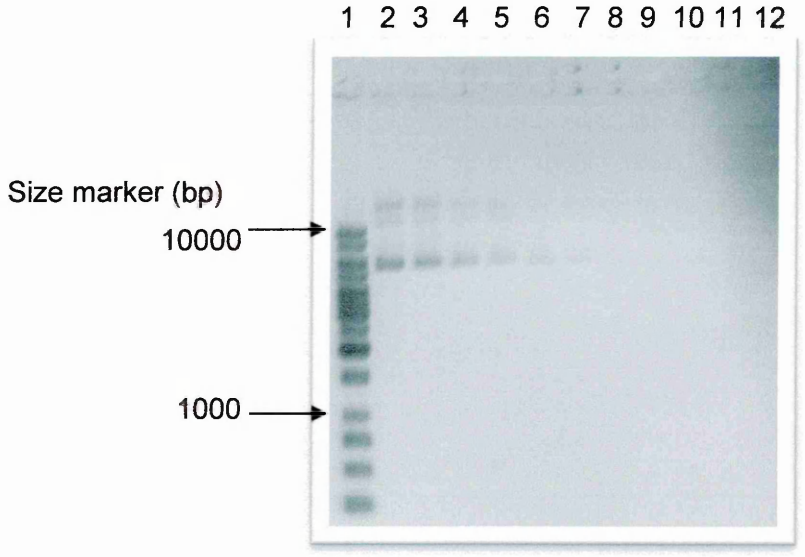


Figure 3.6 Visualisation of plasmid DNA using SybrGreen staining

Lane 1 marker, lane 2 4.6 ng DNA, lane 3 2.3 ng DNA, lane 4 1.15 ng DNA, lane 5 0.575 ng DNA, lane 6 0.288 ng DNA, lane 7 0.144 ng DNA, lane 8 0.07 ng DNA, lane 9 0.04 ng DNA, lane 10 0.02 ng DNA, lane 11 0.01 ng DNA, lane 12 0.005 ng DNA

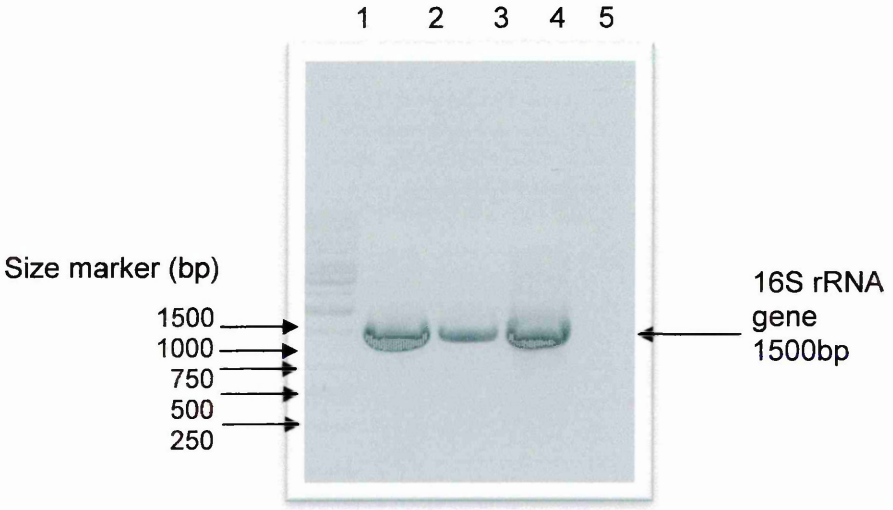


Figure 3.7 Validation of 16S rRNA gene PCR

Lane 1 marker, lane 2 *S. aureus* SH1000, lane 3 *E. coli* XL1, lane 4 purified DNA from environmental sample (plughole slime in the trap on the waste pipe), lane 5 no DNA template control

Amplification of 16S rRNA genes only indicates the presence of eubacterial DNA (prokaryotic cells), therefore DGGE technology was also used to resolve specific GC-clamped nt 341-926 16S rDNA fragments based on the sequence rather than size.

3.3.4 Optimisation of PCR-DGGE

Universal DGGE primers 341F-GC and 901R **(Muyzer *et al.*, 1993; Brinkhoff *et al.*, 1998)** were used to amplify the 341-901 region of 16S rRNA genes, which is conserved in size (600 bp) among prokaryotic cells but varies in sequence. The PCR cycle was optimised using pure cultures of a coagulase-negative *Staphylococcus* (CNS) and *K. oxytoca* (from ICU clinical strains) and a mix of the two. After PCR the products were run on a 0.8 % agarose gel to observe the expected 600 bp amplicon (Figure 3.8). Products were then run on 8 % acrylamide 30-70 % denaturant gels at 60 V for 16 h. Products from mixed template PCR resolved/migrated separately on the gel (Figure 3.9).

3.4 Fungal identification

With the incidence of fungal nosocomial infections rising, it was necessary to detect environmental fungal isolates by culture followed by further analysis to identify medically relevant yeasts.

3.4.1 Detection of fungal organisms

All fungal species possess internal transcribed spacer regions (ITS) 1 and 2 between the DNA sequences encoding the mature forms of the 18S and 25-28S subunits of

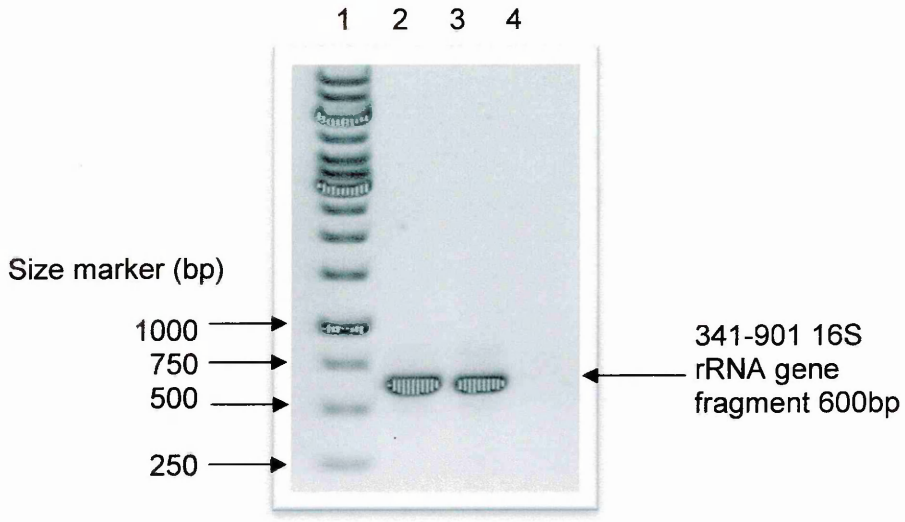


Figure 3.8 Optimisation of DGGE PCR

Lane 1 marker, lane 2 purified clinical CNS DNA, lane 3 purified clinical *K. oxytoca* DNA, lane 4 no DNA template control

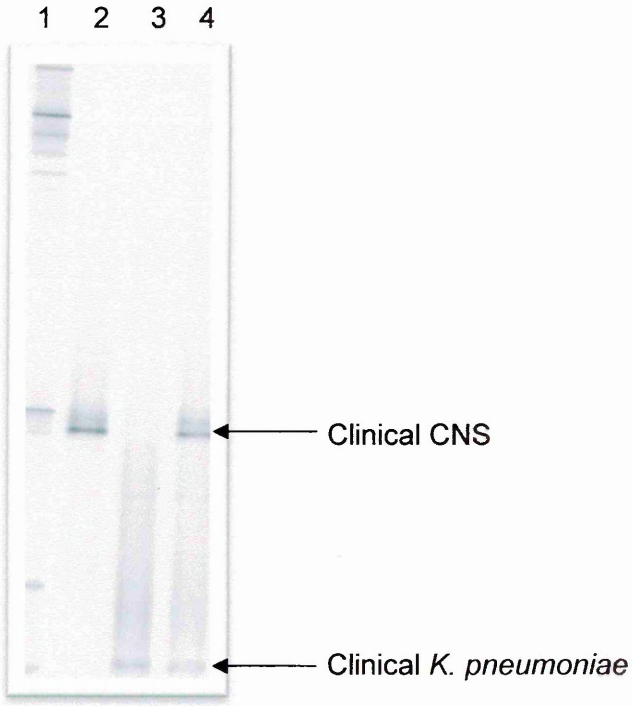


Figure 3.9 Representative DGGE gel

Lane 1 marker, Lane 2 purified clinical CNS DNA, lane 3 purified clinical *K. pneumoniae* DNA, lane 4 mix purified clinical CNS and *K. pneumoniae* DNA

rDNA. Primers were used from Trost *et al.* (2004) to amplify this region and the 5.8S rDNA of the most common human fungal pathogens **(Trost *et al.*, 2004)**. The PCR products resulted in altered sizes for different *Candida* species. The PCR was optimised using RHH ICU clinical fungal strains of *C. glabrata, C. guillermondii, C. parapsilosis, C. tropicalis* and *S. cerevisiae* (data not shown). The optimum annealing temperature was 50 °C (Figure 3.10). This PCR was used to identify medically relevant yeasts from environmental isolates, however further analysis was required to identify to species level.

3.4.2 Identification of fungal species

To identify the species of medically relevant yeasts detected, Trost *et al.* (2004) established a restriction digest using *Mwo*I, which involved each yeast amplicon possessing at least one cleavage site **(Trost *et al.*, 2004)**. Using *C. glabrata* and *C. parapsilosis* the digests were run using the method by Trost *et al.*, 2004 (Figure 3.11).

It was confirmed using known *Candida* species that the digest could be used to identify the species of several medically relevant yeasts and a further digest with *Bsl*I enabled the differentiation of *C. parapsilosis* and *C. tropicalis*.

3.5 Antibiotic resistance determinants

3.5.1 mecA gene detection

PCR for the *mec*A gene was optimised using a clinical strain of MRSA. Primers mecA1 and mecA2 **(Geha *et al.*, 1994)** were used to amplify the 533 bp conserved region of the *mec*A gene. PCR cycles were performed at 55, 60 and 65 °C annealing

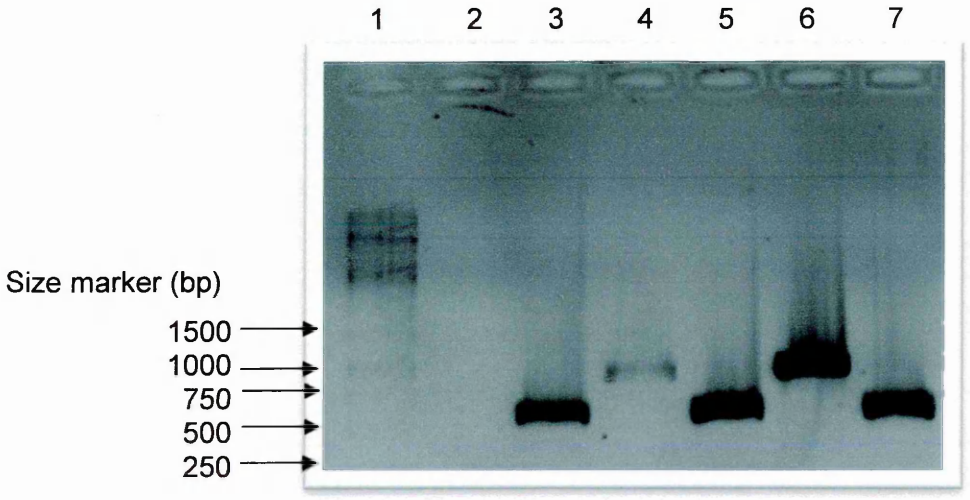


Figure 3.10 Identification of medically relevant yeasts by PCR

Lane 1 marker, lane 2 no DNA template control, lane 3 *C. parapsilosis*, lane 4 *C. glabrata*, lane 5 *C. guillermondii*, lane 6 *S. cerevisiae*, lane 7 *C. tropicalis*

(Using 18S-25/28S rDNA fragment specific primers detailed in materials and methods. All templates were purified DNA from clinical fungal isolates)

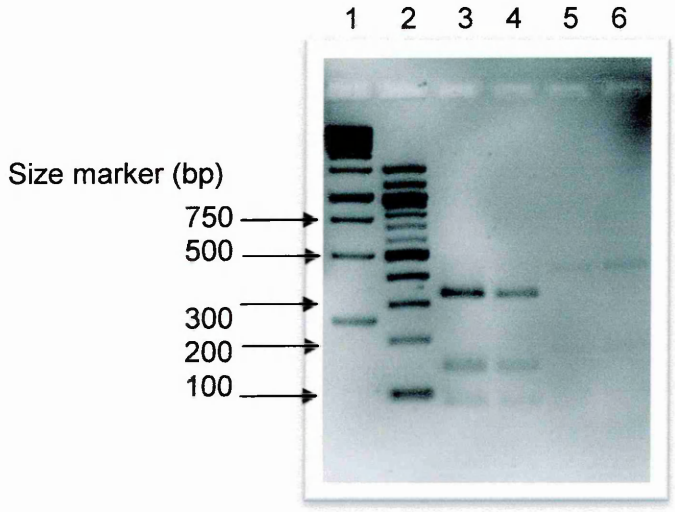


Figure 3.11 *Mwo*1 restriction digestion

Lane 1-2 markers, lane 3 1 U *Mwo*1 enzyme, lane 4 1/5 dilution, lane 5 1/10 dilution, lane 6 1/100 dilution

(PCR products from the amplification of 18S-25/28S rDNA fragments were digested with *Mwo*I. The enzyme was diluted and lanes 3-4 were *C. parapsilosis* and lanes 5-6 were *C. glabrata*)

temperatures. Products were generated at 533 bp for 60 °C annealing temperature, but not for 55 and 65 °C (Figure 3.12). The optimum annealing temperature was 60 °C.

3.5.2 bla_{CTX-M} gene detection

bla_{CTX-M}-specific PCR was optimised using *E. coli* strains which possessed bla_{CTX-M} genes fof CTX-M (-2, 14, 15, 26). CTX-M-F and CTX-M-R primers **(Weill *et al.*, 2004)** were used to amplify the 540 bp conserved fragment of the bla_{CTX-M} gene. The PCR cycle was run at:

[10min 94°C] x1 [30s 94°C, 30s 55°C, 1min 72°C] x35 [10min 72°C] x1

This yielded a band of approximately 540 bp which was the expected size (Figure 3.13).

3.5.3 bla_{SHV} gene detection

bla_{SHV}-specific PCR was optimised using *E. coli* which possessed the SHV-2 ESBL gene. SHV-F and SHV-R primers **(Naiemi *et al.*, 2005)** were used to amplify the 827 bp fragment of the bla_{SHV} gene. The PCR cycle was run at 50, 55 and 60 °C annealing temperature and positive amplification was seen at 827 bp for 50 and 55 °C cycle (Figure 3.14). The optimum annealing temperature for bla_{SHV} genes was 55 °C.

3.5.4 bla_{TEM} gene detection

bla_{TEM}-specific PCR was optimised using the plasmid pTJS140 **(Smith *et* al., 2002)** known to possess a TEM beta-lactamase gene. TEM-F and TEM-R primers **(Naiemi *et al.*, 2005)** were used to amplify the highly conserved 862 bp fragment of the bla_{TEM} gene. The PCR cycle was run at 50 and 55 °C annealing temperatures and positive

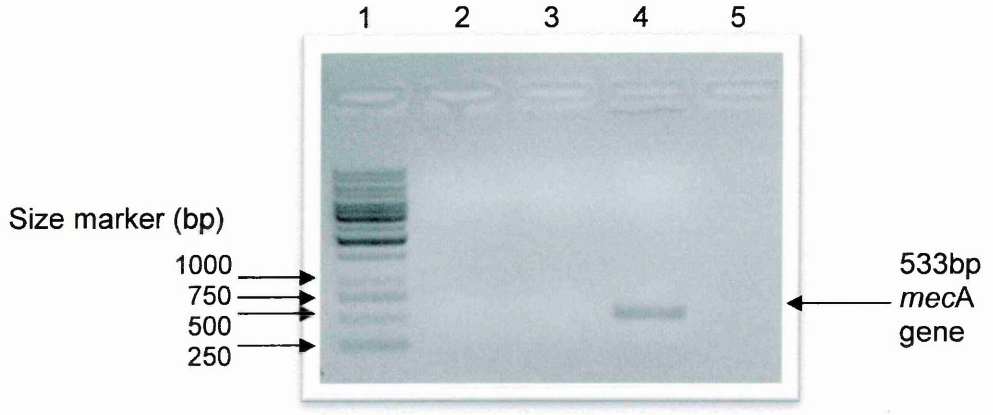


Figure 3.12 Optimisation of *mec*A gene PCR

Lane 1 marker, lane 2 no DNA template control, lanes 3-5 PCR products from template DNA purified from an RHH ICU clinical MRSA strain at 55°C, 60°C and 65°C AT (Using *mec*A gene specific primers detailed in the materials and methods)

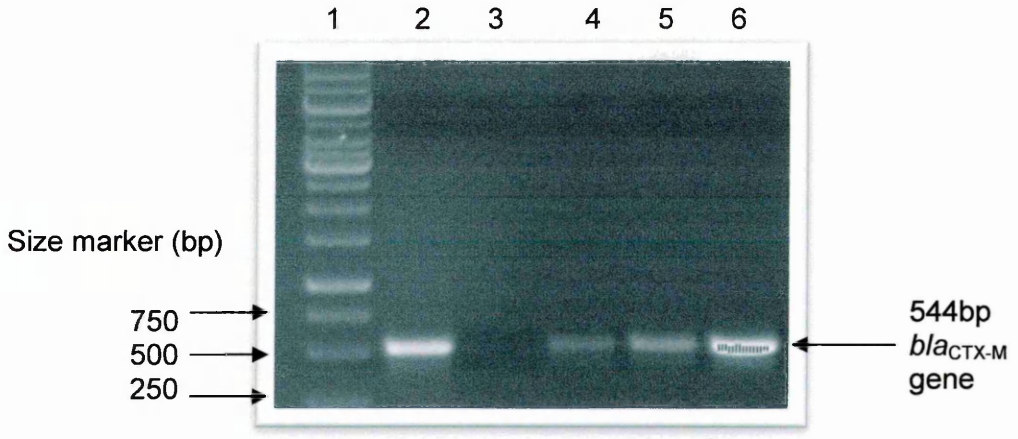


Figure 3.13 *bla*$_{CTX-M}$ gene detection

Lane 1 marker, lane 2 PCR product from template DNA purified from cultured *E. coli* clinical isolate, Lane 3 no DNA template control, lane 4 PCR product from template DNA purified from *E. coli* CTX-M-2, lane 5 PCR product from template DNA purified from *E. coli* CTX-M-14, Lane 6 PCR product from template DNA purified from *E. coli* CTX-M-15

(Using *bla*$_{CTX-M}$ gene specific primers detailed in the materials and methods)

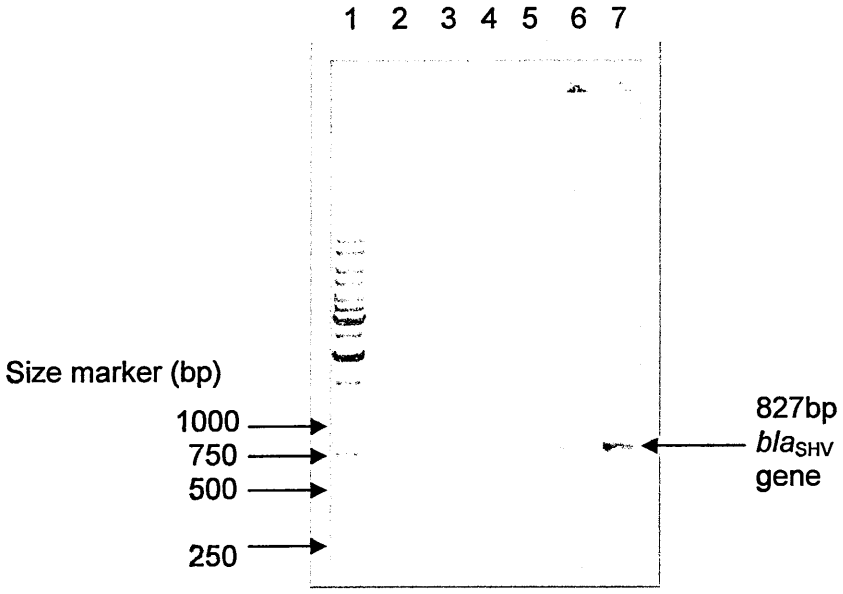


Figure 3.14 *bla*$_{SHV}$ gene detection

Lane 1 marker, lane 2 no DNA template control, lanes 3-4 PCR products from template DNA purified from *E. coli* SHV-2 at 50°C AT, lane 5 negative control, lanes 6-7 PCR products from template DNA from *E. coli* SHV-2 at 55°C AT

(Using *bla*$_{SHV}$ gene specific primers detailed in materials and methods)

amplification was seen at 862bp for the 50 °C cycle (Figure 3.15). The optimum annealing temperature for bla_{TEM} genes was 50 °C.

An environmental DNA sample obtained by swabbing slime from the trap of the waste pipe (from the molecular biology laboratory) was used as template for *bla* gene-specific PCR. No products were visualised except a faint positive result for bla_{TEM}. The environmental DNA sample was spiked with 1, 0.1 and 0.01 ng of plasmid pTJS140 DNA to check whether low concentrations of DNA template could be amplified from a mixed background. From this PCR 0.01 ng of DNA template could be detected from spiked samples by PCR (Figure 3.16).

3.5.5 Detection of tet genes

A *Bacillus subtilis* strain known to possess the *tet*(M) gene and *E. coli* strains known to possess the *tet*(O) and *tet*(W) genes were provided by Dr P Mullany. The PCR annealing temperatures were tested at 45, 50, 52, 55 and 60 °C. The optimum AT was determined as 52 °C for *tet*(M)/*tet*(O) and 60 °C for *tet*(W) (data not shown).

3.6 Detection levels of ESBL-producing organisms

Since it was possible that no environmental sites would yield ESBL-producing organisms it was required to know the detection rates of the methods used. In order for bacteria to survive in the environment they have evolved complex starvation survival patterns to enable persistence **(Clements *et al.*, 1999)**. *E. coli* SHV-2 and *E. coli* CTX-M-2 were cultured in reduced nutrient broth (1/10 dilution) to create long-term starved cells. Microscopy enabled cell enumeration and dilutions were carried out to provide 25 µl aliquots containing 1, 10, 100 and 1000 cells. The ICU floor piece was

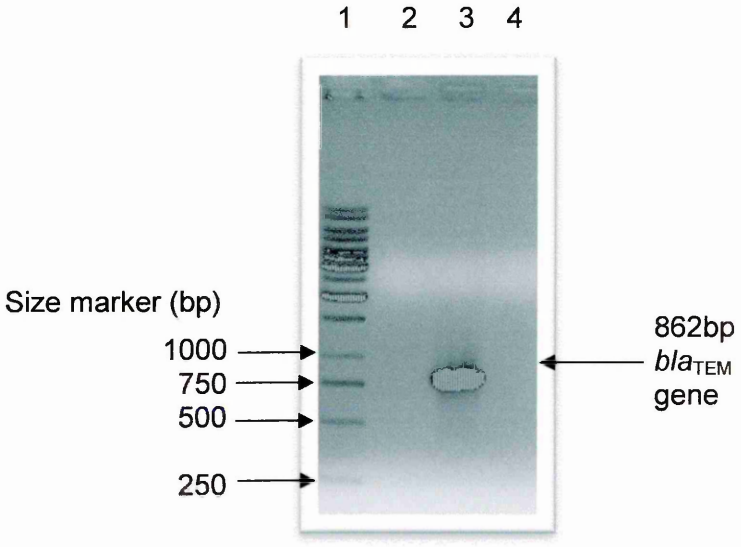


Figure 3.15 *bla*$_{TEM}$ gene detection

Lane 1 marker, lane 2 no DNA template control, lane 3-4 PCR products from template DNA purified from *E. coli* clinical isolate at 50°C and 55°C AT

(Using *bla*$_{TEM}$ gene specific primers detailed in materials and methods)

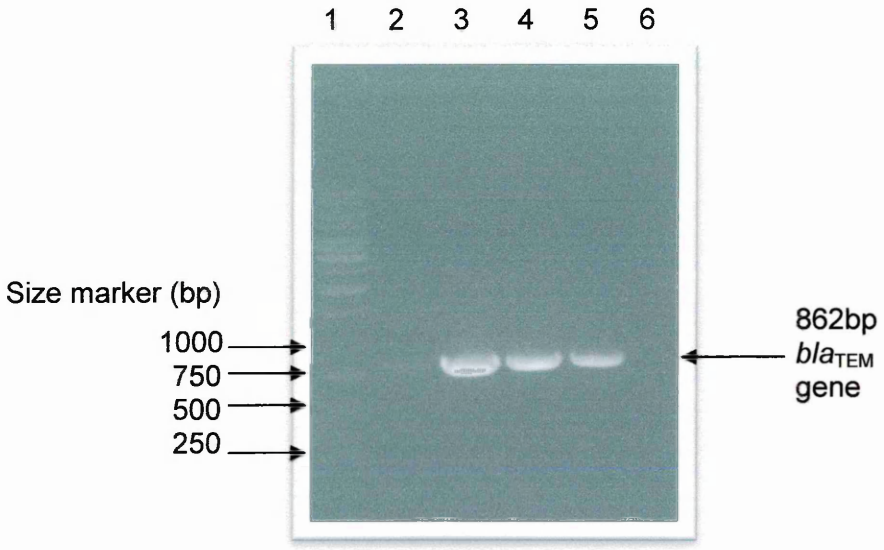


Figure 3.16 Threshold of detection of *bla*$_{TEM}$ gene in environmental samples

Lane 1 marker, lane 2 PCR product from template DNA purified from environmental plughole sample, lane 3-5 PCR products from template DNA from environmental plughole sample spiked with 1, 0.1 and 0.01 ng *bla*$_{TEM}$ positive DNA, lane 6 negative control

(Using *bla*$_{TEM}$ gene-specifc primers detailed in materials and methods)

cleaned with bactericidal wipes and domestic washing liquid to simulate ward cleaning. Aliquots of cells were spread onto the floor piece, allowed to dry and the area was swabbed. DNA was extracted directly from the swab samples and used as the template for bla_{CTX-M} and bla_{SHV} gene specific PCRs (as described in Chapter 2).

After PCR, amplicons of bla_{CTX-M} and bla_{SHV} genes were recovered from approximately $1x10^3$ SHV and CTX-M-producing cells. No amplicons were obtained when less than 1000 cells were added (Figure 3.17). If efficiency of DNA recovery and PCR amplification of beta-lactamase genes from the hospital environment are similar to those observed from this laboratory study, this result would mean that if no ESBL or native β-lactamase genes were detected from a particular environmental site, then there were possibly $1x10^3$ ESBL-producing cells present and therefore would not cause a significant clinical infection threat.

3.7 Results summary

After extensive method optimisation a robust sampling regime was established, which included sample areas, a swabbing technique, DNA extraction, detection of eubacterial-specific genes, detection of medically relevant yeasts, bacterial and fungal species identification and the detection of antibiotic resistance determinants.

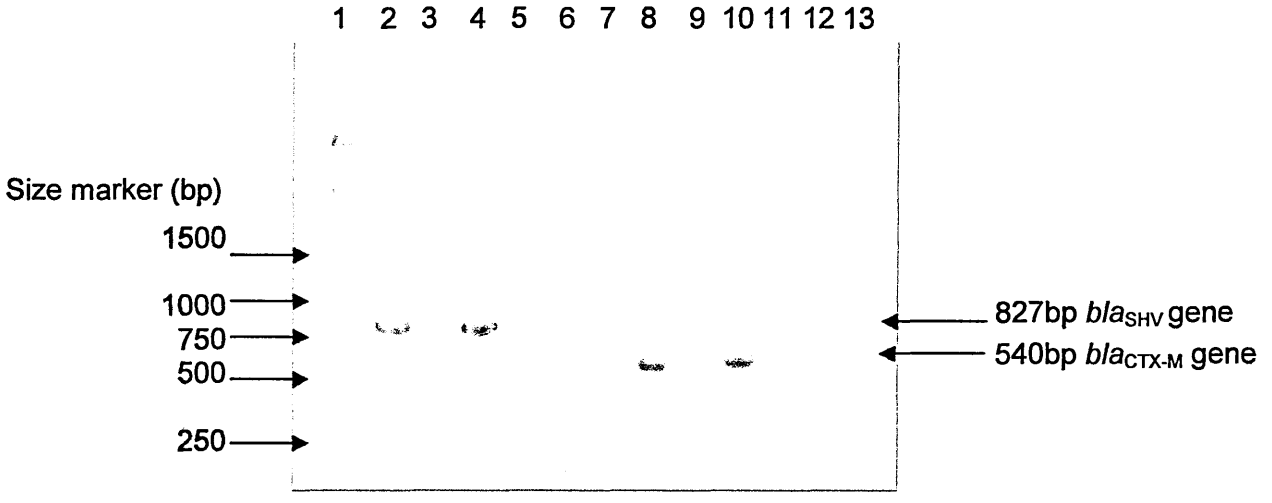


Figure 3.17 Detection levels of *bla*$_{CTX-M}$ and $_{SHV}$ producing organisms

Lane 1 marker, lane 2 *E. coli* SHV-2 positive control, lane 3 no DNA template control, lane 4-7 PCR products from purified template DNA representing 1000, 100, 10 and 1 individual SHV-producing cells, lane 8 *E. coli* CTX-M positive control, lane 9 no DNA template control, lane 10-13 PCR products from purified template DNA representing 1000, 100, 10 and 1 individual CTX-M producing cells

(Using *bla*$_{SHV}$ and *bla*$_{CTX-M}$ gene specific primers detailed in the materials and methods)

4. Distribution of bacteria in the ICU and HDU environment

4.1 Background and aims

The aim of this chapter was to determine the distribution of bacteria in the ICU and HDU environment. In order to determine the presence of and identify bacteria in the environment a molecular approach was used. PCR was used to detect bacterial 16S rRNA genes in the environment and PCR-DGGE enabled bacterial species identification on the basis of 16S rRNA gene fragment mobility. Parallel isolation of culturable bacteria was also performed.

The objectives were to: -

- detect bacteria in the ICU and HDU environment from swab samples by PCR using eubacterial 16S rRNA gene-specific primers (as detailed in materials and methods)
- isolate bacteria from the ICU and HDU environment by cultivation from swab, dipslide and contact plate samples
- determine environmental sites where bacteria could most frequently be detected
- identify species present and diversity using PCR-DGGE (as detailed in materials and methods)
- compare bacterial species from environmental sites with those isolated from clinical samples

4.2 Overview of materials and methods

The ICU and HDU departments were sampled over 12 sessions from the RHH (12/05/08 – 15/12/08) and the NGH (11/03/08 – 09/12/08). In total 252 environmental

swab samples were taken from both the RHH and NGH (Table 2.2a/b). At the RHH, 15 environmental sites were sampled and 13 environmental sites at the NGH. After sampling, DNA was extracted from each swab sample and the remaining swab solution was plated out onto various solid media (NA, blood and CLED) for the isolation of culturable bacteria. Contact plates were used to sample and culture bacteria from two textured surfaces at the RHH (HDU patient chair and ICU curtain) and two similar surfaces at the NGH (ICU patient chair and ICU curtain) (Table 2.3a/b). Dipslides were used to sample and culture bacteria from four small hard surfaces at the RHH and six at the NGH (Table 2.4a/b) (full details of the sampling regime are provided in Section 2.2 Materials and Methods).

4.3 Results

ICU and HDU at the Royal Hallamshire Hospital

4.3.1 Detection of prokaryotic cells from RHH environment

In total, eubacterial DNA was detected in 42.1% (106/252) of swab samples (Table 4.1 and representative gels are shown in Figure 4.1a/b).

Bacteria were isolated from swab, contact plate and dipslide samples (Table 4.1, 4.2 and 4.3). In total bacteria were isolated from 43.7% (110/252) of swab samples (Table 4.1), 100% (36/36) of contact plate samples (Table 4.2) and 60.4% (29/48) of dipslide samples (Table 4.3).

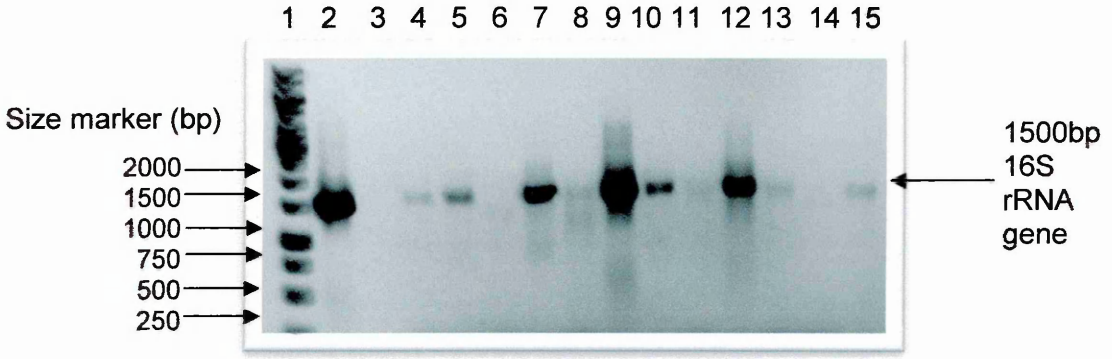
Table 4.1 Proportion of ICU and HDU swab samples positive for eubacterial 16S rRNA genes and culturable bacteria per sample session at RHH[1]

Sample session	Proportion of swabs positive for eubacterial 16S rRNA gene (%) (n = 252)*	Proportion of swabs that yielded culturable bacteria (%) (n = 252)*
1 (12/05/08)	61.9	76.2
2 (02/06/08)	9.5	38.1
3 (30/06/08)	4.8	28.6
4 (14/07/08)	19.0	33.3
5 (15/09/08)	47.6	28.6
6 (29/09/08)	57.1	61.9
7 (13/10/08)	66.7	61.9
8 (03/11/08)	19.0	47.6
9 (17/11/08)	71.4	33.3
10 (01/12/08)	47.6	38.1
11 (08/12/08)	38.1	14.3
12 (15/12/08)	61.9	61.9
Average (%)	42.1	43.7

[1] The data was obtained from molecular and cultural studies from ICU and HDU environmental swab samples

* n = 21 per sample session

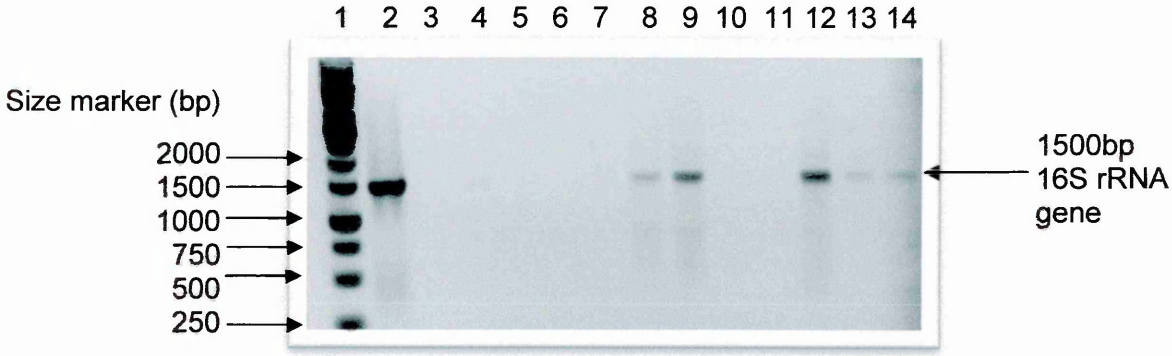
Figure 4.1 Amplification of eubacterial DNA from RHH environmental swab samples



(a)

Lane 1 marker, lane 2 *E. coli* XL1 positive control, lane 3 no DNA template control, lanes 4-5, 7-13, 15 PCR products from template DNA purified from environmental swab samples (sites – ICU floor, ICU bedside, ICU window ledge from 01/12/08 session 10, sites – ICU trolley, ICU ward sink plughole, ICU computer keyboard, ICU ward sink tap from 13/10/08 session 7, site – ICU picture from 12/05/08 session 1 and site – ICU fan from 13/10/08 session 7 respectively)

(Using the eubacterial 16S rRNA gene-specific primers detailed in the materials and methods)



(b)

Lane 1 marker, lane 2 *E. coli* XL1 positive control, lane 3 no DNA template control, lanes 8-9, 12-14 PCR products from template DNA purified from environmental swab samples (sites – ICU ward sink plughole, ICU computer keyboard, ICU ward sink tap, ICU picture from 12/05/08 session 1 and site - fan from 29/09/08 session 6 respectively)

(Using the eubacterial 16S rRNA gene-specific primers detailed in the materials and methods)

Table 4.2 Number of bacterial colonies cultured from RHH ICU and HDU contact plate samples[1]

Sample site[1]	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Chair back (HDU)	21	5	2	21	9	1	111	42	1	5	2	2
Chair base (HDU)	152	8	1	13	36	1	11	29	5	33	2	5
Curtain (ICU)	1	5	5	7	7	3	2	1	25	3	1	8

[1] The data were obtained from cultural studies from ICU and HDU contact plate samples

Table 4.3 Number of bacterial colonies cultured from RHH ICU and HDU dipslide samples[1]

Sample site[1]	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Door handle (ICU)	0	1	1	0	0	0	1	0	1	0	1	0
Handwash bottle (ICU)	1	1	1	11	4	41	48	1	9	5	1	5
Handwash bottle (HDU)	2	1	1	7	1	1	0	6	1	1	0	46
Light switch (ICU)	0	0	0	0	0	1	0	0	1	0	0	0

[1] The data were obtained from cultural studies from ICU and HDU contact plate samples

Eubacterial DNA was most frequently detected in swab samples from the ICU ward sink plughole (70.8% of samples), HDU patient chair (52.1%) and ICU floor (50%) (Figure 4.2). Bacteria were most frequently isolated from HDU patient chair contact plate samples (Table 4.2) and ICU/HDU handwash bottle dipslides (Table 4.3).

Eubacterial DNA was much less frequently detected in swab samples from the sluice room sink plughole, hard surfaces (ICU bedside, ICU computer keyboard, ICU fan, HDU computer keyboard, HDU computer stand, ICU ward sink taps, ICU trolley and ICU window ledge) and pictures (ICU and HDU) (Figure 4.2). Bacteria were detected with only very low frequency from ICU curtain contact plates compared to HDU patient contact plates (Table 4.2). The majority of dipslide samples from the ICU door handle (58.3%) and ICU light switch (83.3%) did not yield culturable bacteria (Table 4.3).

All (36) contact plate samples (HDU patient chair and ICU curtain) and ICU handwash bottle dipslide samples yielded at least one bacterial colony (Table 4.2 and 4.3). The total number of bacterial colonies from the sites of most frequent isolation, varied between sample sessions; HDU patient chair contact plates yielded 1 to 152 colonies (Table 4.2), ICU handwash bottle dipslides yielded 1 to 48 colonies and HDU handwash bottle dipslides yielded 0 to 46 colonies (Table 4.3). Interestingly the majority (75%) of ICU handwash bottle dipslides yielded <10 colonies, however on sessions 6 (29/09/08) and 7 (13/10/08) the number of colonies increased more than four fold (41 and 48 colonies respectively) (Table 4.3).

Bacterial sequences of the nt 341-926 16S rRNA gene fragment were retrieved from environmental swab samples of the RHH ICU ward sink plughole, ICU floor and HDU paitent chair by PCR-DGGE, excised and sequenced. Each sequence was obtained from a single representative band (Table 4.4a/b/c and representative gel shown in Figure 4.3) where the number of samples with persistant sequence refers to the

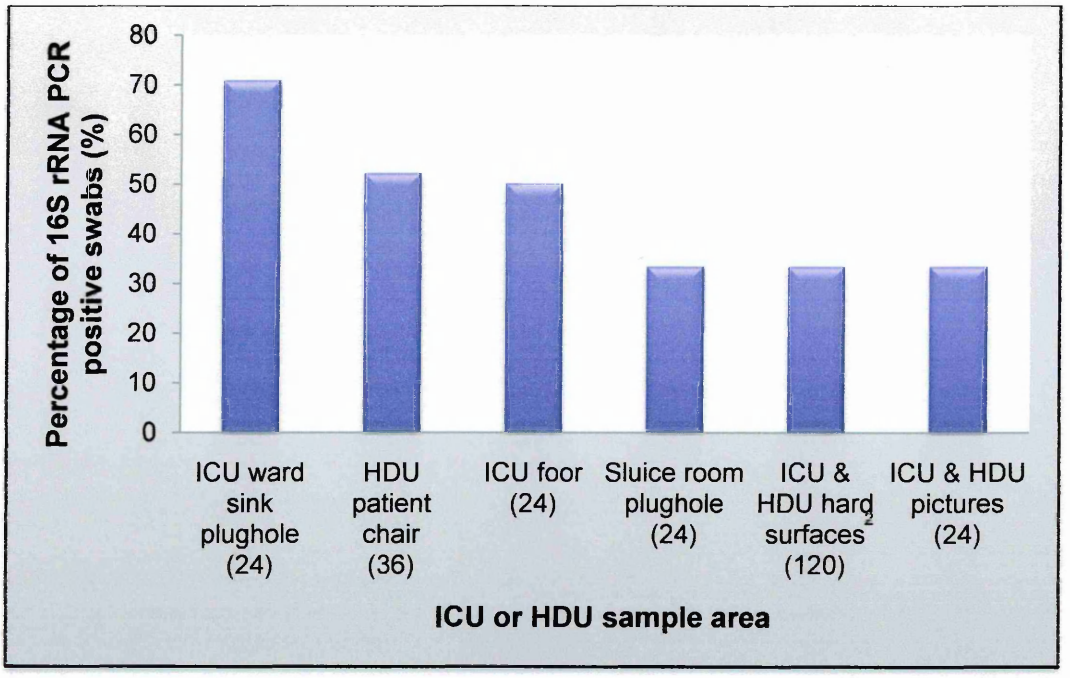


Figure 4.2 Proportion of swab samples positive for eubacterial 16S rRNA genes from RHH ICU and HDU environmental sites[1]

(number) = total number of swab samples taken

[1] The data were obtained from PCR of 16S rRNA gene in DNA from ICU and HDU environmental swab samples

[2] Hard surfaces – ICU bedside, ICU computer keyboard, ICU fan, HDU computer keyboard, HDU computer stand, ICU ward sink taps, ICU trolley and ICU window ledge

Table 4.4 Retrieval of sequences from RHH environmental swab samples (ICU ward sink plughole, ICU floor and HDU patient chair) generated after PCR-DGGE from representative DGGE bands: -

(a) ICU ward sink plughole

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Luteibacter spp.	9	99	432*	AM930508
Soil bacterium	9	97	432*	DQ490030
Lactobacillus salivarius	9	99	411*	GU357500
Ralstonia metallidurans	9	98	384*	CP000352
Burkholderia cepacia	8	99	405*	NR029209
Burkholderia cenocepacia	8	97	414*	FJ947055
Pseudomonas aeruginosa	8	86	294**	DQ864493
Cupriavidus metallidurans	5	97	390*	CP000353
Cupriavidus gilardii	3	96	432**	EF114428
Ralstonia spp.	3	96	450*	GQ417782
FR2_C116 *Ralstonia* spp.	3	84 84	420** 366**	EU888560 FJ774001

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

(b) ICU floor

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Staphylococcus epidermidis	4	50	168**	EF558734
Ralstonia spp.	3	100	477*	GQ417854
nbw555e03cl *Trochodendron aralioides*	1	99 99	417** 327**	GQ107864 DQ629469
nbw402g08cl *Ralstonia* spp.	1	70 68	420* 273*	GQ098212 EU440055
Burkholderia cepacia	1	96	393*	AF097530
Leifsonia spp.	1	99	476*	FJ872398

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

(c) HDU patient chair

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Leifsonia spp.	6	89	330**	FJ872398
Variovorax spp.	3	98	414*	GQ332345
Burkholderia cepacia	2	100	474*	NR029209
TSPB_37 Gamma proteobacterium	2	39 43	255** 126**	FJ213492 EF111071

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

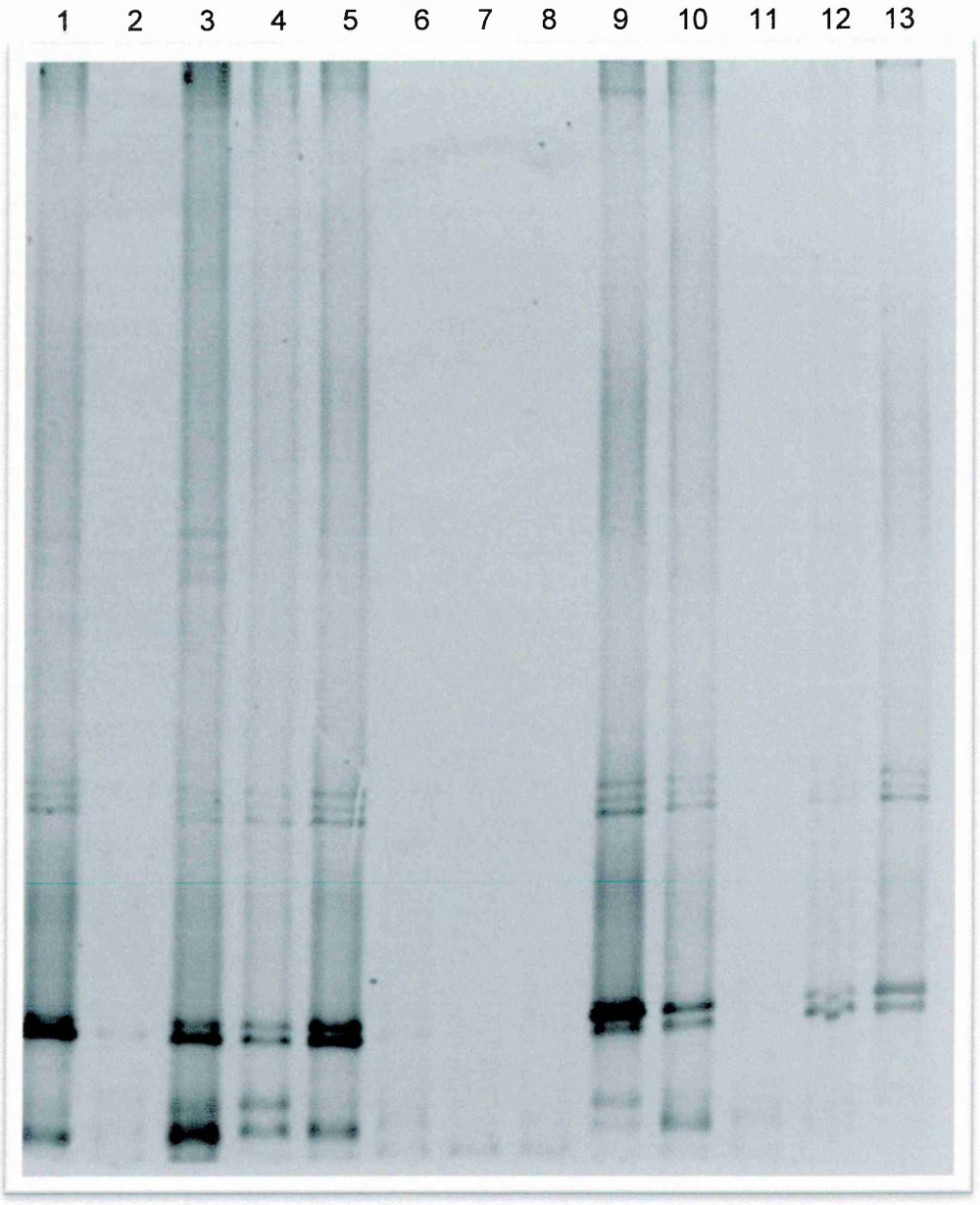


Figure 4.3 DGGE gel showing bacterial diversity and community from RHH ICU ward sink plughole swab samples

Lanes 1-13 PCR products from template DNA purified from RHH environmental ICU ward sink plughole swab samples (from 12/05/08 session 1 to 17/11/08 session 9) (Using nt 341-926 16S rDNA fragment-specific primers detailed in the materials and methods)

DGGE bands that have migrated the same distance from multiple swab samples. A number of samples had clearly defined chromatograms resulting in a high percentage identity against other sequences in the GenBank database. However, after sequencing several samples did not contain enough clear sequence of the 16S rRNA gene that could be unambiguously assigned to enable a clear identification of the organism, resulting in lower percentage identity values against sequences in the GenBank database. A number of chromatograms showed mixed samples or conceivably bleeding from neighbouring lanes after loading of sequencing gel.

Bacterial diversity was greatest in ICU ward sink plughole swab samples compared to the ICU floor and HDU patient chair swab samples (Table 4.4a/b/c). From ICU ward sink plughole swab samples 11 different DGGE bands were retrieved. From ICU floor and HDU patient chair swab samples six and four different DGGE bands were retrieved respectively.

The number of representative DGGE bands indicates the presence of the same or similar organism from the same site on different sample sessions. The ICU ward sink plughole swab samples contained the highest number of similar DGGE bands from multiple samples compared to ICU floor and HDU patient chair swab samples (Table 4.4a/b/c). From nine ICU ward sink plughole swab samples, four similar DGGE bands were retrieved and from seven ICU ward sink plughole swab samples three similar DGGE bands were retrieved. From six HDU patients chair swab samples, one similar DGGE band was retrieved and from four ICU floor swab samples, 1 DGGE band was retrieved.

The explanation of types of bacteria present is specultative because precise identification was not possible from all sequencing data. In some cases as indicated in Table 4.4a/b/c the fact that sequences can not be identified precisely may be due at least in part to inaccuracies and ambiguity in the sequences. In the majority of cases

however, analysis of the sequencing gel chromatograms showed that the sequence is accurate and there are closely matching sequences in the GenBank database. Despite a number of low percentage identities all retrieved sequences had e values ranging from 0.0 to 9e-12. This suggests that although the organism identification could not always be precise the sequence must be closely related and not a completely different organism. The PCR-DGGE results presented do provide complementary information from other techniques of organisms present in the ICU environment.

Sequences most frequently retrieved were similar to common environmental or commensal organisms; *Luteibacter* spp., *Lactobacillus salivarius* and *Ralstonia metallidurans* from ICU ward sink plughole swab samples (Table 4.4a). *Staphylococcus epidermidis* and *Ralstonia* spp. from ICU floor swab samples (Table 4.4b) and *Leifsonia* spp. from HDU patient chair swab samples (Table 4.4c). Sequences with relatively high similarity to the opportunistic pathogen *P. aeruginosa* were frequently retrieved from ICU ward sink plughole swab samples.

Sequences that were less frequently retrieved include those with high similarity to opportunistic organisms, for example members of the genus *Burkholderia* (*B. cepacia* and *B. cenocepacia*). However, representative *Burkholderia* spp. sequences were retrieved from each of the three environmental sites (ICU ward sink plughole, ICU floor and HDU patient chair) suggesting a wide environmental distribution of *Burkholderia* spp.

Occassionally sequences were retrieved with relative similarity to organisms associated with human skin and blood (according to the annotation of the GenBank database entry); nbw555e03cl (*Trochodendron aralioides*) and nbw402g08cl (*Ralstonia* spp.) from ICU floor swab samples and TSPB_37 (Gamma proteobacterium) from HDU patient chair swab samples. Sequences were also retrieved similar to environmental organisms; *Cupriavidus gilardii*, *Ralstonia* spp., *Cupriavidus metallidurans* and

FR2_C116 (*Ralstonia* spp.) from ICU ward sink plughole swab samples and *Leifsonia* spp., and *Ralstonia* spp. from ICU floor swab samples.

From environmental HDU patient chair and ICU curtain contact plate samples, isolated organisms were identified by PCR of the eubacterial 16S rRNA gene from purified DNA (Table 4.5). After representative colony picks, *S. epidermidis* and *Micrococcus* spp. were identified from ICU curtain and HDU patient chair.

Only one organism, *S. epidermidis* was detected using both molecular and cultural methods. *S. epidermidis* was isolated from ICU curtain and HDU patient chair contact plate samples and sequences were retrieved from ICU floor swab samples that had low percentage identity to *S. epidermidis*.

4.3.2 RHH ICU bacterial clinical isolates

All clinical samples were routinely taken by medical staff and culturable microorganisms identified by routine laboratory analysis. A total of 24 bacterial species were identified from clinical samples during the period that environmental sampling was taking place (12/05/08-15/12/08) (Table 4.6). Multiple samples were taken from patients, resulting in more positive cultures and isolates than the number of infected or colonised patients. The most commonly isolated organisms were CNS, *E. coli* and *S. aureus*. From 403 clinical isolates 55% were Gram-negative pathogens.

Table 4.5 Identification of bacterial species from RHH contact plates (HDU patient chair and ICU curtain) after representative colony picks, from DNA sequences generated after PCR of the eubacterial 16S rRNA gene[1]

Sample site	Organism identified	Percentage identity (%)	Number of aligned residues (max 1500)	Accession number
Curtain (ICU)	*Micrococcus luteus*	99	820	EU196531
	Staphylococcus epidermidis	99	1433	FN393820
HDU patient chair	*Micrococcus spp.*	99	1035	FJ357605
	Staphylococcus epidermidis	98	819	DQ870761

[1] The data were obtained from cultured organisms from ICU and HDU contact plate samples after molecular sequencing

Table 4.6 Diversity of RHH bacterial clinical isolates and frequency of infected patients

Organism	Number of clinical isolates	Number of infected/colonised patients
Coagulase-negative *Staphylococci* (CNS)	85	73
Escherichia coli	53	46
Staphylococcus aureus	51	43
Pseudomonas aeruginosa	37	27
Klebsiella pneumonia	31	24
Enterococcus spp.	30	24
Klebsiella oxytoca	18	17
Enterobacter cloacae	16	16
Proteus mirabilis	15	11
MRSA	14	8
Stenotrophomonas maltophilia	13	10
Pseudomonas spp.	12	12
Enterobacter aerogenes	5	4
Serratia spp.	4	4
Acinetobacter baumanii	3	3
Citrobacter freundii	3	3
Serratia liquefaciens	3	2
Citrobacter koseri	2	2
Enterobacter agglomerans	2	2
Proteus vulgaris	2	2
Enterococcus faecium	1	1
Morganella morganii	1	1
Pantoea agglomerans	1	1
Providencia rettgeri	1	1

4.3.3 Detection of prokaryotic cells from NGH environment

Similar to the RHH, 40.1% (101/252) of swab samples were positive for eubacterial 16S rRNA genes (Table 4.7 and representative gels are shown in Figure 4.4a/b).

Bacteria were isolated from 37.7% (95/252) of swab samples (Table 4.7). All (36) contact plate samples (ICU curtain and ICU patient chair) yielded culturable bacteria (Table 4.8), similar to the RHH, and bacteria were isolated from 38.9% (28/72) of dipslide samples (Table 4.9).

Similarly to the RHH data, eubacterial 16S rRNA genes were most frequently detected in swab samples from the ICU ward sink plughole (62.5%), sluice room sink plughole (58.3%) and ICU floor (52.1%) (Figure 4.5). Again, similar to the RHH, bacteria were most frequently isolated from ICU patient chair contact plates (Table 4.8) and ICU handwash bottle dipslides (Table 4.9).

The number of sample sessions when bacteria were isolated from ICU handwash bottle dipslides was much lower at the NGH than RHH. Handwash bottles were sampled an equal number of times (12) at both sites, however no bacteria could be isolated from 66.7% (8/12) of sample sessions at the NGH (Table 4.9).

Eubacterial DNA was infrequently detected from hard surfaces (ICU computer keyboard, HDU computer keyboard and ICU window ledge) and switches (ICU door handle, HDU door handle and ICU machine handles). In contrast to the RHH, eubacterial DNA was least frequently detected from ICU patient chair swab samples (Figure 4.5).

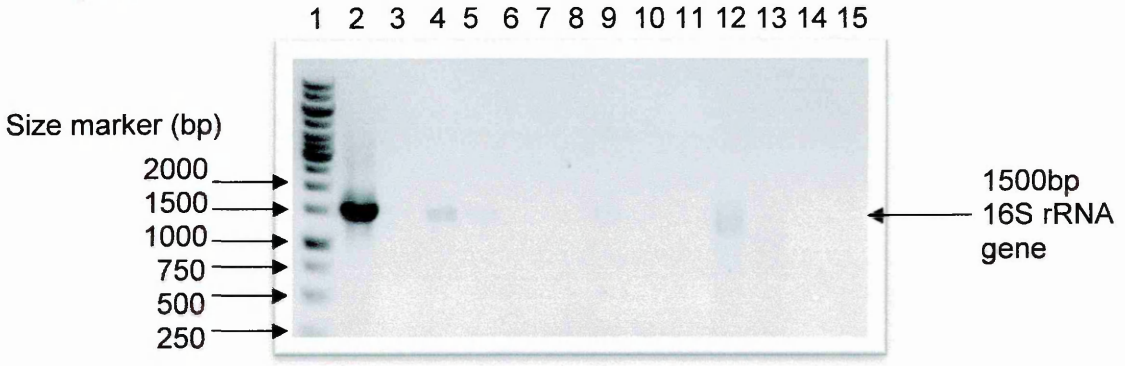
Table 4.7 Proportion of ICU and HDU swab samples positive for eubacterial 16S rRNA genes and culturable bacteria per NGH sample session[1]

Sample session	Proportion of swabs positive for eubacterial 16S rRNA gene (%) (n = 252)*	Proportion of swabs that yielded culturable bacteria (%) (n = 252)*
1 (11/03/08)	23.8	33.3
2 (08/04/08)	42.9	38.1
3 (15/05/08)	81.0	61.9
4 (03/06/08)	14.3	28.6
5 (01/07/08)	23.8	33.3
6 (15/07/080	42.9	33.3
7 (16/09/08)	19.0	19.0
8 (30/09/08)	47.6	38.1
9 (14/10/08)	38.1	38.1
10 (04/11/08)	42.9	47.6
11 (18/11/08)	38.1	28.6
12 (09/12/08)	66.7	52.4
Average (%)	40.1	37.7

[1] The data were obtained from molecular and cultural studies from ICU and HDU environmental swab samples

* n = 21 per sample session

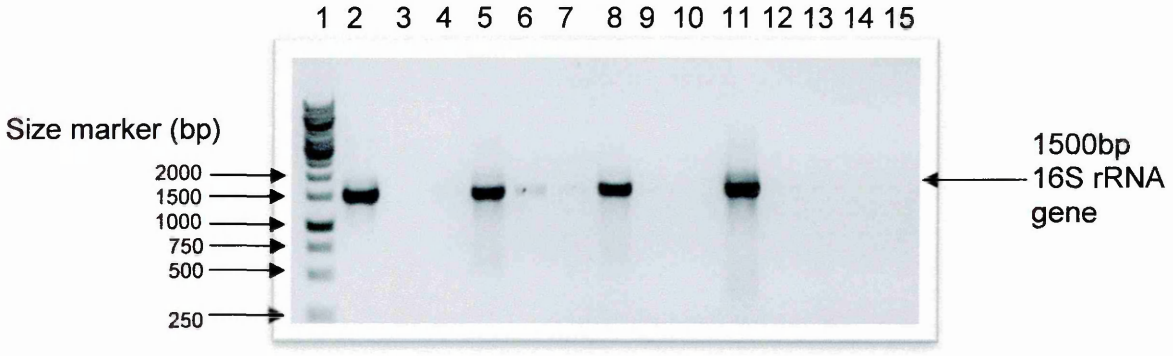
Figure 4.4 Amplification of eubacterial DNA from NGH environmental swab samples



(a)

Lane 1 marker, lane 2 *E. coli* XL1 positive control, lane 3 no DNA template control, lanes 4, 5, 9, 12 PCR products from template DNA purified from environmental swab samples (sites – ICU machine handle x2, ICU computer keyboard, and ICU floor-ward respectively from 15/05/08 session 3)

(Using the eubacterial 16S rRNA gene-specific primers detailed in the materials and methods)



(b)

Lane 1 marker, lane 2 *E. coli* XL1 positive control, lane 3 no DNA template control, lanes 5-6, 8, 11 PCR products from template DNA purified from environmental swab samples (sites - HDU floor, HDU computer keyboard, ICU floor, ICU ward sink plughole respectively from 08/04/08 session 2)

(Using the eubacterial 16S rRNA gene-specific primers detailed in the materials and methods)

Table 4.8 Number of bacterial colonies cultured from NGH ICU contact plate samples[1]

Sample site[1]	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Patient chair back (ICU)	2	1	16	2	29	2	9	1	61	104	110	37
Patient chair base (ICU)	15	4	22	28	36	17	18	2	112	78	175	74
Curtain (ICU)	5	43	4	10	3	6	9	9	3	5	17	8

[1] The data were obtained from cultural studies from ICU contact plate samples

Table 4.9 Number of bacterial colonies cultured from NGH ICU dipslide samples[1]

Sample site[1]	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Blind switch (ICU)	0	0	0	0	1	1	0	0	9	1	0	0
Handwash bottle corridor (ICU)	150	1	50	14	2	0	1	6	1	1	0	0
Handwash bottle isolation room (ICU)	0	2	0	0	35	48	2	0	27	0	8	1
Inner window switch (ICU)	0	1	0	0	1	0	0	0	0	0	0	0
Intercom button (ICU)	0	0	1	0	0	1	0	0	0	0	1	0
Light switch (ICU)	0	0	13	0	4	0	0	0	1	0	0	0

[1] The data were obtained from cultural studies from ICU and HDU contact plate samples

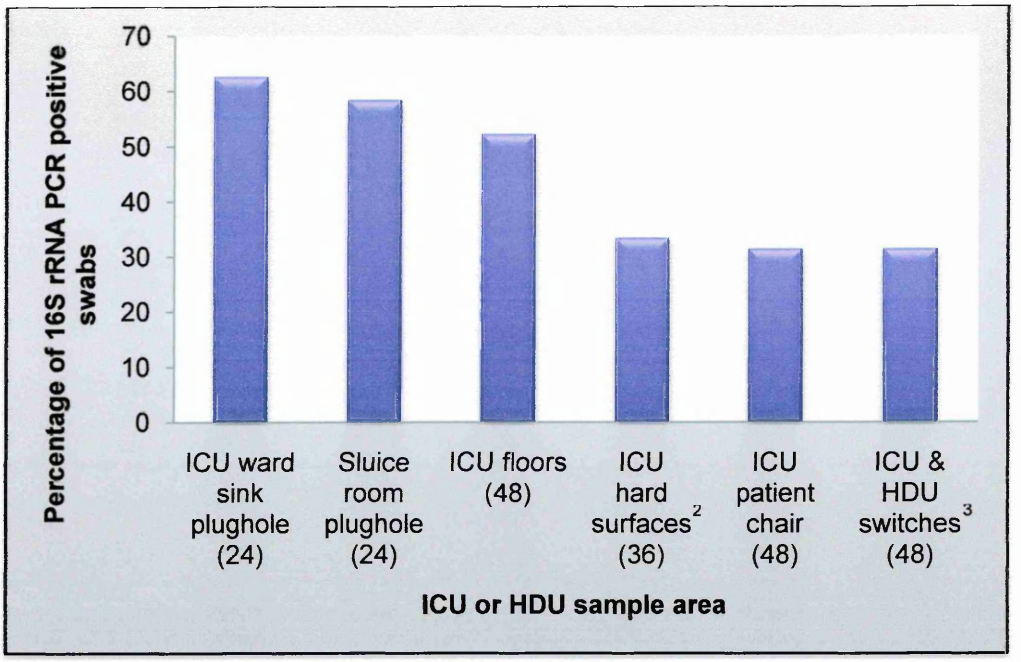


Figure 4.5 Proportion of swab samples positive for eubacterial 16S rRNA genes from NGH ICU and HDU environmental sites[1]

(number) = total number of swabs taken

[1] The data were obtained from PCR of eubacterial 16S rRNA gene in DNA from ICU and HDU environmental swab samples

[2] Hard surfaces – ICU computer keyboard, HDU computer keyboard and ICU window ledge

[3] Switches – ICU door handle, HDU door handle and ICU machine handles

Similar to the RHH data, bacteria were least frequently isolated from ICU curtain contact plates compared to ICU patient chair contact plates (Table 4.8) and 75% of ICU light switch dipslides yielded no culturable bacteria (Table 4.9). In addition, the majority of other dipslide samples from the ICU blind switch (66.7%), ICU inner window switch (83.3%) and intercom button (75%) also yielded no culturable bacteria (Table 4.9).

Bacterial sequences were retrieved using the same technique as the RHH from environmental swab samples of the NGH ICU ward sink plughole, ICU floor and ICU patient chair (Table 4.10a/b/c and representative gel shown in Figure 4.6). Similar to the RHH, precise identification of bacteria was not always possible from the sequencing data however, the results do provide information about the microbial ecology and diversity in sites harbouring bacteria.

ICU ward sink plughole swab samples had the greatest bacterial diversity (17 different DGGE bands were retrieved) and ICU patient chair swab samples had the lowest (three different DGGE bands were retrieved). However in contrast to the RHH, the NGH ICU floor swab samples had a greater bacterial diversity (10 different DGGE bands were retrieved) (Table 4.10a/b/c).

The number of similar DGGE bands present in multiple ICU ward sink plughole swab samples was higher at the NGH compared to the RHH. From ten, eight and seven ICU ward sink plughole swab samples two, one and four similar DGGE bands were retrieved respectively (Table 4.10a). From eight and five ICU floor swab samples one and two similar DGGE bands were retrieved respectively (Table 4.10b).

Similar to the RHH, the most prevalent DGGE bands had sequence similarity to environmental or commensal organisms. Sequences retrieved from ICU ward sink plughole swab samples were similar to *Pseudomonas putida*, *Aquabacterium* spp., *Variovorax* spp. and nbw533b03cl (Beta-proteobacterium) (associated with human skin

Table 4.10 Retrieval of sequences from NGH environmental swab samples (ICU ward sink plughole, ICU floor and ICU patient chair) generated after PCR-DGGE from representative DGGE bands: -

(a) ICU ward sink plughole

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Serratia marcescens	10	38	201**	FJ919562
nbw533b03cl Beta-proteobacterium	10	80 80	225** 222**	GQ106160 AB252912
Pseudomonas putida	8	87	312**	DQ313383
Burkholderia cepacia	7	98	453*	GQ359110
Peptostreptococcus spp.	7	95	495*	GU401462
Aquabacterium spp.	7	98	417*	AF523022
Klebsiella spp.	7	50	303**	EU360123
Variovorax spp.	6	97	399*	EU593268
Enterobacter spp.	5	45	252**	CP000653
Stenotrophomonas maltophilia	4	100	511*	GU391033
Alcaligenes spp.	4	94	408*	AB046605
Pseudomonas spp.	4	99	324*	GU198110
Delftia spp.	4	99	384*	GQ205102
T529_a01f06 *Coriobacterium* spp.	3	79 79	396** 369**	FJ367280 AJ131150
Beta-proteobacterium	2	99	462*	AB252902
Burkholderia pyrrocinia	2	99	450*	NR029210
Soil bacterium	2	65	378**	EU515500

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

(b) ICU floor

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Variovorax paradoxus	8	100	468*	GQ332345
Proteus mirabilis	5	94	282*	AM942759
Staphylococcus epidermidis	5	50	180**	AJ581947
Delftia spp.	4	99	444*	AB451538
Burkholderia spp.	4	98	450*	GQ465226
nbw1222e02cl *Cupriavidus metallidurans*	3	59 58	297** 234**	GQ059064 FJ644635
Stenotrophomonas maltophilia	3	82	330**	FJ393299
Burkholderia cepacia	2	95	420*	GQ383907
nbw775b04cl *Wautersiella falsenii*	1	79 79	426* 429*	GQ009331 FM162560
nbt171c11 *Empedobacter* spp.	1	61 61	369** 156**	EU535130 EU276091

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

(c) ICU patient chair

Sequences similar to	Number of samples with persistant sequence	Percentage identity (%)	Number of aligned residues (max 600)	Accession number
Delftia spp.	3	98	465*	GQ329375
Stenotrophomonas maltophilia	2	98	480*	EF114176
Delftia spp.	2	100	485*	AB451538

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

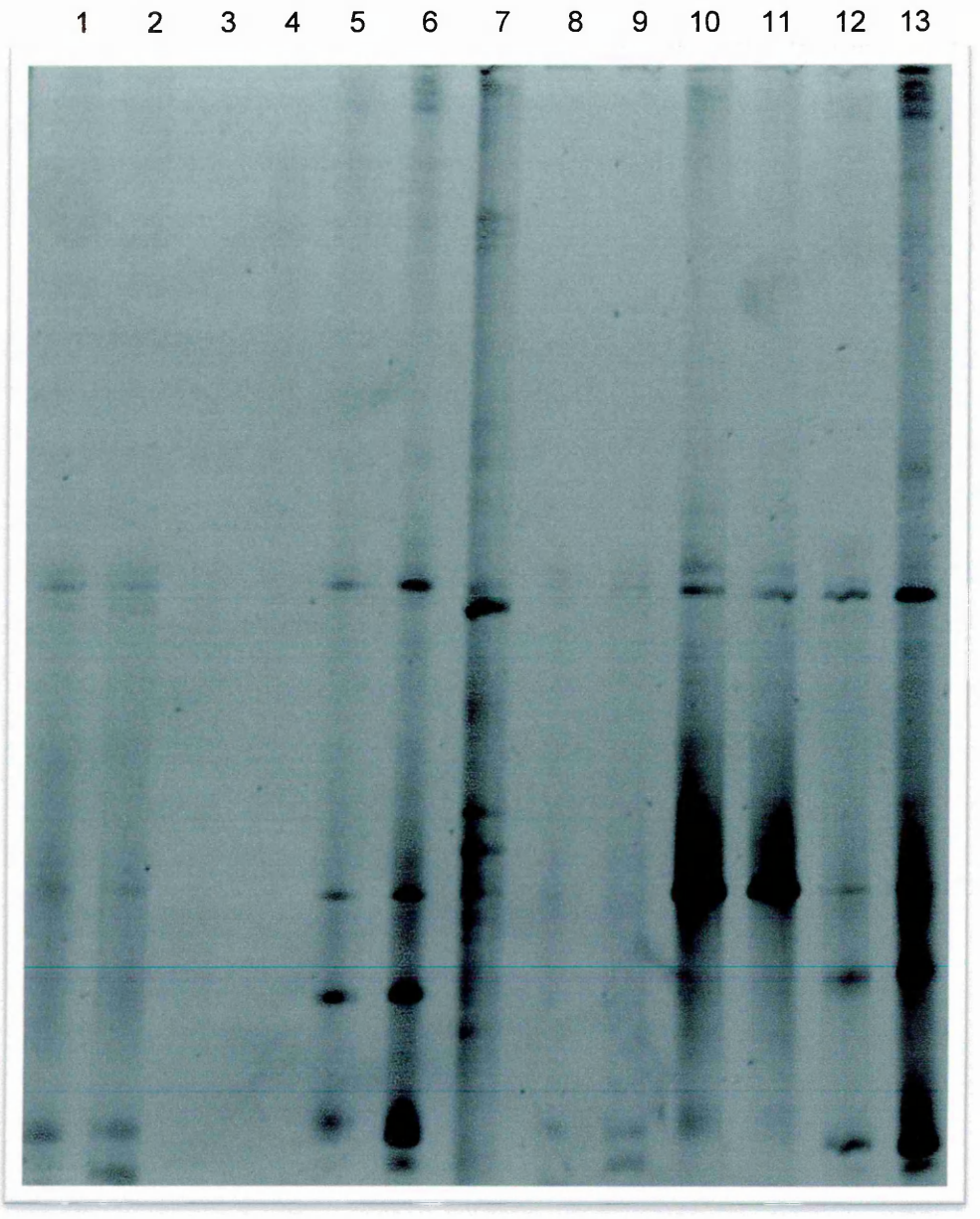


Figure 4.6 DGGE gel showing bacterial diversity and community from NGH ICU ward sink plughole swab samples

Lanes 1-13 PCR products from template DNA purified from NGH environmental ICU ward sink plughole swab samples (from 15/07/08 session 6 to 09/12/08 session 12) (Using nt 341-926 16S rDNA fragment-specific primers detailed in the materials and methods)

and blood) (Table 4.10a) and from ICU floor swab samples were similar to *Variovorax paradoxus* and *S. epidermidis* (Table 4.10b).

Sequences retrieved from ICU ward sink plughole swab samples had the highest similarity to opportunistic species; *Serratia marcescens, Peptostreptococcus* spp., *Klebsiella* spp. and *Burkholderia cepacia* (Table 4.10a) and from ICU floor swab samples sequences were relativley similar to *Proteus mirabilis* (Table 4.10b).

It is interesting that, as in the ICU at the RHH, swab samples from the ward sink plughole and floor in the ICU at the NGH contained sequences similar to *Burkholderia* spp. (*B. cepacia* and *B. cenocepacia*). In addition, at the NGH sequences similar to *Stenotrophomonas maltophilia* and *Delftia* spp. were also retrieved from all environmental sites (ICU ward sink plughole, ICU floor and ICU patient chair), suggesting a wide distribution of these sequences in the NGH ICU environment.

Sequences retrieved infrequently were similar to environmental organisms; Bacteroidetes, beta-proteobacterium, *Burkholderia pyrrocinia*, T529_a01f06 (*Coriobacterium* spp.), *Alcaligenes* spp, *Pseudomonas* spp., soil bacterium and *Enterobacter* spp. retrieved from ICU ward sink plughole swab samples (Table 4.10a). Sequences retrieved relatively similar to nbw775b04cl (*Wautersiella falsenii*), nbt171c11 (*Empedobacter* spp.) and nbw1222e02cl (*Cupriavidus metallidurans*) (from samples associated with human blood and skin as detailed in the annotation on GenBank database) from ICU floor swab samples (Table 4.10b).

Micrococcus spp. were also identified after representative colony picks from NGH ICU curtain and ICU patient chair contact plate samples, similar to the RHH (Table 4.11). However, none of the representative DGGE bands that were sequenced yielded sequences similar to *Micrococcus* spp.

Table 4.11 Identification of bacterial species from NGH ICU contact plates (ICU patient chair and ICU curtain) after representative colony picks, from DNA sequences generated after PCR of the eubacterial 16S rRNA gene[1]

Sample site	Organism identified	Percentage identity (%)	Number of aligned residues (max 1500)	Accession number
Curtain (ICU)	*Micrococcus luteus*	99	1463	FJ999947
Patient chair (ICU)	*Bacillus* spp. *Micrococcus* spp.	98 99	888 828	EU58537 AM990824

[1] The data were obtained from cultured organisms from ICU and HDU contact plate samples after molecular sequencing

4.3.4 NGH ICU bacterial clinical isolates

A total of 22 bacterial species were identified from clinical samples during the period of environmental sampling (11/03/08 – 09/12/08) (Table 4.12). The most common organisms isolated were the same as the RHH (CNS, *E. coli* and *S. aureus*). However, in comparison from 360 clinical isolates 47.8% were Gram-negative pathogens.

4.4 Discussion

HAIs are a continual cause for concern due to high mortality rates and costs to the NHS **(Edbrooke *et al.*, 1999; Dean *et al.*, 2002; Wilcox, 2003; HPA, 2008)**. Extensive research has previously relied upon analysis of clinical isolates to reveal cross-transmission events **(Naiemi *et al.*, 2005; Mammina *et al.*, 2007; Manzur *et al.*, 2007; Khan *et al.*, 2009)** and cultural studies to identify environmental sites harbouring organisms during infection outbreaks **(Bures *et al.*, 2000; Devine *et al.*, 2001; Drees *et al.*, 2008)**. This chapter has investigated the bacterial ecology of the ICU environment during a non-outbreak situation, using a combination of cultural and molecular ecology techniques. To the authors knowledge this is the first example of PCR-DGGE being used in the hospital environment.

In this present study, a number of environmental sites yielded bacteria. Areas of highest bacterial detection were: ICU handwash bottles, ICU ward sink plugholes, ICU floors, HDU patient chairs and sluice room sink plugholes. The same sites had the highest bacterial detection at both the RHH and NGH, indicating that these sites commonly yield bacteria in hospitals. It is widely acknowledged that plugholes and sinks have been associated with outbreak situations **(Bures *et al.*, 2000; Naas *et al.*, 2002; Hota *et al.*, 2009)** (as detailed in Chapter 8).

Table 4.12 Diversity of NGH clinical bacterial isolates and frequency of infected patients

Organism	Number of clinical isolates	Number of infected/colonised patients
Coagulase-negative *Staphylococci* (CNS)	108	57
Escherichia coli	48	33
Staphylococcus aureus	36	21
Enterococcus spp.	29	23
Pseudomonas aeruginosa	27	13
Enterobacter cloacae	16	11
Klebsiella pneumoniae	15	7
Enterobacter aerogenes	11	6
Stenotrophomonas maltophilia	10	5
MRSA	9	8
Proteus mirabilis	9	7
Acinetobacter baumanii	6	2
Klebsiella oxytoca	6	4
Citrobacter freundii	4	4
Citrobacter koseri	4	2
Proteus spp.	4	2
Serratia spp.	4	1
Klebsiella spp.	3	3
Micrococcus spp.	3	2
Peptostreptococcus spp.	3	2
Serratia liquefaciens	3	2
Serratia marcescens	2	2

It is extensively reported that Gram-positive organisms (e.g. CNS) are present in the environment particularly due to hand contact and skin shedding **(Andersson *et al.*, 1999; Kampfer *et al.*, 1999; Tsai and Macher, 2005; Lee *et al.*, 2007; Rintala *et al.*, 2008)**. In the present study, *S. epidermidis* and *Micrococcus* spp. were frequently isolated. However, PCR-DGGE yielded sequences that could be classified as Gram-negative more frequently than those which were clearly Gram-positive.

During this present study a limitation was the inability to precisely identify bacterial species from all DNA sequencing after PCR-DGGE. This was due to unclear sequences and not mis-alignment against bacterial sequences in the GenBank database.

There are also general limitations to PCR-DGGE and primer bias has been well documented **(Muyzer *et al.*, 1993; Muyzer and Smalla, 1998; Ishii and Fukui, 2001)**. There are different types of primer bias, especially from multi-template PCRs: -

- The PCR products to bacterial cells ratio in multi-template PCRs often differ due to the different copy numbers of rDNA in organisms **(Farrelly *et al.*, 1995; Fogel *et al.*, 1999; Klappenbach *et al.*, 2000)**
- Difference in primer binding energies **(Polz *et al.*, 1998)**
- Frequent cycling of template re-annealing **(Suzuki *et al.*, 1996)**
- High GC rich sequences may not be amplified due to low efficiency of template dissociation **(Reysenbach *et al.*, 1992; Muyzer and Smalla, 1998)**

Other limitations to PCR-DGGE are the formation of multiple bands from single genomes after gene amplification **(Brosius *et al.*, 1981; Nubel *et al.*, 1996; Ercolini, 2004)**, co-migration of DNA in the same DGGE band from different species **(Sekiguchi *et al.*, 2001; Speksnijder *et al.*, 2001; Gafan and Spratt, 2005)** and reproducable and

efficient DNA extraction from environmental samples **(Muyzer and Smalla, 1998; Niemi *et al.*, 2001)**.

Recent research by Araujo and Schneider (2008) showed the ability of PCR-DGGE for the identification of important community members from a three member consortium (*E. coli*, *B. cepacia* and *S. maltophilia*) but could not identify the most dominant organisms. It was concluded that PCR-DGGE from template genomic DNA was not appropriate for identifying dominant organisms (and was only marginally improved using template 16S rDNA) but was extremely useful for demonstrating bacterial diversity. Inefficiencies in the PCR reaction are most likely to effect the identification of dominant organisms.

Despite these limitations, PCR-DGGE using genomic DNA as template was necessary to provide knowledge of the bacterial diversity and ecology where samples yielded no culturable bacteria. Using universal PCR-DGGE primers **(Muyzer *et al.*, 1993; Brinkhoff *et al.*, 1998)** enabled a wide variety of bacterial sequences to be retrieved from samples.

During this present study, results from PCR-DGGE identified hospital sinks and floors to have a high bacterial diversity. Particular bacterial communities were shown to persist in hospital sinks and floors over a prolonged period of time. An explanation for this observation could be the presence of biofilms which are commonly associated with sinks **(Davies *et al.*, 1998; Costerton *et al.*, 1999; Conway *et al.*, 2002)**. Alternatively, it could be due to the effect of routine ICU ward cleaning on the bacterial species present in the hospital sinks and on the floor (as detailed in Chapter 7).

The results from PCR-DGGE indicate the potential presence of opportunistic and environmental spp. Several opportunistic spp. (*Enterobacter* spp., *Klebsiella* spp., *Peptostreptococcus* spp., *Pseudomonas aeruginosa*, *Serratia marcescens* and *Stenotrophomonas maltophilia*) which are causative agents of infections, have been

isolated from clinical samples during this study. This implies that ICU environmental sites can harbour organisms that cause disease, however further investigation would be required to isolate these organisms using selective media to identify any potential transmission routes and confirm sequences from the environment.

Burkholderia spp. are common environmental organisms which frequently persist in biofilms **(Conway *et al.*, 2002)**. While their association with sinks has been established in previous work **(O'Toole *et al.*, 2000; Conway *et al.*, 2002; Yoshida *et al.*, 2009)** their association with hard surfaces has not previously been reported in the literature.

There was also a widespread distribution of *S. maltophilia* in the NGH ICU environment. *S. maltophilia* has recently been described as a new 'superbug' **(Batty, 2008)** and is a common cause of nosocomial pneumonia and bacteraemia **(Denton *et al.*, 1998; Micozzi *et al.*, 2000; Hanes *et al.*, 2002)** thus detection of *S. maltophilia* is of potentially great clinical significance. *S. maltophilia* was isolated from clinical samples throughout this study (at both the RHH and NGH).

Identification of *Burkholderia* spp. and *S. maltophilia* in all environmental sites implies the possibility of transmission. Recent research has established the spread of *P. aeruginosa* from hospital sinks onto nearby medical equipment and hospital staff hands **(Brooke, 2008; Hota *et al.*, 2009)**. *Burkholderia* spp. and *S. maltophilia* may have been transferred from the ICU ward sink onto patient chairs and floors during this present study. However, further work would be necessary to determine if the same organism is present in these sites which would require isolation of these organisms and clonal studies (e.g. using PFGE).

Throughout this study samples were taken to establish the microbial ecology of the ICU and HDU environments and care was taken not to influence the normal operation of the

wards. In an effort to remove any potential 'Hawthorne effect', whereby staff may alter their cleaning pattern, samples were taken at different times of the day.

After a review of the data across the 12 sample sessions it was revealed that the detection of eubacterial DNA varied at both the RHH and NGH ICU/HDU between sample sessions. In order to determine whether this routine ICU ward cleaning regime could account for these differences, a study was designed to compare the detection of eubacterial DNA on occasions before and after routine ICU ward cleaning (detailed in Chapter 7).

5. Detection of antibiotic resistance determinants in the ICU and HDU environment

5.1 Background and aims

The aim of this chapter was to characterise environmental reservoirs of antibiotic resistance determinants in the ICU and HDU. Initially MRSA and ESBL-producing organisms were chosen as the focus of this study, because these two resistance determinants represent two of the major molecular mechanisms of resistance in organisms causing HAIs. Although of less clinical significance, tetracycline resistance genes were included in this study to act as markers of antibiotic resistance, as they are commonly detected in the environment.

The objectives were to: -

- detect antibiotic resistance determinants in the ICU and HDU environment by PCR using gene-specific primers for *mec*A, $bla_{CTX\text{-}M,\ SHV,\ TEM}$ and *tet* O, M, W genes (as detailed in materials and methods)
- characterise native and ESBL genes from ICU and HDU environmental swab samples and clinical isolates
- identify bacterial species carrying target genes
- identify the sites of antibiotic resistance determinant detection
- relate the distribution of MRSA and ESBL infected patients and rate of MRSA isolation from clinical samples

5.2 Overview of materials and methods

The RHH and NGH ICU/HDU departments were sampled over 12 sessions (RHH 12/05/08 – 15/12/08 and NGH 11/03/08 – 09/12/08). Only samples which were positive for eubacterial DNA by PCR were tested for antibiotic resistance determinants (RHH = 106 swab samples, NGH = 101 swab samples) (full details of the sampling regimes are provided in Section 2.2 Materials and Methods, swab samples were screened by molecular methods and dipslide samples by cultural methods).

5.3 Results

ICU and HDU at the Royal Hallamshire Hospital

5.3.1 Detection of mecA and bla genes in the RHH environment

No $bla_{CTX\text{-}M}$ or bla_{SHV} genes were detected in DNA from environmental swab samples from the RHH wards (a selection of these negative results are shown in Figure 5.1 and 5.2).

However, bla_{TEM} genes were identified from 11.3% (12/106) of swab samples screened. The sites of detection included the ICU floor, ICU ward sink plughole, HDU ward sink plughole, HDU patient chair and ICU ward sink taps, which had previously shown the highest detection of eubacterial 16S rRNA genes. bla_{TEM} genes were also detected on hard surfaces of low bacterial detection (ICU bedside, ICU computer keyboard and ICU window ledge) (Figure 5.3a/b/c).

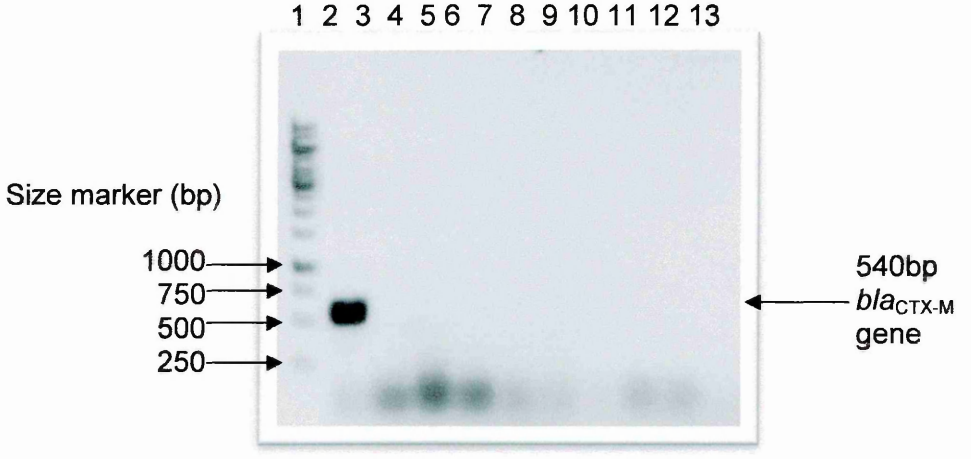


Figure 5.1 bla_{CTX-M} gene detection from RHH environmental swab samples

Lane 1 marker, lane 2 *E. coli* CTX-M positive control, lane 3 no DNA template control, lanes 4-13 PCRs from template DNA purified from swab samples (sites – ICU floor, ICU trolley, ICU ward sink plughole, ICU floor, ICU computer keyboard, ICU ward sink taps, ICU fan and HDU poster respectively)

(Using bla_{CTX-M} gene-specific primers as detailed in the materials and methods)

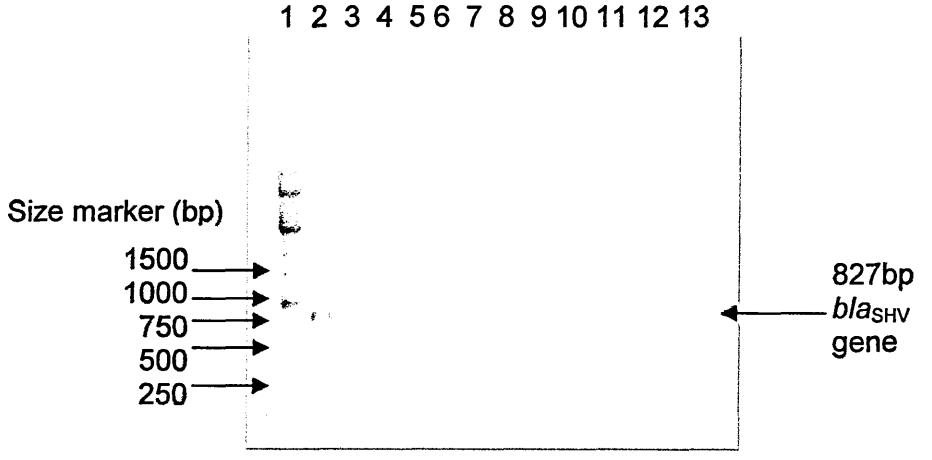
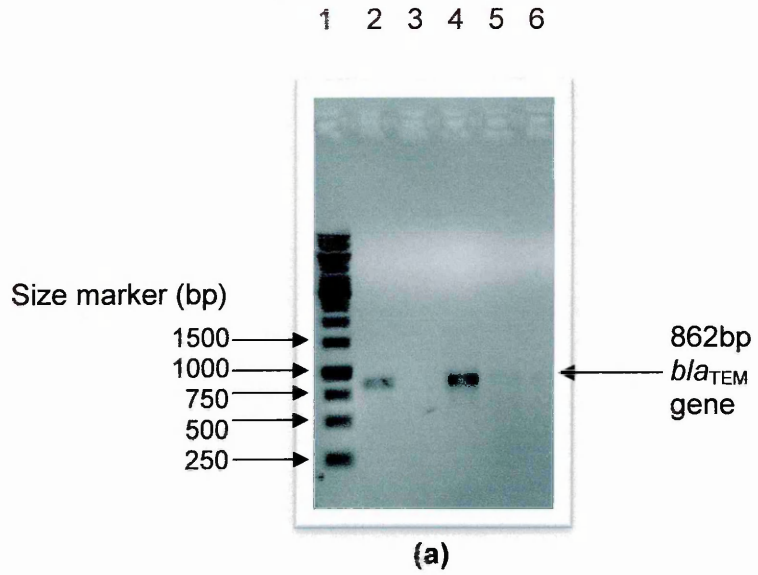


Figure 5.2 bla_{SHV} gene detection from RHH environmental swab samples

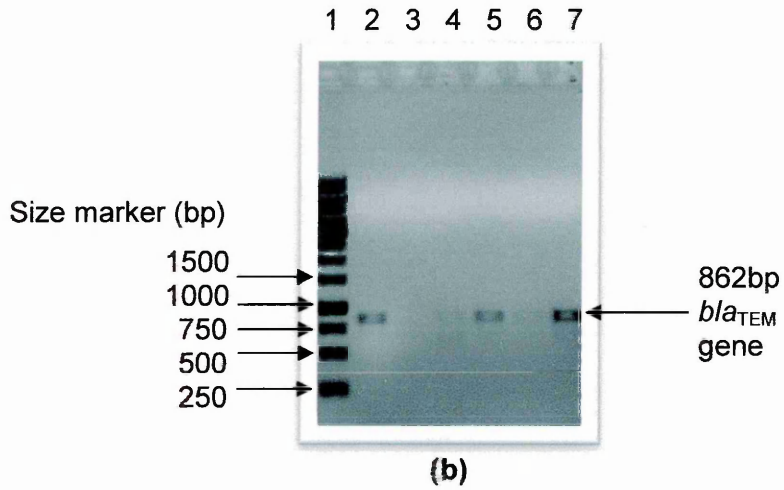
Lane 1 marker, lane 2 *E. coli* SHV-2 positive control, lane 3 no DNA template control, lanes 4-13 PCRs from template DNA purified from swab samples (sites – ICU computer keyboard, ICU ward sink taps, sluice room sink plughole, HDU ward sink plughole, HDU poster, HDU computer stand and HDU patient chair x2 respectively) (Using bla_{SHV} gene-specific primers as detailed in the materials and methods)

Figure 5.3 Amplification of *bla*$_{TEM}$ from RHH environmental swab samples



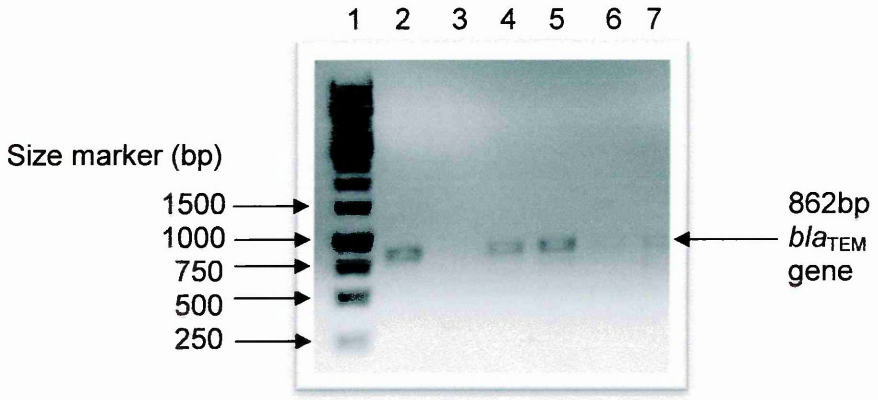
(a)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4-6 PCR products from template DNA purified from swab samples (sites – ICU bedside, ICU ward sink plughole and ICU computer keyboard respectively) (Using *bla*$_{TEM}$ gene-specific primers detailed in the materials and methods)



(b)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4-7 PCR products from template DNA purified from swab samples (sites – ICU ward sink taps, HDU ward sink plughole and HDU chair respectively) (Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)



(c)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4-7 PCR products from template DNA purified from swab samples (sites – ICU floor, ICU bedside, ICU window ledge and ICU computer keyboard respectively) (Using bla_{TEM} gene-specific primers as detailed in the materials and methods)

Interestingly, bla_{TEM} genes were detected on five consecutive sampling sessions (13/10/08 - 08/12/08) although from different sites. However, bla_{TEM} genes were only detected from one site (ICU ward sink taps) on consecutive sampling sessions (Table 5.1).

DNA sequencing was used to characterise native and ESBL TEM genes amplified directly from environmental samples. All positive amplicons were identified as native TEM beta-lactamases and not ESBLs (Table 5.1).

No organisms carrying bla_{TEM} genes could be identified from bacterial isolates from bla_{TEM} positive swab samples.

One of 106 DNA samples (from the ICU ward sink plughole) was positive for *mec*A (0.94%) (Figure 5.4). No *mec*A gene-carrying organisms could be identified from bacterial isolates from the ICU ward sink plughole swab sample.

A *mec*A positive coagulase-negative *Staphylococcus* was cultured (confirmed by Gram stain, coagulase test and culturing on MRSA selective media) from the ICU handwash bottle (previously identified as a site of high bacterial isolation) using dipslides (Figure 5.5).

To confirm the amplified products were *mec*A, they were sequenced. Both amplicons were confirmed to be altered PBP, PBP2a (*mec*A gene) commonly associated with MRSA (Table 5.1). Interestingly, MRSA infected patients were present on the ward during the sampling sessions positive for *mec*A gene detection (02/06/08 and 13/10/08) (Table 5.1).

Table 5.1 Distribution and classification of *mec*A, *bla* and *tet* genes in the RHH ICU/HDU environment (continued over page)

Sample site	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Bedside (ICU)							TEM-1 Native[3] 7 Tet colonies[4] 2 Tet(M) 1 Tet(O)			TEM-1 Native[3]	-	
Computer keyboard (ICU)	-				-		S21 Native[3]			TEM-1 Native[3]		-
Fan (ICU)						-	-		-	-		-
Floor (ICU)	-			-	1 Tet colony[4]	11 Tet colonies[4]	2 Tet colonies[4] 1 Tet(O)	-	-	Bt48 Native[3] 1 Tet colony[4]	-	-
HDU computer keyboard	-				-	-	-		-			
HDU computer stand	-				-				-	1 Tet colony[4]		-
HDU patient chair	-	-		-	-	-	1 Tet colony[4]		TEM-1 Native[3]	-	-	-
HDU picture	-				-	-	-			-		-
HDU plughole											TEM-1 Native[3]	
HDU staff chair	-					3 Tet colonies[4]	1 Tet colony[4]		-	-	-	-
Plughole (ICU ward sink)	-	-	-	-		-	PBP2a[1] TEM-1 Native[3] 2 Tet colonies[4]	1 Tet colony[4] 1 Tet(W)	TEM-1 Native[3] 2 Tet colonies[4]	1 Tet colony[4] 1 Tet(W)	-	2 Tet colonies[4]

Sample site	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Tap (ICU)	-				3 Tet colonies[4]	1 Tet colony[4]	-	TEM-1 Native[3]	TEM-1 Native[3] 1 Tet(M) colony[4]			
Trolley (ICU)							-					-
Sluice room sink plughole	Tet(O)			-	-	-	-		-			
Window ledge (ICU)						1 Tet colony[4]		1 Tet colony[4]		TEM-1 Native[3]		-
Door handle dipslide (ICU)												
Handwash bottle dipslide (ICU)		PBP2a[1] CNS[2]										
HDU handwash bottle dipslide												
Light switch dipslide (ICU)												

[1] PBP2a is the altered penicillin binding protein encoded by the *mecA* gene responsible for the MRSA phenotype

[2] Coagulase-negative *Staphylococcus* (CNS) was identified as the gene-carrying organism

[3] Native beta-lactamase genes were identified not ESBLs

[4] Number of tetracycline resistant colonies cultured from environmental swab samples

- Negative samples

Blank boxes = sample not screened for antibiotic resistance determinants

Background colour:- Blue MRSA infected patient on the ward

Purple *E. coli* or *Klebsiella* spp. carrying native TEM beta-lactamase genes identified from clinical isolates

Red MRSA infected patient on the ward and *E. coli* or *Klebsiella* spp. carrying native TEM beta-lactamase genes identified from clinical isolate

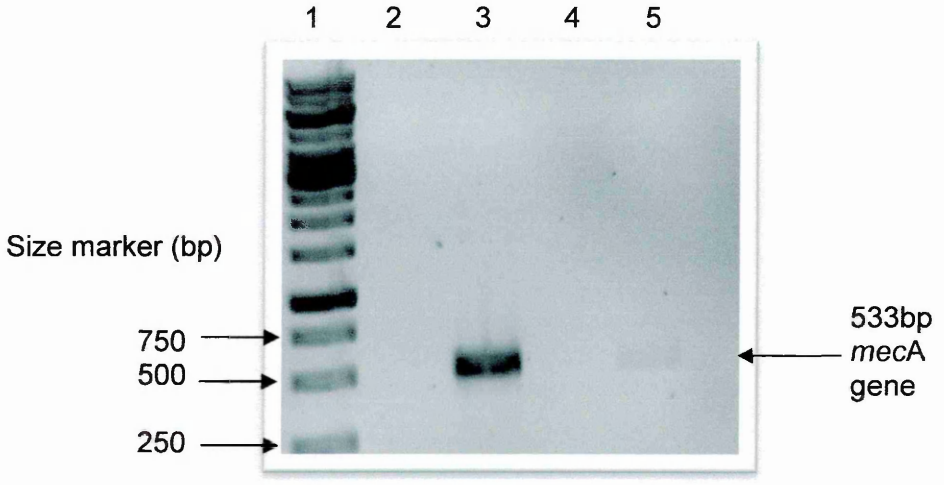


Figure 5.4 Amplification of *mecA* gene from swab sample of ICU ward sink plughole RHH

Lane 1 marker, lane 2 no DNA template control, lane 3 MRSA positive control, lane 4 no DNA template control, lane 5 PCR product from template DNA purified from swab sample of ICU ward sink plughole

(Using *mecA* gene-specific primers as detailed in the materials and methods)

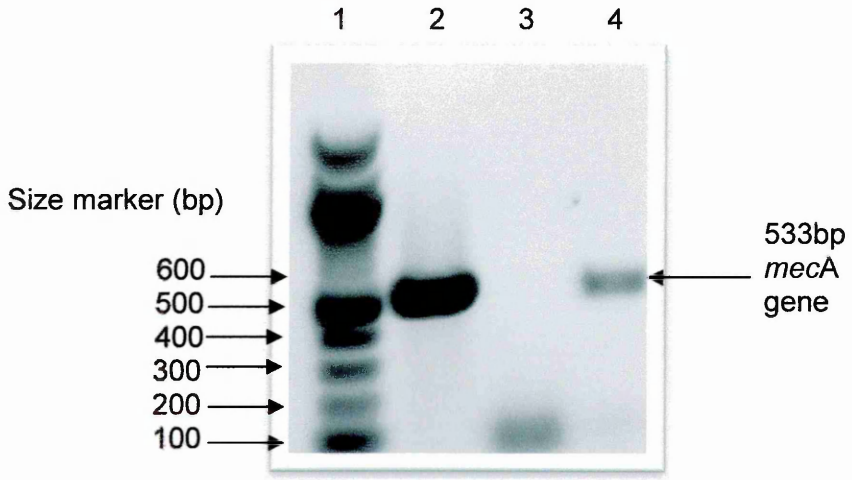


Figure 5.5 Amplification of *mec*A gene from cultured bacterium of ICU handwash bottle dipslide sample RHH

Lane 1 marker, lane 2 MRSA positive control, lane 3 no DNA template control, lane 4 PCR product from template DNA purified from cultured bacterium of ICU handwash bottle dipslide sample

(Using *mec*A gene-specific primers as detailed in the materials and methods)

5.3.2 Rate of MRSA and ESBL isolation from clinical samples at RHH

From 181 Gram-positive clinical isolates cultured from routine patient samples on the ICU by the clinical microbiology staff, only 14 MRSA isolates were identified. Only eight individual patients had an MRSA infection, totalling 40 patient days. MRSA infected patients were present on the ward during sampling sessions 2-3 (02/06/08 – 30/06/08), 6-7 (29/09/08 – 13/10/08), 10 (01/12/08) and 12 (15/12/08) (Table 5.1).

Routinely, Gram-negative clinical isolates are screened for ESBLs phenotypically at the RHH (**Derbyshire *et al.*, 2009**); during this study, there were 222 Gram-negative clinical isolates. Using gene-specific PCR, $bla_{CTX\text{-}M}$ genes were detected from two ESBL-producing clinical isolates (*E. coli* and *K. pneumoniae*) (Figure 5.6) from two separate patients. These patients were not on the ward on the date of sampling.

Following the detection of native bla_{TEM} genes in the RHH environment, all (20) *Klebsiella* spp. and *E. coli* clinical isolates were screened for the presence of native bla_{TEM} genes. Native bla_{TEM} genes were identified from eight clinical isolates (Figure 5.7a/b). These organisms were isolated during sampling sessions 1-4 (12/05/08 – 14/07/08), 7 (13/10/08) and 10 (01/12/08) (Table 5.1).

5.3.3 Tet gene detection in the RHH environment

PCR was used to amplify *tet*(O, M and W) genes directly from environmental swab samples. From the first four sample sessions (12/05/08 – 14/07/08), only *tet* O was detected on one occasion from the sluice room sink plughole swab sample by PCR (Figure 5.8). No *tet* M or W genes were detected from any environmental swab samples by PCR (a few of these negative results are shown in Figure 5.9 and 5.10).

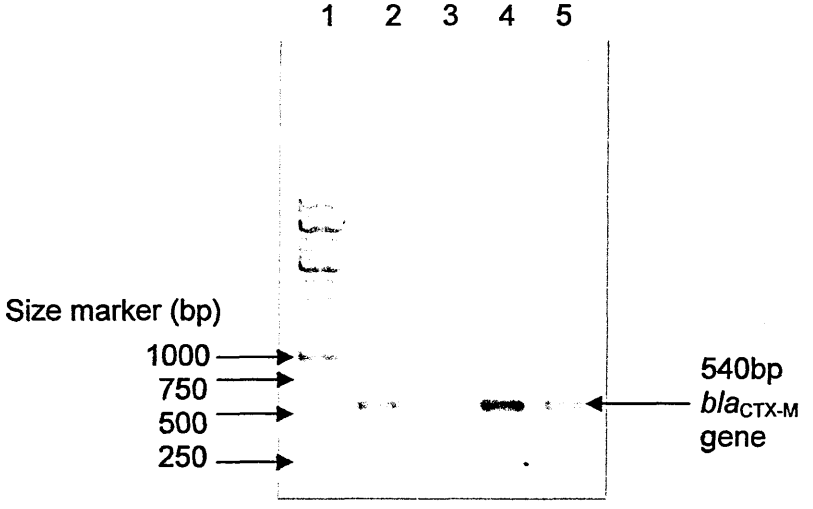
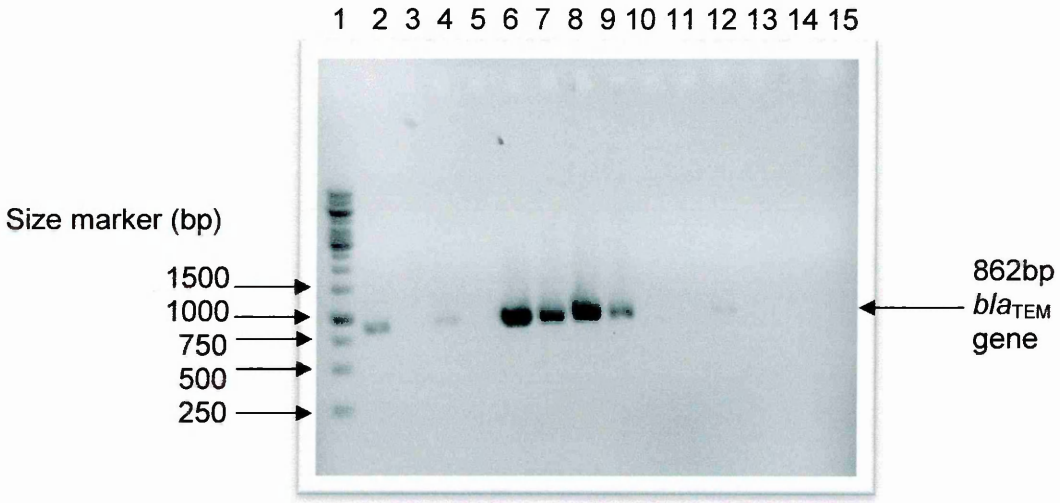


Figure 5.6 Detection of CTX-M ESBL from two RHH clinical isolates

Lane 1 marker, lane 2 *E. coli* CTX-M positive control, lane 3 no DNA template control, lane 4 PCR product from template DNA purified from cultured *Escherichia coli* clinical isolate, lane 5 positive PCR product from template DNA purified from cultured *Klebsiella pneumoniae* clinical isolate

(Using bla_{CTX-M} gene-specific primers as detailed in materials and methods)

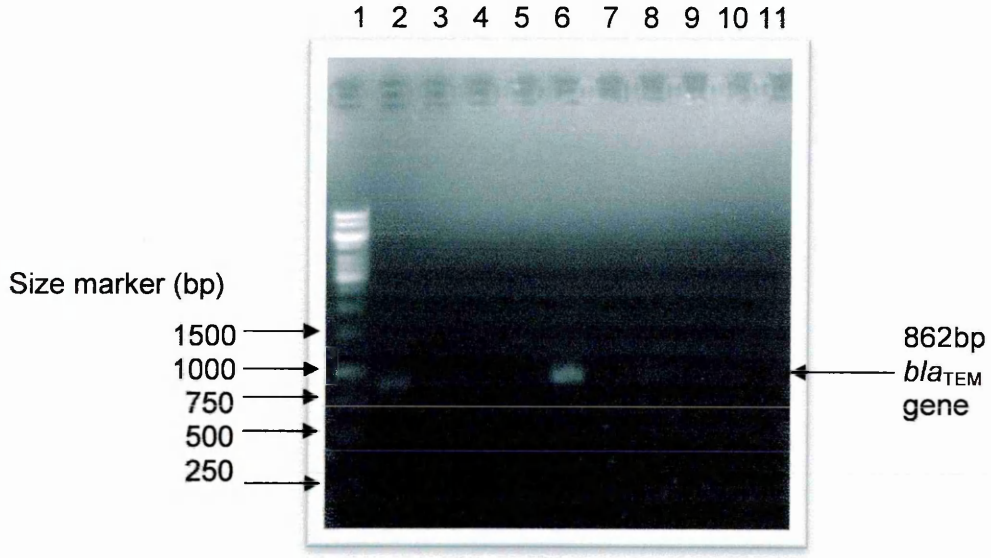
Figure 5.7 *bla*$_{TEM}$ gene detection from RHH clinical isolates



(a)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4, 6-9, 12 PCR products from template DNA purified from cultured *Klebsiella* spp. and *E. coli* clinical isolates

(Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)



(b)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 6, 8 PCR products from template DNA purified from cultured *Klebsiella* spp. and *E. coli* clinical isolates

(Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)

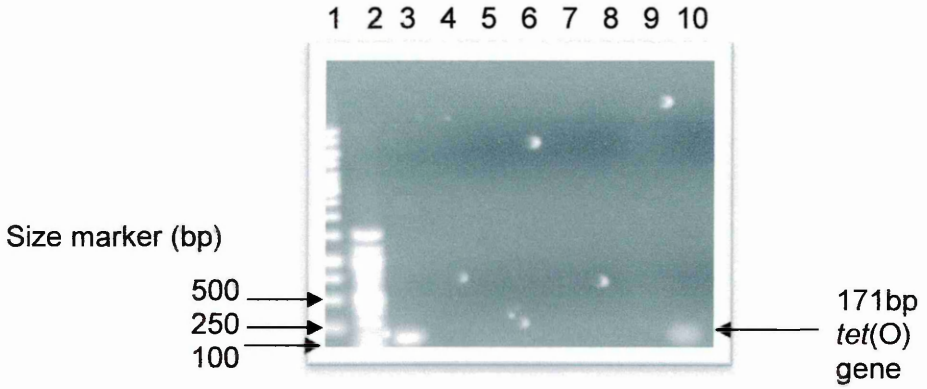


Figure 5.8 *tet*(O) gene detection from RHH environmental swab samples

Lane 1-2 markers, lane 3 *E. coli tet* O positive control, lane 4 no DNA template control, lanes 5-9 PCRs from template DNA purified from swab samples (sites – ICU floor, ICU computer keyboard, ICU ward sink tap and ICU picture respectively), lane 10 PCR product from template DNA purified from swab sample of sluice room sink plughole (Using *tet*O gene-specific primers as detailed in the materials and methods)

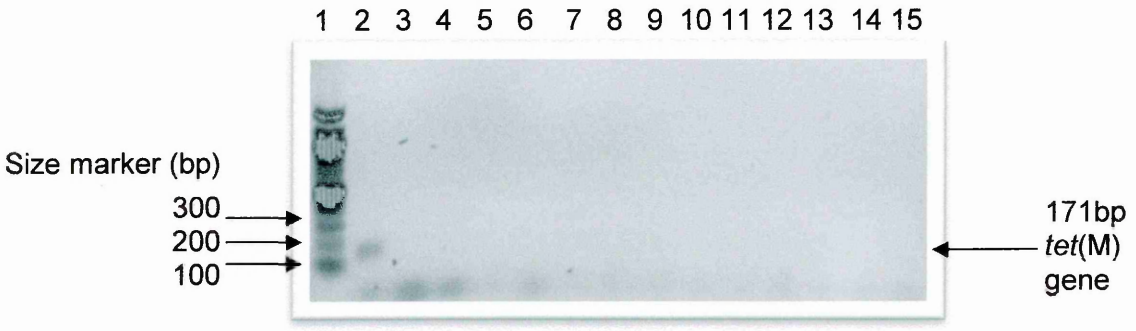


Figure 5.9 *tet*(M) gene detection from RHH environmental swab samples

Lane 1 marker, lane 2 *Bacillus subtilis tet* M positive control, lane 3 no DNA template control, lanes 4-15 PCRs products from colony picks from environmental dipslide and contact plate samples (HDU handwash bottle x2, ICU handwash bottle x4, ICU door handle, HDU patient chair x2, ICU curtain x3 and HDU curtain respectively) (Using *tet*M gene-specific primers as detailed in the materials and methods)

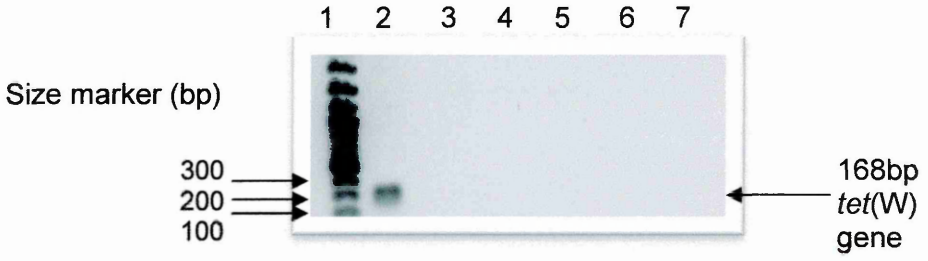


Figure 5.10 *tet*(W) gene detection from RHH environmental swab samples

Lane 1 marker, lane 2 *E. coli tet* W positive control, lane 3 no DNA template control, lanes 4-7 PCRs from template DNA purified from swab samples (sites – ICU ward sink plughole, HDU patient chair, ICU ward sink plughole x2 and ICU floor respectively) (Using *tet*W gene-specific primers as detailed in the materials and methods)

Due to the low detection of *tet* genes by PCR, a cultural method was introduced during the last eight sessions (15/09/08 – 15/12/08), during which period 49 tetracycline resistant colonies were cultured from swab samples. Tetracyline resistant colonies were cultured most frequently from the ICU ward sink plughole, ICU floor and ICU bedside trolley (Table 5.1). *tet*(M) was identified from three tetracycline resistant colonies, *tet*(O) from two and *tet*(W) from two. There were 42 tetracycline resistant colonies that did not possess any of the *tet* genes tested for, and so were presumed to carry other *tet* resistance determinants (Table 5.1).

ICU and HDU at the Northern General Hospital

5.3.4 Detection of mecA and bla genes in the NGH environment

Similar to the RHH, no $bla_{CTX\text{-}M}$ or bla_{SHV} genes were detected in DNA from environmental swab samples, however, no *mec*A genes were identified from either swab or dipslide samples (a small number of these negative results are shown in Figure 5.11, 5.12, 5.13).

A similar proportion of swab samples yielded bla_{TEM} genes (10.9%). The sites of detection were similar to the RHH; the ICU floor-ward, ICU ward sink plughole, sluice room sink plughole and ICU patient chair. Other sites were hard surfaces of low bacterial detection; the ICU computer keyboard, ICU machine handle, ICU door handle and ICU window ledge (Figure 5.14a/b).

In contrast to the RHH, bla_{TEM} genes were detected on two non-consecutive sessions (15/07/08 and 04/11/08). Interestingly, on session 10 (04/11/08) bla_{TEM} genes were detected in swab samples from five different sites; the ICU floor, two from the sluice

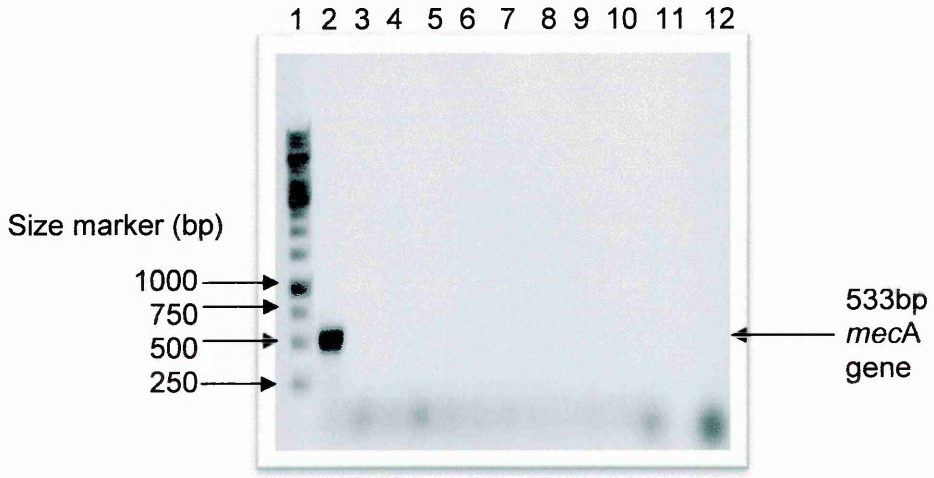


Figure 5.11 *mec*A gene detection from NGH environmental swab samples

Lane 1 marker, lane 2 MRSA positive control, lane 3 no DNA template control, lanes 4-12 PCRs from template DNA purified from swab samples (sites – ICU machine handle, ICU computer keyboard, ICU window ledge, ICU door handle, ICU floor and sluice room sink plughole respectively)

(Using *mec*A gene-specific primers as detailed in the materials and methods)

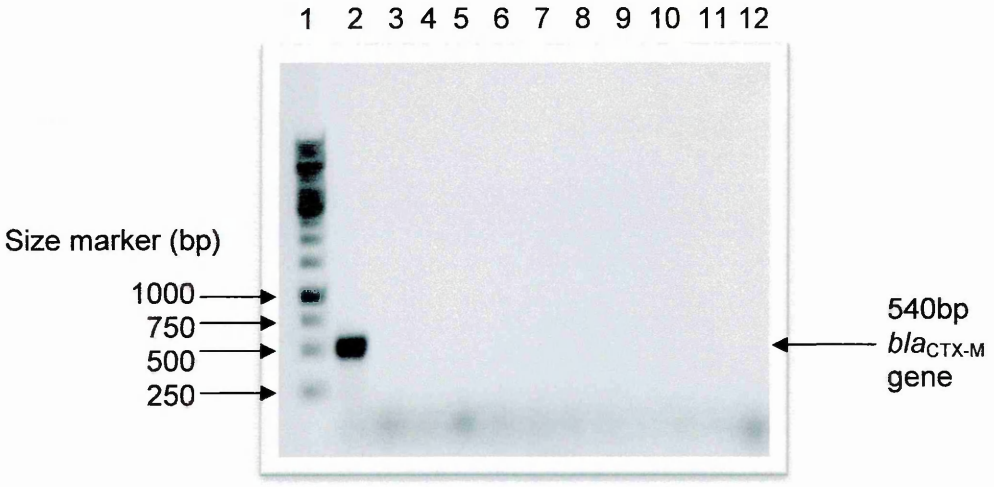


Figure 5.12 *bla*$_{CTX-M}$ gene detection from NGH environmental swab samples

Lane 1 marker, lane 2 *E. coli* CTX-M positive control, lane 3 no DNA template control, lanes 4-12 PCRs from template DNA purified from swab samples (sites – ICU machine handle, ICU computer keyboard, ICU window ledge, ICU door handle, ICU floor and sluice room sink plughole respectively)

(Using *bla*$_{CTX-M}$ gene-specific primers as detailed in the materials and methods)

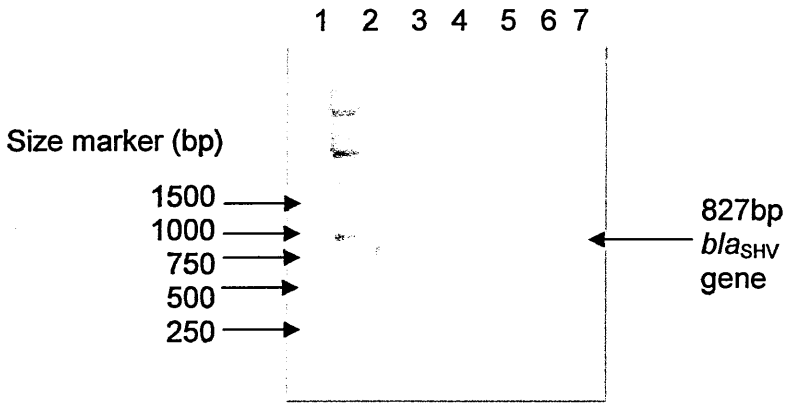
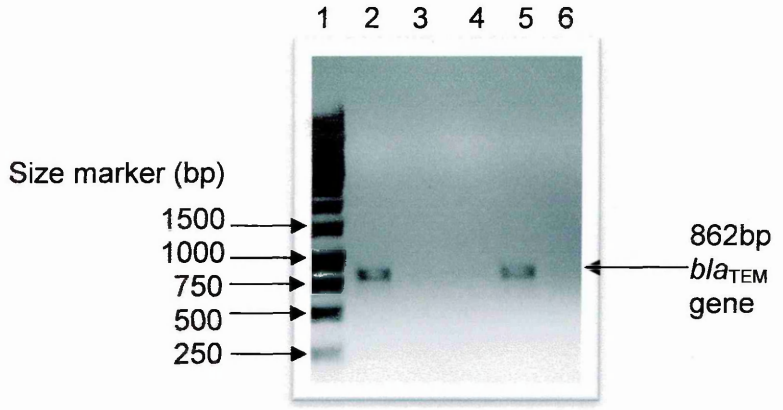


Figure 5.13 *bla*$_{SHV}$ gene detection from NGH environmental swab samples

Lane 1 marker, lane 2 *E. coli* SHV-2 positive control, lane 3 no DNA template control, lanes 4-7 PCRs from template DNA purified from swab samples (site – ICU patient chair, ICU floors and sluice room sink plughole respectively)

(Using *bla*$_{SHV}$ gene-specific primers as detailed in the materials and methods)

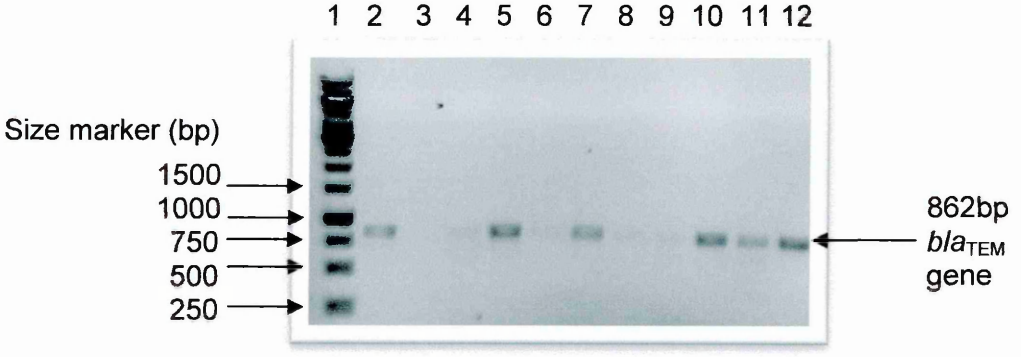
Figure 5.14 *bla*$_{TEM}$ gene detection from NGH environmental swab samples



(a)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4-6 PCR products from template DNA purified from swab samples (ICU ward sink plughole and ICU patient chairs respectively)

(Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)



(b)

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 4-12 PCR products from template DNA purified from swab samples (sites – ICU machine handles, ICU computer keyboard, ICU window ledge, ICU door handle, ICU floors-ward, ICU ward sink plughole respectively)

(Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)

room plughole, left and right ICU machine handles, ICU computer keyboard and ICU window ledge (Table 5.2).

Characterisation of native and ESBL bla_{TEM} genes amplified during all 12 sampling sessions revealed that 10 out of 11 amplicons were native bla_{TEM} and one was a TEM-116 ESBL (from session 10 04/11/08) (Table 5.2). No bla_{TEM} gene-carrying organisms were identified from isolated bacteria cultured from swab samples.

5.3.5 Rate of MRSA and ESBL isolation from clinical samples at NGH

The rate of MRSA isolation from clinical samples was similar to the RHH. From 188 Gram-positive clinical isolates (cultured routinely by clinical microbiologists), only nine MRSA isolates were identified. In total five MRSA infected patients were present on the ward during sampling; sessions 5 (01/07/08), 7-8 (16/09/08 – 30/09/08) and 10 (04/11/08) (Table 5.2).

A similarly low proportion of ESBL-producing Gram-negatives were identified from NGH clinical isolates (1/172). The ESBL-producing *E. coli* was identified as a CTX-M ESBL by gene-specific PCR (Figure 5.15). This patient was on the ward for one day (02/11/08) and was present two days prior to sampling session 10 (04/11/08).

After detecting native bla_{TEM} genes in the NGH environment, similar to the RHH, three clinical isolates were carrying native bla_{TEM} genes (from 11 *Klebsiella* spp. and *E. coli* clinical isolates) (Figure 5.16). They were isolated during sampling sessions 7-8 (16/09/08 - 30/09/08) and 10 (04/11/08) (Table 5.2).

Table 5.2 Distribution and classification of *mec*A, *bla* and *tet* genes in the NGH ICU/HDU environment (continued over page)

Sample	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10*	18/11/08 Sample session 11	09/12/08 Sample session 12
Computer keyboard (ICU)			-		-					TEM-116 ESBL[2]		
Door handle (ICU)			-			-		-	-	-	1 Tet colony[3]	-
Floor - isolation room (ICU)		-	-	-				-	2 Tet colonies[3] 2 Tet(W)		-	-
Floor-ward (ICU)	-		-		-	-	-	-		TEM-1 Native[1]	-	6 Tet colonies[3] 1 Tet(M)
Floor-ward (ICU)	-		-		-	-	-	-		TEM-1 Native[1]	-	-
HDU computer keyboard		-	-					-			-	-
HDU door switch		-	-					-				-
HDU floor		-						-			-	-
HDU plughole		-	-						-			-
Machine handle (ICU)			-			-		-	-	TEM-1 Native[1]		
Machine handle (ICU)			-			-		-	-	TEM-1 Native[1]		
Patient chair (ICU)		-	-		-	TEM-1 Native[1]	-	-	-	1 Tet colony[3]	-	-
Patient chair (ICU)		-	-		-	TEM-1 Native[1]	-	-	-		-	-
Plughole (ICU ward sink)	-	-	-	-		TEM-1 Native[1]		-	-		-	-

Sample	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10*	18/11/08 Sample session 11	09/12/08 Sample session 12
Sluice room sink plughole	-	-	-		-	-	-		-	TEM-168 Native[1]		-
Sluice room sink plughole	-	-	-		-	-	-		-	TEM-168 Native[1]		-
Window ledge (ICU)			-					-		S21 Native[1]		

[1] Native beta-lactamase genes were identified not ESBLs

[2] ESBL gene identified

[3] Number of tetracycline resistant colonies cultured from environmental swab samples

* Clinical isolate identified as a CTX-M ESBL-producing organism (02/11/08)

- Negative samples

Blank boxes = sample not screened for antibiotic resistance determinants

Background colour: -

Blue = MRSA infected patient on the ward

Purple = *E. coli* or *Klebsiella* spp. carrying native TEM beta-lactamase genes identified from clinical isolates and MRSA infected patient on the ward

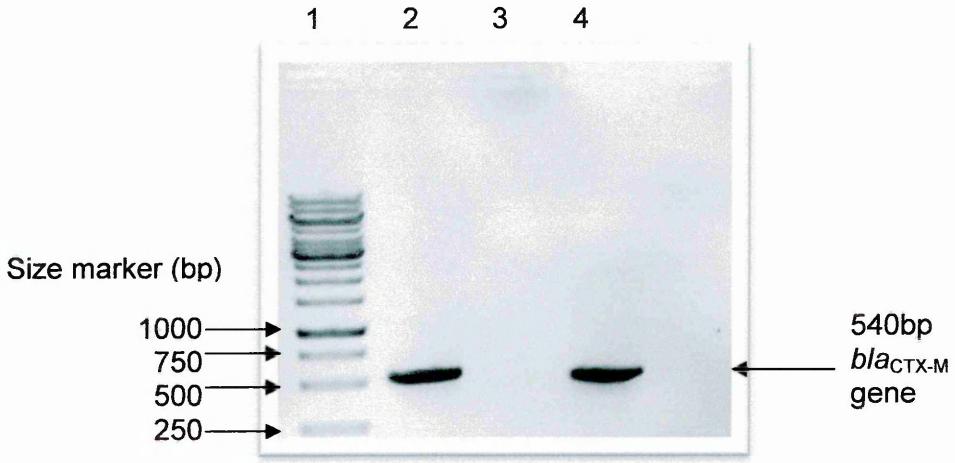


Figure 5.15 Detection of CTX-M ESBL from NGH *E. coli* clinical isolate

Lane 1 marker, lane 2 *E. coli* CTX-M positive control, lane 3 no DNA template control, lane 4 PCR product from template DNA purified from cultured *E. coli* clinical isolate (Using bla_{CTX-M} gene-specific primers as detailed in the materials and methods)

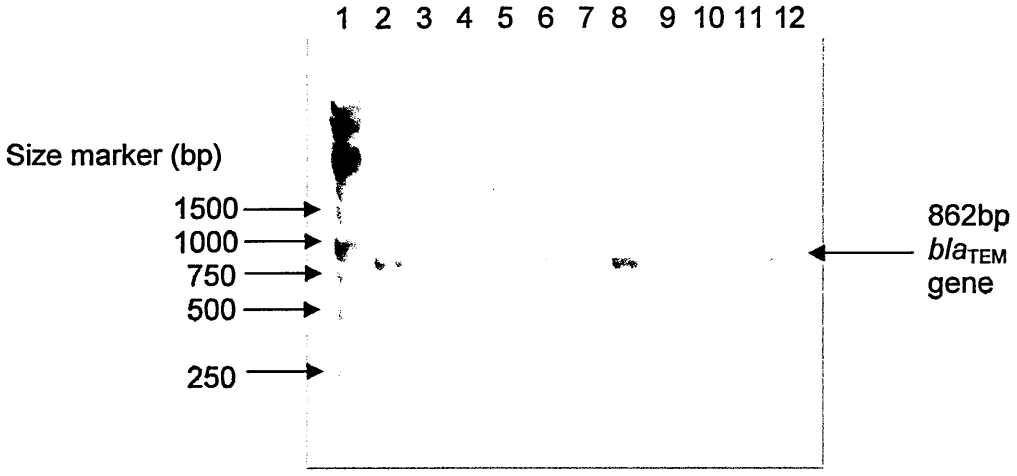


Figure 5.16 *bla*$_{TEM}$ gene detection from NGH clinical isolates

Lane 1 marker, lane 2 *E. coli* TEM positive control, lane 3 no DNA template control, lanes 6, 8, 12 PCR products from template DNA purified from cultured *Klebsiella* spp. and *E. coli* clinical isolates

(Using *bla*$_{TEM}$ gene-specific primers as detailed in the materials and methods)

5.3.6 Detection of tet genes in the NGH environment

In contrast to the RHH, from the first seven sessions (11/03/08 – 16/09/08) no *tet* M, O or W genes were identified from environmental swab samples by PCR (some of these negative results are shown in Figure 5.17, 5.18 and 5.19).

From the last five sampling sessions (30/09/08 – 09/12/08) 13 tetracycline resistant colonies were cultured from swab samples, which was much lower than the RHH. However, the tetracycline resistant colonies were most frequently cultured from the same environmental sites; ICU floors (isolation rooms and main bed ward). *tet*(W) was identified from two tetracycline resistant colonies and *tet*(M) from one; similar to the RHH, a proportion of colonies (10) were carrying different *tet* resistance genes (Table 5.2).

5.4 Discussion

The impact of nosocomial infections caused by antibiotic resistant organisms has been high on the political and public agenda. Previous culturing studies have identified MRSA and ESBL-producing organisms in clinical samples and the ICU environment during infection outbreaks **(Naas *et al.*, 2002; Naiemi *et al.*, 2005; Manzur *et al.*, 2007; Khan *et al.*, 2009)**. In this present study, antibiotic resistance determinants were detected in the ICU and HDU environments at a low frequency.

Antibiotics are extensively used to treat patients on ICUs. It is known that antibiotic resistance determinants (particularly *tet* genes) are commonly found in the environment due to the extensive use of antibiotics in human/animal therapy and agriculture **(Aminov *et al.*, 2001; Chee-Sanford *et al.*, 2001; Bryan *et al.*, 2004; Borjesson *et***

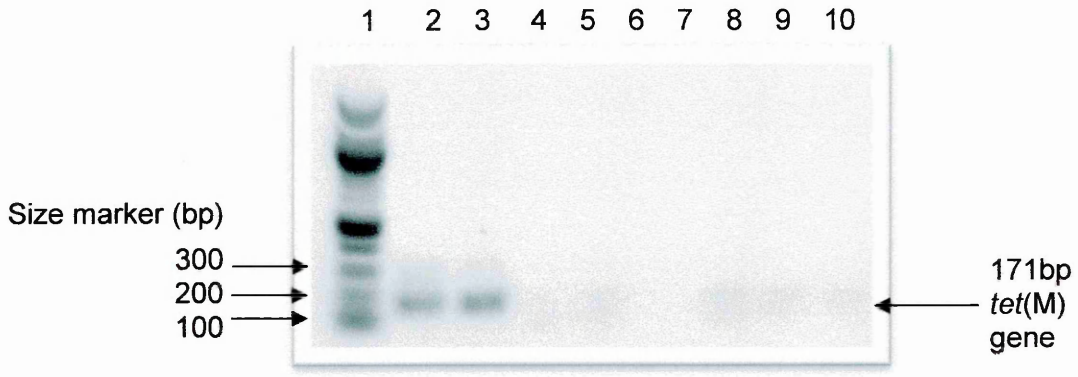


Figure 5.17 *tet*(M) gene detection from NGH environmental swab samples

Lane 1 marker, lane 2 and 3 *Bacillus subtilis tet* M positive control, lane 4 no DNA template control, lane 5-10 PCRs from template DNA purified from swab samples (site – ICU ward sink plughole, ICU patient chair, ICU computer keyboard, ICU floor-ward and sluice room sink plughole x2 respectively)

(Using *tet*M gene-specific primers as detailed in the materials and methods)

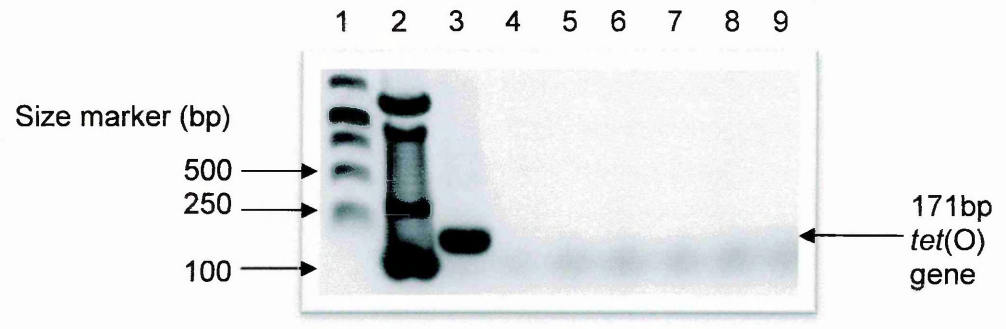


Figure 5.18 *tet*(O) gene detection from NGH environmental swab samples

Lane 1-2 markers, lane 3 *E. coli tet* O positive control, lane 4 no DNA template control, lanes 5-9 PCRs from template DNA purified from swab samples (site – ICU ward sink plughole, ICU floor-ward x2 and sluice room sink plughole x2 respectively) (Using *tet*O gene-specific primers as detailed in the materials and methods)

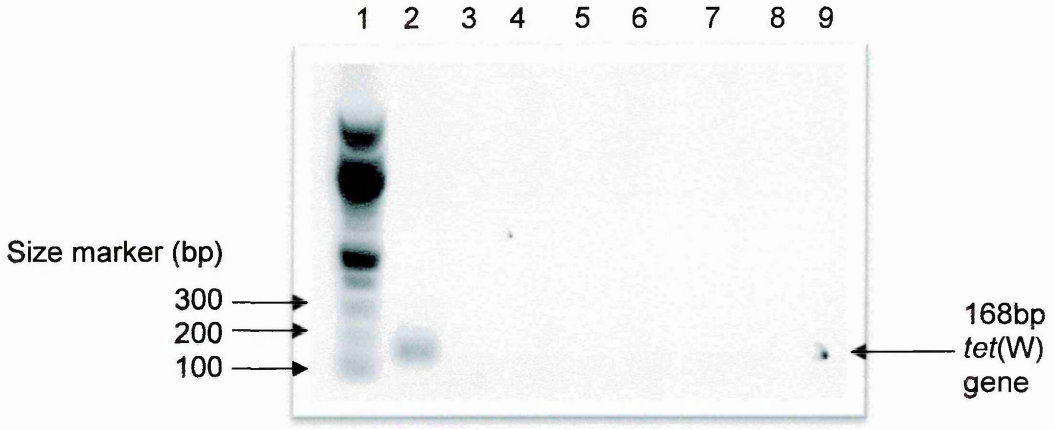


Figure 5.19 *tet*(W) gene detection from NGH environmental swab samples

Lane 1 marker, lane 2 *E. coli tet* W positive control, lane 3 no DNA template control, lanes 4-9 PCRs from template DNA purified from swab samples (site – ICU ward sink plughole, ICU patient chair, ICU computer keyboard, ICU floor-ward, sluice room sink plughole x2 respectively)

(Using *tet*W gene-specific primers as detailed in the materials and methods)

al., **2009; Martinez, 2009)**. Previous research using culture methods has shown the detection of MRSA and ESBL-producing organisms in the ICU environment **(Naas *et al.*, 2002; Naiemi *et al.*, 2005; Manzur *et al.*, 2007; Khan *et al.*, 2009)**. However, from this present study *mec*A and bla_{TEM} genes were detected in low numbers from the ICU and HDU environments.

During this study, PCR was used to detect antibiotic resistance determinants. There are limitations to the use of molecular techniques for detecting antibiotic resistance determinants. Single bacterial cells or single copies of antibiotic resistance genes can not be harvested from swab samples and thus the determinants would not be amplified by PCR **(Bartlett and Stirling, 2003)**. However, in this present study a PCR approach enabled antibiotic resistance determinants to be detected in the absence of the host organism. This was of great importance when samples did not yield culturable bacteria.

In contrast to culture-based studies **(Shiomori *et al.*, 2002; NHS Estates, 2003; Howie and Ridley, 2008; Khan *et al.*, 2009)** there was a low frequency of *mec*A genes detected in the ICU environment even in the presence of MRSA infected patients during this present study. Although the *mec*A gene is commonly associated with *S. aureus* **(McKeegan *et al.*, 2002)**, in this present study the two *mec*A-gene carrying organisms were CNS. Methicillin resistant CNS have been reported in the literature and are common commensal organisms **(Suzuki *et al.*, 1992; Hussain *et al.*, 2000; Schulin and Voss, 2001; Tee *et al.*, 2003)**. Although during this present study the frequency of *mec*A gene detection was low, CNS were isolated by culturing.

There is a wide distribution of the *mec*A gene among CNS species **(Suzuki *et al.*, 1992; Kobayashi *et al.*, 1994; Hanssen *et al.*, 2004)**. Previously two hypotheses have been put forward for why CNS carry the *mec*A gene:-

1 – The *mec*A gene has been inherited by CNS and *S. aureus* from a common ancestor cell

2 – The *mec*A gene has been transferred between *Staphylococci* species **(Suzuki *et al.*, 1992)**

Only 17 ICU and HDU patients were found to be colonised or infected with MRSA during this study and the Sheffield Teaching Hospitals NHS Foundation Trust as a whole have seen only low numbers of MRSA bacteraemias compared with other specialist trusts **(HPA, 2006a)**. The Sheffield Trust had 103 MRSA bacteraemias in a one year period between April 2004 and March 2005, which when compared with the Leeds Trust (200 cases), Brighton and Sussex Trust (129) and Cambridge Trust (123), is relatively low **(HPA, 2006a)**. These data suggest that MRSA is seldom found in the ICU and HDU ward environments at the Sheffield Teaching Hospitals and this may account for the low numbers of MRSA infected patients.

Interestingly, all bla_{TEM} genes detected in the ICU and HDU environment were native beta-lactamases not ESBLs, except one (TEM-116 from the NGH ICU). The number of ESBL-producing organisms isolated from clinical samples was low, however they were all CTX-M, the most common ESBL in the general hospital setting **(Bonnedahl *et al.*, 2009)**. The low rate of ESBLs and MRSA may in part be due to the high standard of infection control in operation within these two ICU departments. Strict control measures have been shown in other studies to halt the spread of ESBL-producing organisms **(Naiemi *et al.*, 2005; Mammina *et al.*, 2007)**.

To the author's knowledge, this is the only study to report native bla_{TEM} genes in the ICU environment and clinical isolates during the same time period. The possibility cannot be excluded that wild-type bla_{TEM} genes were detected in previous studies but were not reported since they may not have been perceived as a clinical priority. Although the frequency of antibiotic resistance determinant detection was low, there

was a widespread distribution with *bla*$_{TEM}$ genes being detected in various samples. Gram-negative organisms (including Enterobacteriaceae) frequently carry beta-lactam resistance genes (e.g. native *bla*$_{TEM}$ genes, TEM-1 and TEM-2) **(Tristram *et al.*, 2005)**. However, detection of native *bla*$_{TEM}$ in clinical isolates is of clinical significance as organisms are not routinely screened for native beta-lactamases and there is the potential for ESBL conversion under antibiotic selective pressure **(Hammond *et al.*, 2005; Paterson and Bonomo, 2005; Pfaller and Segreti, 2006)**.

During this present study *Klebsiella* spp. and *E. coli* ICU clinical isolates were screened for *bla*$_{TEM}$ genes. Future work could be carried out to screen other Gram-negative clinical isolates for *bla*$_{TEM}$ genes and isolate *bla*$_{TEM}$ gene-carrying organisms from the ICU environment. This would enable clonal work to be carried out to identify if the same *bla*$_{TEM}$ gene-carrying organism is present in the ICU environment and clinical isolates, which could indicate transmission between the environment and patients (or *vice versa*).

Tetracycline resistance genes are commonly found in the environment e.g. in soil and water **(Aminov *et al.*, 2001; Chee-Sanford *et al.*, 2001; Bryan *et al.*, 2004)**, therefore they were used in this present study as an environmental marker for antibiotic resistance. During this study a number of tetracycline resistant colonies were cultured, indicating the ICU and HDU environment does yield common antibiotic resistance determinants.

The detection of antibiotic resistance determinants in the ICU and HDU environment was investigated. Low frequencies of *bla*$_{TEM}$ and *mec*A genes were detected in the ICU and HDU environments. However, there is a potential for gene transfer among bacteria in areas of high density. The detection of *bla*$_{TEM}$ genes in clinical isolates is of clinical significance due to the potential for mutation to give ESBLs under particular antibiotic selective pressure.

All bla_{TEM} genes were detected in the absence of culturable organisms. However, no culturable organisms does not mean no viable organisms. It may be that the medium used did not enable organism growth, the ICU environmental site was not sufficient to enable the organisms' survival or the bacterial cells are in a dormant state **(Barer, 1997)**. From the data presented in Chapter 4, it is known that viable organisms are present in the ICU environment. Therefore the absence of culturable gene-carrying organisms could be due to the effect of routine ICU ward cleaning. Cell lysis after cleaning could enable the detection of the antibiotic resistance determinant in the absence of culturable organisms. To investigate this possibility and persistence of antibiotic resistance in the ICU environment, swab samples were taken on occasions before and after routine ICU ward cleaning (detailed in Chapter 7).

6. Identification of environmental fungal reservoirs in the ICU and HDU departments

6.1 Background and aims

The aim of this chapter was to identify environmental reservoirs of fungi in the ICU and HDU departments. In order to detect environmental fungi a culture method was used. A molecular approach was used to identify yeasts amongst fungal isolates. *Candida* spp. and *S. cerevisiae* were identified by PCR of yeast-specific 18S-25S/28S rDNA gene fragments. Restriction digestion of amplified products enabled identification to species level.

The objectives were to: -

- isolate fungi from the ICU and HDU environment
- identify the sites where fungi were most frequently detected
- quantify the level of fungal growth
- identify yeasts to species level, including *C. albicans, C. glabrata, C. guillermondii, C. tropicalis* and *C. parapsilosis*
- compare fungal species from environmental sites with clinical isolates from patients in the RHH

6.2 Overview of materials and methods

The ICU and HDU departments were sampled over 12 sessions from the RHH (12/05/08 – 15/12/08) and the NGH (11/03/08 – 09/12/08). Fungi were cultured from swab samples positive for eubacterial DNA. Samples were taken from 17 sites in the

RHH (Table 6.1); and from 13 sites in the NGH (Table 6.4) (full details of the sampling regime are provided in Section 2.2 Materials and Methods).

6.3 Results

ICU and HDU at the Royal Hallamshire Hospital

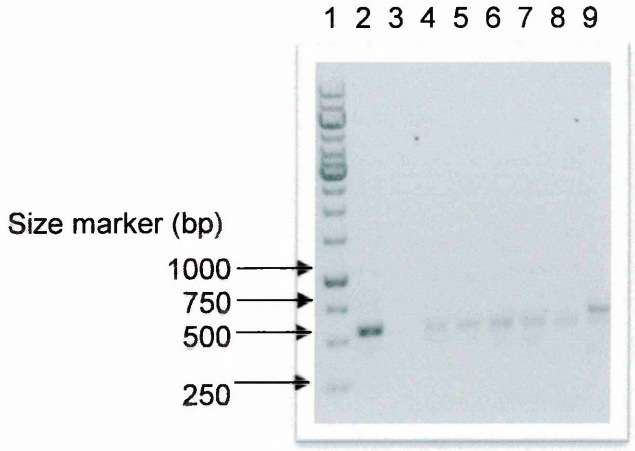
6.3.1 Isolation of fungi from the RHH environment

Medically relevant yeasts (*Candida* spp. and *S. cerevisiae*) were isolated from 46.8% of 95 RHH environmental swab samples (representative gels used for identification of cultured organisms are shown in Figure 6.1a/b).

Fungi were isolated from samples from 16 environmental sites (Table 6.1). At least one sample from each environmental site was positive for fungal growth except the sluice room sink plughole, where no fungi could be isolated (Table 6.1). Each of the sampling sites except the HDU bedside trolley yielded culturable yeasts on at least one occasion (Figure 6.2). All samples from the ICU ward sink plughole yielded fungal isolates; the majority were members of the *Candida* spp. (66.7%).

Candida spp. were most frequently cultured from samples of the ICU ward sink plughole, ICU ward sink taps and ICU floors. However, fungi were much less frequently cultured from all other sites (the ICU bedside, ICU bedside trolley, ICU computer keyboard, HDU bedside trolley, HDU patient chair, HDU computer stand, HDU floor, HDU picture, HDU plughole, HDU window ledge, ICU picture, ICU staff chair and ICU window ledge) (Figure 6.3).

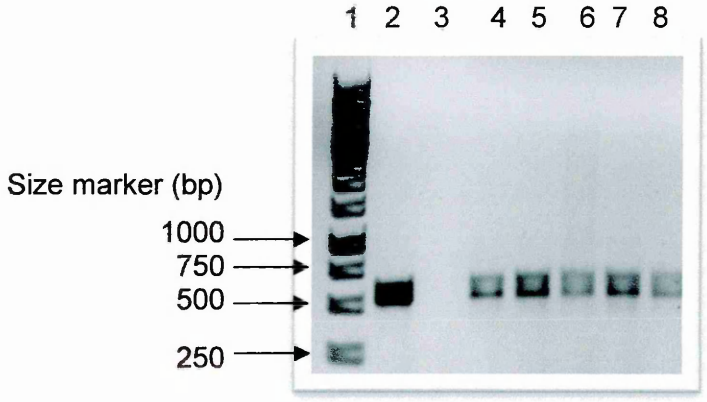
Figure 6.1 Amplification of the yeast-specific 18S-25/28S rRNA gene fragment from medically relevant yeasts isolated from RHH environment



(a)

Lane 1 marker, lane 2 *C. tropicalis* positive control, lane 3 no DNA template control, lanes 4-9 PCR products from template DNA purified from fungal isolates (sites - HDU computer stand, HDU chair, HDU picture, HDU computer stand from 14/07/08 session 4, sites – ICU bedside, ICU bedside trolley from 03/11/08 session 8 and site – ICU computer keyboard from 30/06/08 session 3 respectively)

(Using yeast specific 18S-25/28S rRNA gene fragment primers as detailed in materials and methods)



(b)

Lane 1 marker, lane 2 *C. tropicalis* positive control, lane 3 no DNA template control, lanes 4-8 PCR products from template DNA purified from fungal isolates (sites – ICU staff chair, ICU floor x2, HDU chair x2 respectively from 14/07/08 session 4)

(Using yeast specific 18S-25/28S rRNA gene fragment primers as detailed in materials and methods)

Table 6.1 Distribution and growth of fungal isolates from RHH environment (samples as detailed in Table 2.2a)[1] (continued over page)

Sample site	12/05/08 Session 1	02/06/08 Session 2	30/06/08 Session 3	14/07/08 Session 4	15/09/08 Session 5	29/09/08 Session 6	13/10/08 Session 7	03/11/08 Session 8	17/11/08 Session 9	01/12/08 Session 10	08/12/08 Session 11	15/12/08 Session 12
Bedside (ICU)								X +++		X ++		
Bedside trolley (ICU)								X +		X +++		
Computer keyboard (ICU)	(X) ++		X ++	(X) ++					(X) ++	(X) ++		X ++
Floor (ICU)		X +++		X ++		X +++	(X) ++	X ++	X ++++	X +++		X ++
HDU bedside trolley												(X) +
HDU patient chair				X ++					X +			
HDU computer stand				X +	X +							
HDU floor				X +++								
HDU picture				X +	X ++							
HDU ward sink plughole						X ++++	(X) ++++	(X) ++++				

Sample site	12/05/08 Session 1	02/06/08 Session 2	30/06/08 Session 3	14/07/08 Session 4	15/09/08 Session 5	29/09/08 Session 6	13/10/08 Session 7	03/11/08 Session 8	17/11/08 Session 9	01/12/08 Session 10	08/12/08 Session 11	15/12/08 Session 12
HDU window ledge	X ++++											
Picture (ICU)			X ++++	X ++++	(X) ++++							
Plughole (ICU ward sink)	X ++++	(X) ++++	(X) ++++	X ++++	(X) ++++	X ++++	(X) ++++	(X) ++++	(X) ++++	X ++++	(X) ++++	(X) ++++
Sluice room sink plughole												
Staff chair (ICU)				X ++					(X) +			
Taps (ICU)	X ++++	(X) ++++	X ++++	(X) ++++	X ++++	X ++++		(X) ++++		X ++++	X ++++	X ++++
Window ledge (ICU)				(X) ++++		(X) +++	(X) +++		(X) +++	(X) +++		

[1] All fungi were isolated on PDG agar (as described in materials and methods)

X = *Candida* spp. and *S. cerevisiae* identified (X) = Non-*Candida* spp. identified X(X) = *Candida* and non-*Candida* spp. identified

Fungal growth:

+	single colony
++	2-10 colonies
+++	>10 colonies
++++	mould/overgrowth

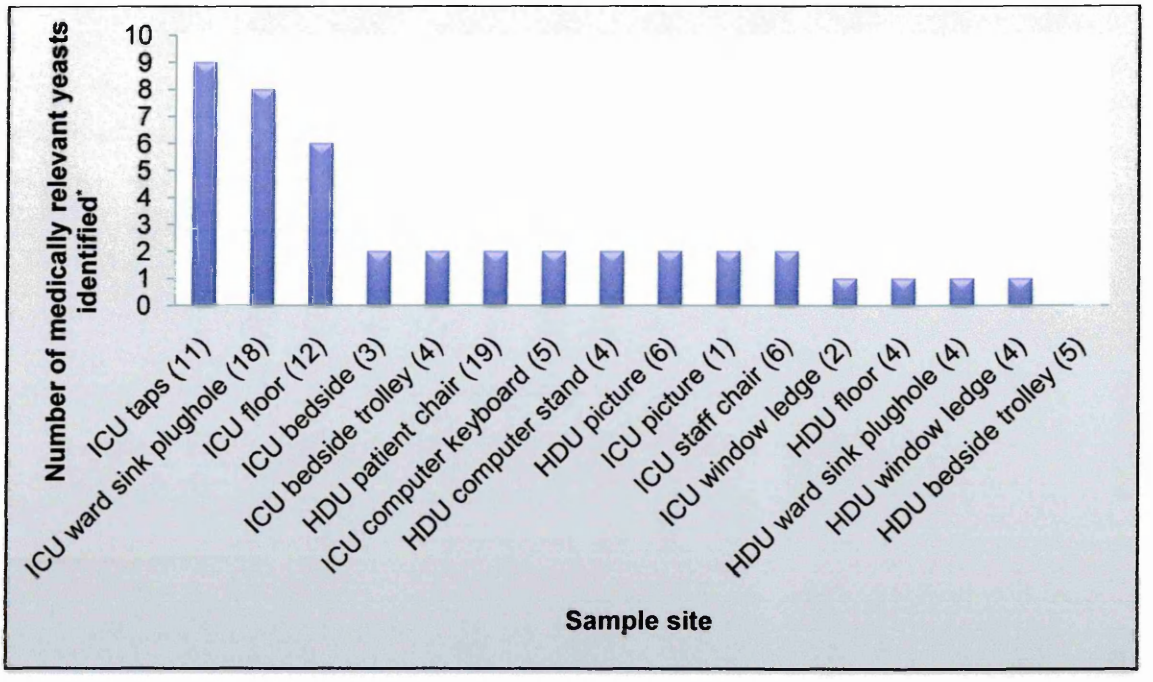


Figure 6.2 Sites of detection for medically relevant yeasts from the RHH[1]

(number) = total number of swab samples taken

[1] The data was obtained from PCRs using DNA extracted from the cultured strains

* Each sample was spread on PDG and incubated at 30°C for a maximum of five days. One colony of each colony type from each PDG plate was subcultured and identified by 18S-25/28S rRNA PCR. The reported number of yeasts identified from each site is the total number of subcultured yeast strains from that site.

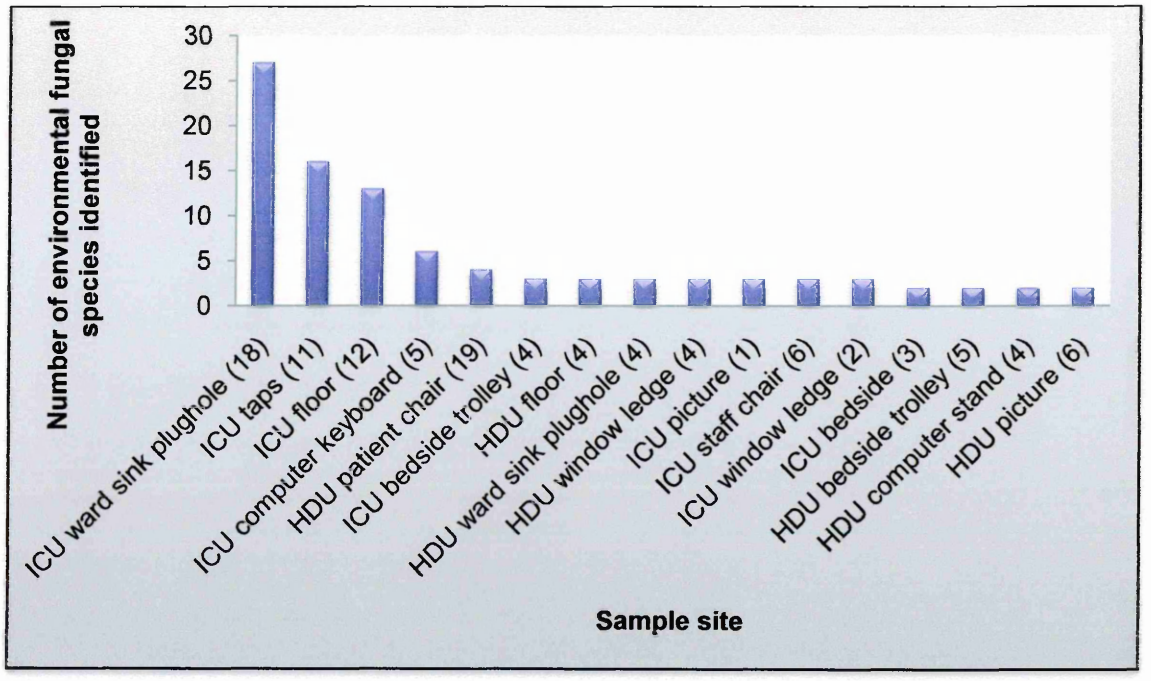


Figure 6.3 Environmental sites of fungal isolation from the RHH[1]

(number) = total number of swab samples taken

[1] This data was obtained from cultural studies from ICU environmental swab samples

The amount of fungal growth on the plate inoculated with each sample differed between environmental sites and sampling sessions (Table 6.1). The majority of fungi isolated formed a confluence of fungal growth on the agar plate (from the ICU floor, HDU window ledge, ICU picture, ICU ward sink plughole, ICU ward sink taps and ICU window ledge). Other fungi isolated were single colonies (from the ICU bedside trolley, HDU chair and HDU picture).

All fungal isolates were identified by 18S-25S/28S rRNA gene fragment specific-PCR as non-*albicans Candida* species (Table 6.2). The majority of these were identified as *C. parapsilosis* (59.1%). *C. guillermondii* (20.5%), *C. tropicalis* (18.2%) and *C. dublinensis* (2.3%) were also identified (Table 6.2). No *C. albicans* were isolated from the ward environment.

6.3.2 Clinical fungal isolates from the RHH ICU

Fungi were cultured from patient samples as part of the standard monitoring and diagnosis performed by clinical staff. During the period of this study (January-May 2008) routine clinical monitoring of patients yielded 119 fungal isolates. The majority of *Candida* positive patients were colonised with *C. albicans* (78.2%). Patients were also colonised with non-*albicans* species; *C. glabrata* (10.9%), *C. parapsilosis* (2.5%), *C. guillermondii* (1.7%), *C. tropicalis* (0.8%) and *C. krusei* (0.8%) (Table 6.3).

Table 6.2 Medically relevant yeasts identified from the RHH ICU environment

Fungal species	Number of environmental isolates	Percentage observed (%) (total 44 samples)
C. parapsilosis	26	59.1
C. guillermondii	9	20.5
C. tropicalis	8	18.2
C. dublinensis	1	2.3
C. albicans	0	0.0
C. glabrata	0	0.0
C. krusei	0	0.0
S. cerevisiae	0	0.0

Table 6.3 Fungal species colonising ICU patients in the RHH ICU

Fungal species	Percentage patient isolates (%) (total 119)
C. albicans	78.2
C. glabrata	10.9
S. cerevisiae	5.0
C. parapsilosis	2.5
C. guillermondii	1.7
C. tropicalis	0.8
C. krusei	0.8
C. dubliniensis	0.0
C. membranefaciens	0.0

6.3.3 Isolation of fungi from the NGH environment

In contrast to the RHH, the proportion of isolates of medically relevant yeasts (*Candida* spp. and *S. cerevisiae*) was higher; 66.2% of 65 NGH environmental swab samples yielded yeasts (a representative gel used for identification of cultured organisms is shown in Figure 6.4).

Fungi were cultured from samples from all (13) ICU and HDU environmental sites at the NGH (Table 6.4). At least one sample from all ICU sites yielded fungi (Figure 6.5). However, in contrast to the results from the RHH HDU, no medically relevant yeasts were isolated from the NGH HDU environment (Figure 6.6).

Candida spp. were most frequently isolated from similar sites to the RHH; from samples of the ICU floor (the ward and isolation room), ICU patient chair and plugholes (ICU ward and sluice room sinks). *Candida* spp. were much less frequently isolated from all other sites (the ICU bedside, ICU computer keyboard, ICU door handle, HDU computer keyboard, HDU door switch, HDU floor, HDU ward sink plughole and ICU window ledge) similar to the RHH (Figure 6.6).

Interestingly, compared to the RHH, fungal isolation from only one sample site (ICU computer keyboard) was consistently an overgrowth on the agar plate (Table 6.4). From the HDU floor and ICU door handle samples fungal growth was consistently low (2-10 colonies and a single colony respectively) (Table 6.4).

The same non-*albicans Candidia* species were identified from fungal isolates at the NGH compared to the RHH; *C. parapsilosis* (39.5%), *C. tropicalis* (20.9%) and

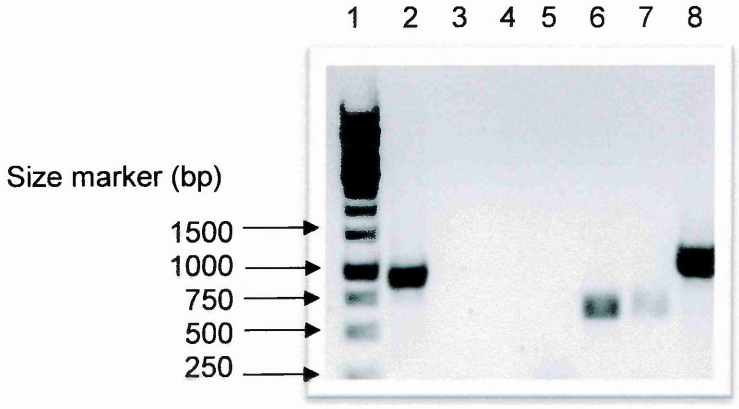


Figure 6.4 Amplification of the yeast-specific 18S-25/28S rRNA gene fragment from medically relevant yeasts isolated from NGH environment

Lane 1 marker, lane 2 *C. glabrata* positive control, lane 3 no DNA template control, lanes 6-8 PCR products from template DNA purified from fungal isolates (sites – ICU ward sink plughole x2, ICU floor, ICU computer keyboard from 08/04/08 session 2 and site - sluice room sink plughole from 03/06/08 session 4 respectively)

(Using yeast specific 18S-25-28S rDNA gene fragment primers as detailed in materials and methods)

Table 6.4 Distribution and growth of fungal isolates from NGH environment (samples detailed in Table 2.2b)[1] (continued over page)

Sample site	11/03/08 Session 1	08/04/08 Session 2	13/05/08 Session 3	03/06/08 Session 4	01/07/08 Session 5	15/07/08 Session 6	16/09/08 Session 7	30/09/08 Session 8	14/10/08 Session 9	04/11/08 Session 10	18/11/08 Session 11	09/12/08 Session 12
Bedside (ICU)		X ++				X ++						
Patient chair (ICU)		X ++			X ++		(X) ++		(X) +++			X ++
Computer keyboard (ICU)				X ++++						(X) ++++		
Door handle (ICU)										X +		
Floor – isolation room (ICU)	X +	X +	X ++	(X) ++			X +++	X +++	X ++++	X +++	X ++	
Floor-ward (ICU)				(X) ++			X +	X +++	X ++	X ++	X ++	X ++++
HDU computer keyboard		(X) +++								(X) ++		(X) ++
HDU door switch		(X) +										
HDU floor												(X) ++
HDU ward sink plughole		(X) +										(X) +

Sample site	11/03/08 Session 1	08/04/08 Session 2	13/05/08 Session 3	03/06/08 Session 4	01/07/08 Session 5	15/07/08 Session 6	16/09/08 Session 7	30/09/08 Session 8	14/10/08 Session 9	04/11/08 Session 10	18/11/08 Session 11	09/12/08 Session 12
Plughole (ICU ward sink)	X +++	X ++	X +					(X) ++++				
Sluice room sink plughole	X ++++		X +				(X) +					X +
Window ledge (ICU)							(X) +					

[1] All fungi were isolated on PDG agar (as described in materials and methods)

X = *Candida* spp. and *S. cerevisiae* identified (X) = Non-*Candida* spp. identified X(X) = *Candidia* and non-*Candida* spp. identified

Fungal growth:

+	single colony
++	2-10 colonies
+++	>10 colonies
++++	mould/overgrowth

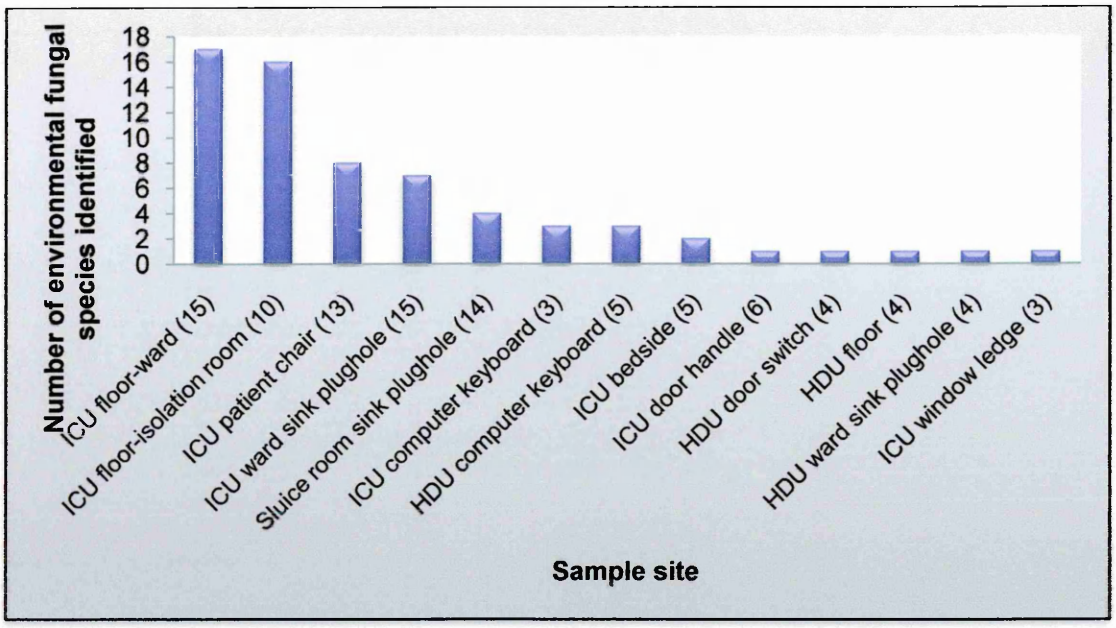


Figure 6.5 Environmental sites of fungal isolation from NGH[1]

(number) = total number of swab samples taken

[1] This data was obtained from cultural studies from ICU environmental swab samples

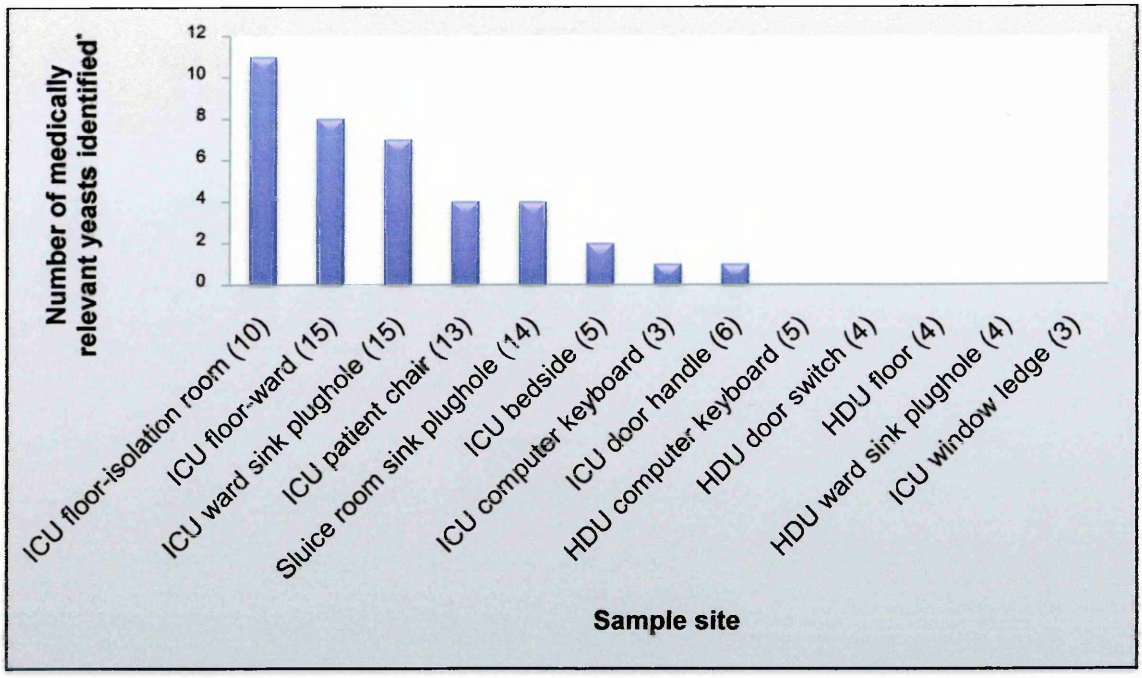


Figure 6.6 Detection of medically relevant yeasts from the NGH environment[1]

(number) = total number of swab samples taken

[1] The data was obtained from PCRs using DNA extracted from the cultured strains

* Each sample was spread on PDG and incubated at 30°C for a maximum of five days. One colony of each colony type from each PDG plate was subcultured and identified by 18S-25/28S rRNA PCR. The reported number of yeasts identified from each site is the total number of subcultured yeast strains from that site.

C. guillermondii (16.3%) (Table 6.5). However in addition *C. glabrata* (20.9%) was also identified from the NGH environment (Table 6.5).

Fungal clinical isolate data was not obtained during this study from the NGH, the focus was on the ICU and HDU environment. Patient data was only obtained for fungal clinical isolates at the RHH.

6.4 Discussion

The incidence of nosocomial yeast infections has risen dramatically, particularly amongst ICU patients **(Verduyn-Lunel *et al.*, 1999; Leone *et al.*, 2003; Bassetti *et al.*, 2006)**. There is limited research on fungal species in the ICU and HDU environment. In this present study the ICU and HDU environment was investigated for the presence of fungal species and *Candida* spp. in particular. Interestingly, the sites of highest fungal isolation were the same as those yielding the highest bacterial detection and where antibiotic resistance determinants were detected (detailed in Chapter 4 and 5).

During this present study the environmental results show the trend towards non-*albicans Candida* spp. which has previously been reported in clinical samples in the literature. For many years *C. albicans* was the most common cause of fungal nosocomial infections, however there is a current switch towards non-*albicans Candida* infections in ICUs **(Nguyen *et al.*, 1996; Hobson, 2003; Bassetti *et al.*, 2006; Shorr *et al.*, 2007)**. This may potentially be due to the extensive use of fluconazole in the 1990's **(Trick *et al.*, 2002; Hobson, 2003)** which has led to an increase in non-*albicans Candida* infections (especially *C. glabrata*) due to their lower susceptibility to azole anti-fungals **(Nguyen *et al.*, 1998; Fidel *et al.*, 1999; Hobson, 2003)**.

Table 6.5 Medically relevant yeasts identified from the NGH ICU environment

Fungal species	Number of environmental isolates	Percentage observed (%) (total 43 samples)
C. parapsilosis	17	39.5
C. glabrata	9	20.9
C. tropicalis	9	20.9
C. guillermondii	7	16.3
S. cerevisae	1	2.3
C. albicans	0	0.0
C. dubliniensis	0	0.0
C. krusei	0	0.0
C. membranefaciens	0	0.0

Non-*albicans Candida* spp. were present in various sample sites within the ICU and HDU environment during this present study. Previous research has demonstrated the presence of *Candida* spp. in the indoor air and on walls in ICUs **(Gniadek and Macura, 2007; Krajewska-Kulak *et al.*, 2007)**. Commensal fungal organisms are typically found on the skin of 10-20% of healthy individuals **(Sullivan *et al.*, 1996)**. The most common environmental yeast isolates (from RHH and NGH) were *C. parapsilosis* which has previously been shown to persist on environmental surfaces for 14 days **(Kramer *et al.*, 2006)**. From the NGH ICU, *C. glabrata* was present which has previously been shown to persist for five months on environmental surfaces **(Kramer *et al.*, 2006)**. This implies that non-*albicans Candida* spp. could persist in the ICU environment on hard surfaces (and sinks).

C. albicans was not isolated in the ICU environment which appears to rule out the possibility of a link between the environment and *C. albicans* infected patients. The reason why no *C. albicans* was present in the ICU environment may be because *C. albicans* is a common commensal of the mouth and gastrointestinal tract **(Cannon and Chaffin, 1999; Hobson, 2003)** in contrast to the most common environmental yeast isolated (*C. parapsilosis*), which is a commensal of the skin (particularly hands) **(Sullivan *et al.*, 1996; Gacser *et al.*, 2007)**. Therefore the lack of *C. albicans* in the ICU environment may be due to good infection control policies around patients infected with *C. albicans*. Insufficient hand hygiene may explain the wide distribution of non-*albicans Candida* spp. due to hand contact with various sites in the ICU environment.

Interestingly, a wider range of *Candida* spp. was isolated from the environment of the NGH than the RHH. Whilst it is not possible to assign the reason for this with certainty, it is possible that the ward design and number of patients/hospital staff could be the cause. There are a greater number of patients and staff on the NGH ICU compared to the RHH ICU. This could explain why more *Candida* spp. were isolated from the NGH. The greater number of patients and hospital staff could result in more *Candida* spp. in

the environment from commensal fungal organisms due to hand contact and skin shedding **(Lee *et al.*, 2007; Rintala *et al.*, 2008)**.

There was a larger number of CFUs per sample at the RHH ICU compared to the NGH ICU. A possible explanation for this observation could be due to patient proximity and ward design. Previous research has shown how patient proximity can contribute to the transmission of MRSA and an increase in environmental contamination **(Howie and Ridley, 2008)**. Besides the NGH ICU having a greater patient intake and number of hospital staff, the ward itself is approminately three times the size (in terms of floor space) compared to the RHH ICU. Therefore, approximately the same number of microorganisms could be present in a larger space, so they are less frequently detected by culturing.

Other differences which could exlplain the larger number of CFUs per sample at the RHH ICU include the age of the ward. The NGH ICU was newly commissioned during this present study therefore a possibility could be that there has not been as much fungal build-up in the NGH ICU environment compared to the RHH ICU. The NGH ICU ward design is also different compared to the RHH ICU. For example the sinks on the NGH ICU have a larger splash guard and no tap handles compared to the RHH ICU. This could limit organism backsplash from the plughole onto hospital staff hands and nearby medical equipment which has previously been reported in the literature **(Doring *et al.*, 1996; Lango *et al.*, 2007; Brooke, 2008; Hota *et al.*, 2009)** (as detailed in Chapter 8).

Non-*albicans Candida* spp. are widely distributed in the ICU environment. There was a wide variation in the number of fungal CFUs isolated over the 12 sample sessions. It was suspected that this variation may be due to routine ICU ward cleaning, therefore the study to compare the samples taken from the ICU environment before and after

cleaning was expanded to include culture media for the isolation of fungal species (as detailed in Chapter 7).

7. Comparison of the microbial ecology and presence of antibiotic resistance determinants before and after routine ICU ward cleaning

7.1 Background and aims

This chapter aims to compare the effect of routine ward cleaning on the detection of prokaryotic and fungal microorganisms and specific antibiotic resistance markers. For a three week period, intensive sampling was carried out on the sites of highest eubacterial DNA detection and fungal isolation (the ICU patient chair, ICU floor, ICU ward sink plughole and sluice room sink plughole).

The objective was to sample on occasions before and after routine ICU ward cleaning and compare the effect on the: -

- detection of eubacterial DNA from ICU environmental swab samples
- presence of bacterial sequences by PCR-DGGE
- isolation of fungi from ICU environmental swab samples
- persistence of antibiotic resistance determinants in the ICU environment by PCR using gene-specific primers for *mec*A and $bla_{\text{CTX-M, SHV, TEM}}$
- isolation and identification of target gene-carrying (*mec*A and bla_{TEM}) bacterial species

7.2 Sampling regime

A total of 180 swab samples were taken over a three week sampling period (02/03/09 – 20/03/09 Monday-Friday) (Table 7.1a/b). Four sample sites were tested; the ICU

Table 7.1a Environmental swab samples tested for eubacterial DNA from the ICU at the RHH

Sample site	Floor (ICU)	Patient chair (ICU)	Plughole (ICU ward sink)	Sluice room sink plughole
Sample session 1 02/03/09	R1M #1/2	R1M #5/6	R1M #3	R1M #4
03/03/09	R1T #1/2	R1T #5/6	R1T #3	R1T #4
04/03/09	R1W #1/2	R1W #5/6	R1W #3	R1W #4
05/03/09	R1Th #1/2	R1Th #5/6	R1Th #3	R1Th #4
06/03/09	R1F #1/2	R1F #5/6	R1F #3	R1F #4
Sample session 2 09/03/09	R2M #1/2	R2M #5/6	R2M #3	R2M #4
10/03/09	R2T #1/2	R2T #5/6	R2T #3	R2T #4
11/03/09	R2W #1/2	R2W #5/6	R2W #3	R2W #4
12/03/09	R2Th #1/2	R2Th #5/6	R2Th #3	R2Th #4
13/03/09	R2F #1/2	R2F #5/6	R2F #3	R2F #4
Sample session 3 16/03/09	R3M #1/2	R3M #5/6	R3M #3	R3M #4
17/03/09	R3T #1/2	R3T #5/6	R3T #3	R3T #4
18/03/09	R3W #1/2	R3W #5/6	R3W #3	R3W #4
19/03/09	R3Th #1/2	R3Th #5/6	R3Th #3	R3Th #4
20/03/09	R3F #1/2	R3F #5/6	R3F #3	R3F #4

Code: -

R [sampling session] [day of the week] # [code number representing site]

Table 7.1b Environmental swab samples tested for eubacterial DNA from the ICU at the NGH

Sample site	Floor (ICU)	Patient chair (ICU)	Plughole (ICU ward sink)	Sluice room sink plughole
Sample session 1 02/03/09	N1M #1/2	N1M #5/6	N1M #3	N1M #4
03/03/09	N1T #1/2	N1T #5/6	N1T #3	N1T #4
04/03/09	N1W #1/2	N1W #5/6	N1W #3	N1W #4
05/03/09	N1Th #1/2	N1Th #5/6	N1Th #3	N1Th #4
06/03/09	N1F #1/2	N1F #5/6	N1F #3	N1F #4
Sample session 2 09/03/09	N2M #1/2	N2M #5/6	N2M #3	N2M #4
10/03/09	2T #1/2	N2T #5/6	ironmenN2T	N2T #4
11/03/09	N2W #1/2	N2W #5/6	N2W #3	N2W #4
12/03/09	N2Th #1/2	N2Th #5/6	N2Th #3	N2Th #4
13/03/09	N2F #1/2	N2F #5/6	N2F #3	N2F #4
Sample session 3 16/03/09	N3M #1/2	N3M #5/6	N3M #3	N3M #4
17/03/09	N3T #1/2	N3T #5/6	N3T #3	N3T #4
18/03/09	N3W #1/2	N3W #5/6	N3W #3	N3W #4
19/03/09	N3Th #1/2	N3Th #5/6	N3Th #3	N3Th #4
20/03/09	N3F #1/2	셀 3F #5/6	N3F #3	N3F #4

Code: -

N [sampling session] [day of the week] # [code number representing site]

patient chair, ICU floor, ICU ward sink plughole and sluice room sink plughole. These sites had previously shown the highest detection and isolation of bacteria and fungi from the extensive sampling period (detailed in Chapter 4 and 6). Samples were taken around the time of cleaning, on each occasion it was determined from the cleaning staff if cleaning had been done. Experience during the whole of the study indicated that the samples taken 'before cleaning' were usually taken within one hour of the cleaning commensing and samples taken 'after' were usually within the hour after cleaning had been completed. In total daily samples were taken on 13 occasions before and 17 occasions after routine ICU ward cleaning. Each sample was given a unique code number that specifies the type of environment sampled and the hospital from which the sample was taken. The DNA was extracted from each swab and stored at -20 °C in the 703/-20/2 freezer in room 703 BMRC labelled with the codes from Table 7.1a/b.

7.3 Results

7.3.1 Detection of eubacterial DNA before and after routine ICU ward cleaning from RHH and NGH environments

There was a 50% reduction in the proportion of ICU environmental swab samples yielding eubacterial DNA after routine ICU ward cleaning. Before the ward was cleaned eubacterial DNA was detected in 65-67% of environmental swab samples; after cleaning eubacterial DNA was detected in 31-33% of environmental swab samples (Figure 7.1). Using chi-squared data analysis the reduction in eubacterial DNA detection after cleaning was significant (Figure 7.1)

The proportion of swab samples positive for eubacterial DNA was lower after routine ICU ward cleaning from all environmental sites (ICU patient chair, ICU floor, ICU ward sink plughole and sluice room sink plughole) (Figure 7.2). There was a 28% reduction

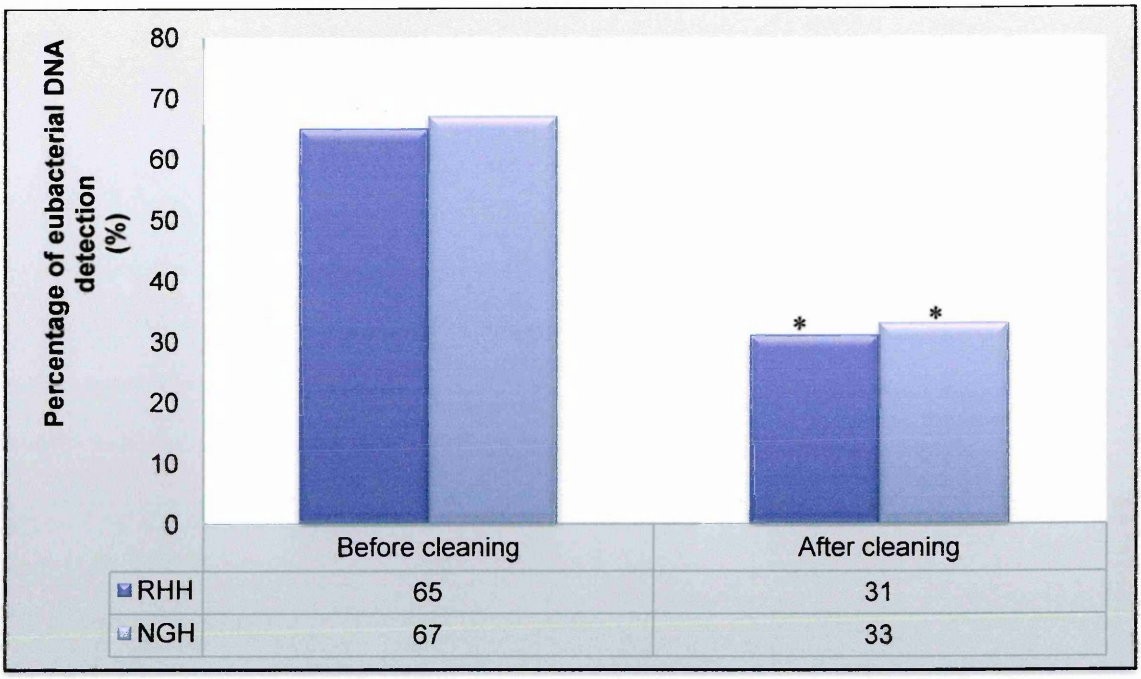


Figure 7.1 PCR detection of eubacterial DNA in RHH and NGH ICU environmental swab samples before and after routine ward cleaning

* p = 0.05

(Chi-squared analysis)

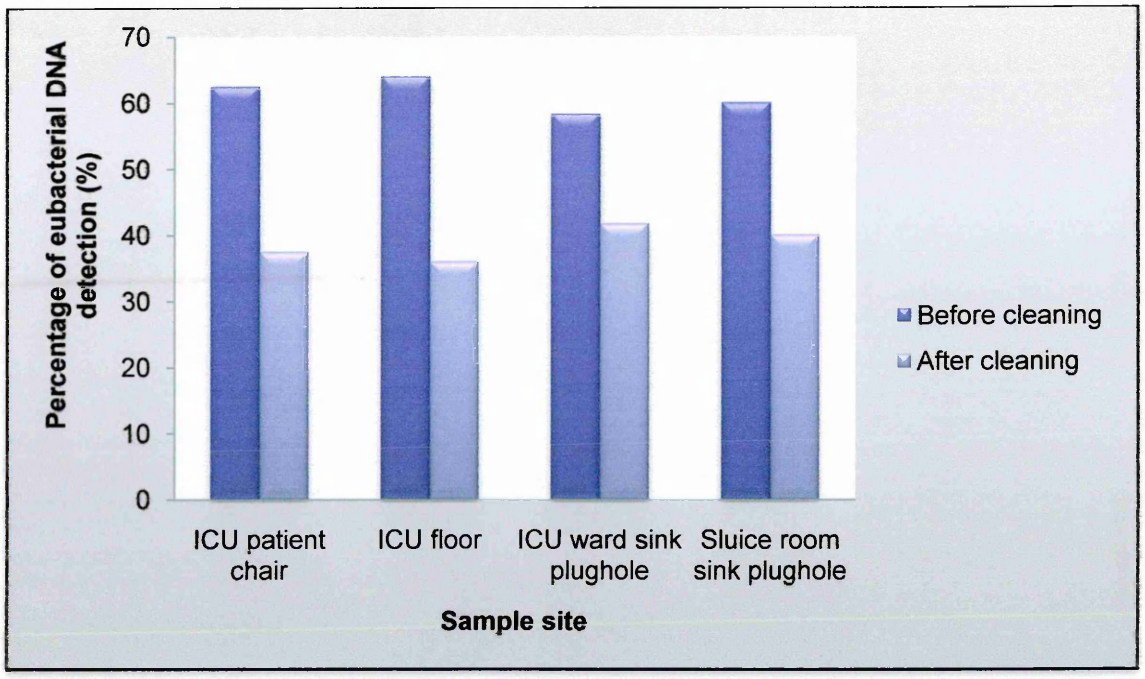


Figure 7.2 PCR detection of eubacterial DNA in RHH and NGH ICU environmental sample sites before and after routine ward cleaning

Total number of swab samples before cleaning: - Chair and floor, each n = 26

Plughole and sluice plughole, each n = 13

Total number of swab samples after cleaning: - Chair and floor, each n = 34

Plughole and sluice plughole, each n = 17

in ICU floor samples yielding eubacterial DNA (before 64%, after 36%), 25% in ICU patient chair samples (before 62.5%, after 37.5%), 20% in sluice room sink plughole samples (before 60%, after 40%) and 17% in ICU ward sink plughole samples (before 58.3%, after 41.7%) (Figure 7.2).

PCR-amplified fragments of 16S rRNA genes were retrieved from environmental swab samples (ICU ward sink plughole, ICU floor and ICU patient chair) taken on occasions before and after routine ICU ward cleaning and analysed by PCR-DGGE (Table 7.2 and representative gel shown in Figure 7.3).

Bacterial diversity was greatest in ICU ward sink plughole swab samples on occasions before and after routine ICU ward cleaning compared to ICU floor and ICU patient chair swab samples (Table 7.2). The retrieval of the same or similar DGGE bands from swab samples taken on occasions before and after routine ICU ward cleaning indicates the presence of the same microorganism. ICU ward sink plughole swab samples contained the most number of similar DGGE bands on occasions before and after routine ICU ward cleaning. From occasions before routine ICU ward cleaning, eight similar DGGE bands were retrieved after cleaning from ICU ward sink plughole swab samples. From ICU floor swab samples, two similar DGGE bands were retrieved on occasions before and after routine ICU ward cleaning (Table 7.2).

Sequences similar to Gram-positive organisms could only be retrieved before routine ward cleaning; Gram-negative sequences could be retrieved from DGGE bands before and after routine ICU ward cleaning (Table 7.2). Sequences most similar to the genus *Burkholderia* were retrieved from ICU floor and ICU ward sink plughole swab samples taken before and after routine ICU ward cleaning. Sequences with highest similarity to uncultured organisms (associated with human skin and blood), *Staphylococcus hominis* (CNS), faecalibacterium and firmicutes (associated with gut flora) could only be retrieved before routine ICU ward cleaning.

Table 7.2 Retrieval of bacterial sequences before and after routine ICU ward cleaning from NGH and RHH environmental swab samples after PCR-DGGE

(continued over page)

Sample site	Representative DGGE band	Retrieval before routine ICU ward cleaning	Retrieval after routine ICU ward cleaning	Gram-positive (+) or Gram-negative (-)	Sequences similar to and percentage identity (%)
ICU patient chair	1	X		-	*Burkholderia* spp. 93**
	2	X		+	*Staphylococcus epidermidis* 78**
ICU floor	1	X	X	-	*Acinetobacter* spp. 49**
	2	X	X	-	*Burkholderia* spp.
	3	X		+	*Propionibacterium* spp. 100*
	4	X		+	*Staphylococcus hominis* 100*
ICU ward sink plughole	1	X	X	-	*Alcaligenes* spp. 69**
	2	X		-	*Bacteroidetes* spp. 96*
	3	X		-	*Beta-proteobacterium* 93*
	4	X	X	-	*Burkholderia* spp. 98*
	5	X	X	-	*Burkholderia cenocepacia* 98*
	6	X	X	-	*Burkholderia cepacia* 99*
	7	X	X	-	*Burkholderia vietnamiensis* 92**
	8	X		+	Faecalibacterium 94**
	9	X		+	Firmicutes 81**

Sample site	Representative DGGE band	Retrieval before routine ICU ward cleaning	Retrieval after routine ICU ward cleaning	Gram-positive (+) or Gram-negative (-)	Sequences similar to and percentage identity (%)
ICU ward sink plughole	10	X	X	-	*Ralstonia* spp. 72**
	11	X	X	-	*Ralstonia metallidurans* 97*
	12	X	X	-	*Stenotrophomonas maltophilia* 92**
	13	X		+	TS13_a03d2 Faecalibacterium 82**
	14	X		-	*Variovorax* spp. 99*

* Clearly defined DNA sequence

** Sequence interrupted due to unassigned residues

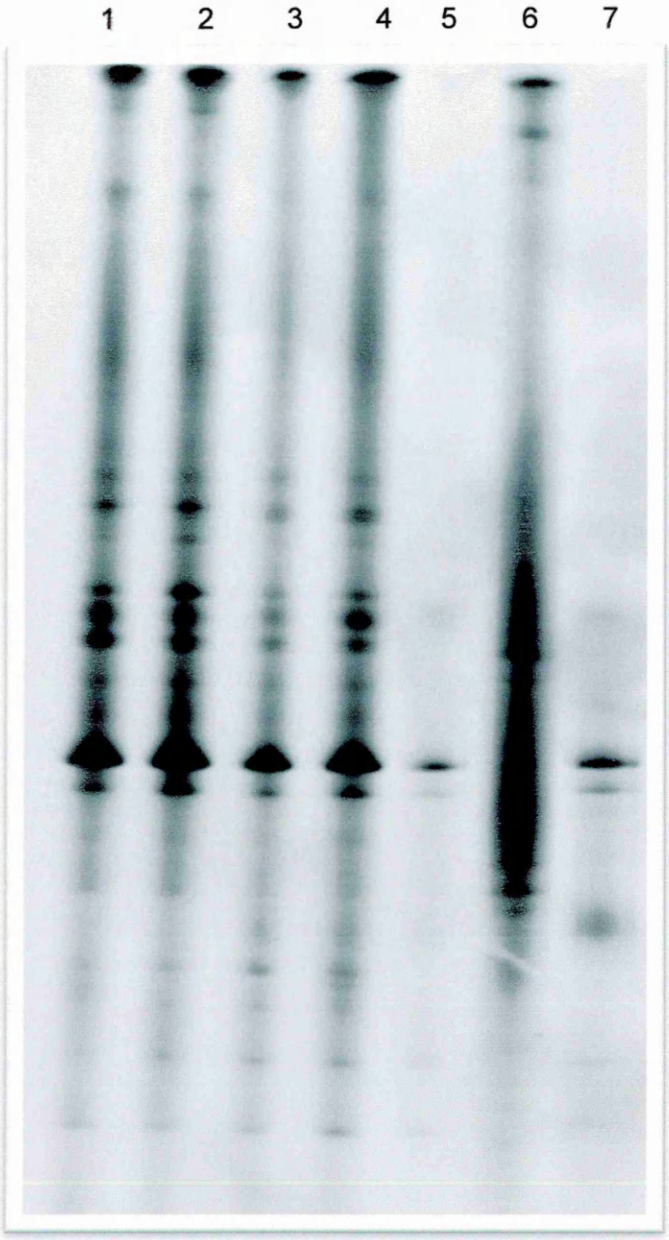


Figure 7.3 DGGE gel showing bacterial diversity from RHH ICU environmental swab samples taken before and after routine ward cleaning

Lanes 1-7 PCR products from template DNA purified from environmental swab samples (sites – ICU ward sink plughole after cleaning x2 from session 2 03/03/09, 04/03/09, ICU ward sink plughole before cleaning x2 from session 2 05/03/09, 06/03/09, sluice room sink plughole after cleaning x2 from session 1 03/03/09 and session 3 18/03/09, sluice room sink plughole before cleaning from session 1 05/03/09 respectively)

(Using nt 341-926 16S rDNA fragment-specific primers detailed in the materials and methods)

Sequences relatively similar to opportunistic species; *Stenotrophomonas maltophilia* and *Acinetobacter* spp. were retrieved before and after routine ICU ward cleaning from ICU floor and ICU ward sink plughole samples. The majority of sequences could not be retrieved after routine ward cleaning from the ICU patient chair and ICU floor; however 57% of sequences detected before cleaning were still retrieved after routine ward cleaning from ICU ward sink plughole swab samples (Table 7.2).

7.3.2 Isolation of fungi from the RHH and NGH ICU environment before and after routine ward cleaning

The number of fungal colonies isolated was reduced after routine ICU ward cleaning from ICU floor samples (before 16, after 3), ICU ward sink plughole samples (before 124, after 47) and sluice room sink plughole samples (before 32, after 14). There was an increase in the number of fungal colonies isolated from ICU patient chair samples after routine ward cleaning (before 9, after 12) (Figure 7.4).

7.3.3 Detection of mecA and bla genes in the RHH and NGH ICU environment before and after routine ward cleaning

Only samples which were positive for eubacterial DNA by PCR were screened for antibiotic resistance determinants (RHH = 45 swab samples, NGH = 44 swab samples).

All (3) bla_{TEM} amplified from NGH swab samples and five (out of eight) RHH swab samples were detected before routine ICU ward cleaning and were not detected in DNA from samples of the same site after 24 hours (Table 7.3a/b). However, bla_{TEM} was amplified in DNA from three (out of eight) RHH swab samples taken after cleaning.

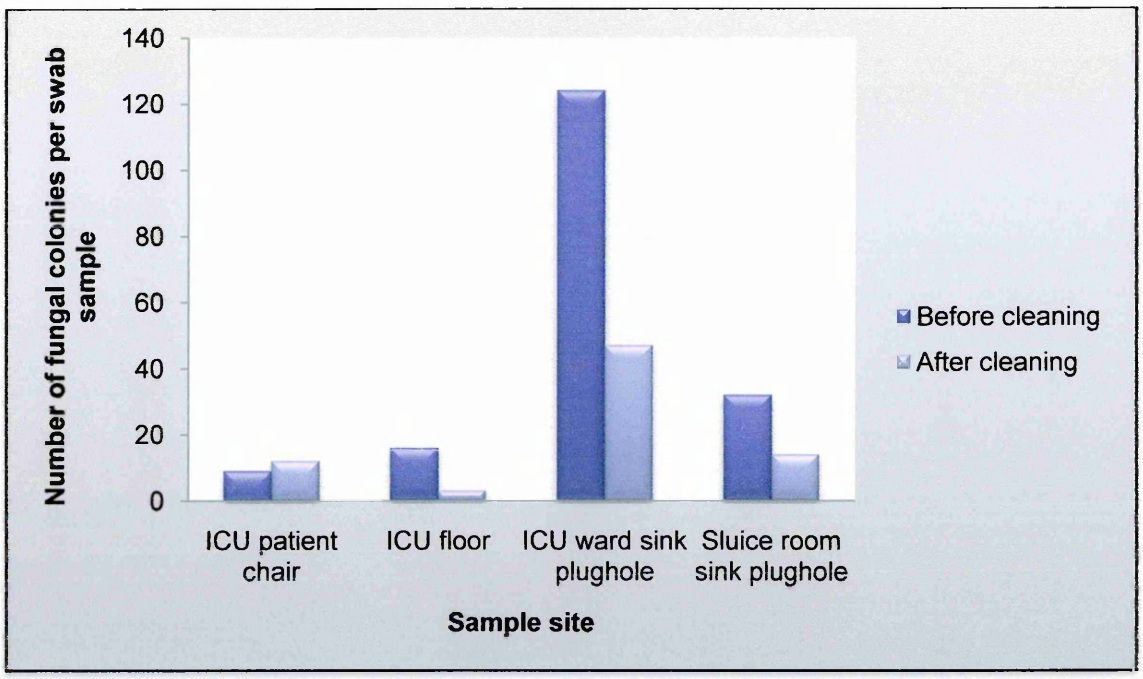


Figure 7.4 Number of fungal colonies before and after routine ward cleaning for each sample site from RHH and NGH ICU environment

Total number of swab samples before cleaning: - Chair and floor, each n = 26

Plughole and sluice plughole, each n = 13

Total number of swab samples after cleaning: - Chair and floor, each n = 34

Plughole and sluice plughole, each n = 17

Table 7.3a Distribution and classification of antibiotic resistance determinants before and after routine ward cleaning in the RHH ICU environment

Sample site	Floor (ICU)	Patient chair (ICU)	Plughole (ICU ward sink)	Sluice room sink plughole
Sample session 1 02/03/09	-	-	-	+ PBP2a[1] *S. hominis*[2]
03/03/09	-	-	-	
04/03/09				
05/03/09	-		-	+ TEM-1 Native[3]
06/03/09	-	-	-	
Sample session 2 09/03/09	-	+ TEM-1 Native[3]	-	-
10/03/09	+ TEM-1 Native[3]		+ TEM-1 Native[3]	
11/03/09			+ TEM-1 Native[3]	
12/03/09	+ TEM-1 Native[3] *Klebsiella* spp.[4]	-	+ TEM-1 Native[3] *Bacillus subtilis*[5]	
13/03/09	-	+ TEM-1 Native[3]	-	
Sample session 3 16/03/09		-	-	
17/03/09		-	-	-
18/03/09				-
19/03/09				
20/03/09	-	-	-	

[1] PBP2a is the altered penicillin binding protein encoded by the *mec*A gene responsible for the MRSA phenotype

[2] *Staphylococcus hominis* was identified as the gene-carrying organism

[3] Native beta-lactamase genes were identified not ESBLs

[4] *Klebsiella* spp. was identified as the gene-carrying organism

[5] *Bacillus subtilis* was identified as the gene-carrying organism

Background colour Blue After routine ward cleaning

Background colour Purple Before routine cleaning

- = negative samples

Blank boxes = sample not tested for antibiotic resistance determinants

Table 7.3b Distribution and classification of antibiotic resistance determinants before and after routine ward cleaning in the NGH ICU environment

Sample site	Floor (ICU)	Patient chair (ICU)	Plughole (ICU ward sink)	Sluice room sink plughole
Sample session 1 02/03/09	-	-	-	-
03/03/09			-	
04/03/09				
05/03/09	-		-	
06/03/09	-		-	-
Sample session 2 09/03/09	-			-
10/03/09		-	-	
11/03/09		-	-	
12/03/09	-	-		
13/03/09		-	-	-
Sample session 3 16/03/09	-	-	+ TEM-1 Native[1]	+ TEM-1 Native[1]
17/03/09	++ TEM-1 Native[1] *Bacillus* spp.[2]	-	-	-
18/03/09			-	-
19/03/09		-	-	-
20/03/09	-	-	-	-

+ One bla_{TEM} PCR positive sample

++ Two bla_{TEM} PCR positive samples

[1] Native beta-lactamase genes were identified not ESBLs

[2] *Bacillus* spp. were identified as the gene-carrying organisms

Background colour Blue = After routine ward cleaning

Background colour Purple = Before routine cleaning

- = negative samples

Blank boxes = sample not tested for antibiotic resistance determinants

bla$_{TEM}$ persisted in the RHH ICU ward sink plughole for approximately 72 hours because *bla*$_{TEM}$ was detected in DNA from three swab samples on consecutive days.

Target gene-carrying organisms could only be cultured from sessions before routine ICU ward cleaning. DNA sequencing of eubacterial DNA identified *Bacillus* spp., *B. subtilis* and *Klebsiella* spp. as *bla*$_{TEM}$ gene-carrying organisms (Table 7.3a/b).

DNA sequencing was used to differentiate between native and ESBL *bla*$_{TEM}$ genes. All positive amplicons were identified as native TEM beta-lactamases and not ESBLs (Table 7.3a/b).

The *mec*A gene was detected after routine ICU ward cleaning in a RHH sluice room sink plughole swab sample (Table 7.3a), and after 24 hours from a sample of the same site *mec*A was not detected. The *mec*A gene-carrying organism was cultured and isolated DNA was used to sequence eubacterial DNA; the organism was identified as *S. hominis* (Table 7.3a).

7.4 Discussion

A number of guidelines have been implemented to combat HAIs particularly in relation to effective cleaning **(Department of Health, 2008)**. Previous research has shown that organisms associated with HAI outbreaks can persist in the hospital environment after cleaning **(Naas *et al.*, 2002; Wilcox *et al.*, 2003; Denton *et al.*, 2004; Whittington *et al.*, 2009)**. This chapter demonstrates that there is a difference in the microbial ecology in the ICU environment on occasions before and after routine ICU ward cleaning. Antibiotic resistance determinants were detected before and after routine ICU ward cleaning, however *mec*A and *bla*$_{TEM}$ gene-carrying organisms could only be isolated on occasions before cleaning.

Previous research has shown that in many environments <1% of bacterial species are culturable **(Amann *et al.*, 1995; Pace, 1997)**. The use of PCR-DGGE enabled sequence based identification of the genera and species present where bacteria could not be cultured on non-selective media (NA, CLED and blood agar). However, retrieval of bacterial sequences implies the presence of bacterial cells, which may or may not be viable.

The routine ICU ward cleaning regime was only effective on hard surfaces. The majority of DGGE bands were not detected from samples on occasions after routine ward cleaning. However, the DGGE bands giving sequences similar to opportunistic species (*Burkholderia* spp., *Acinetobacter* spp. and *Stenotrophomonas* spp.) were detected from samples on occasions before and after routine ICU ward cleaning.

Previous research has shown opportunistic species including *Acinetobacter* spp. to persist in the environment after cleaning **(Wilcox *et al.*, 2003; Denton *et al.*, 2004; HPA, 2006b)**. Sinks have been shown previously to harbour pathogenic bacteria (particularly during outbreaks) and act as a reservoir **(Brooke *et al.*, 2008; Hota *et al.*, 2009)**. These and the results from this present study underline the need for an effective cleaning regime to ensure the removal of organisms to prevent transmission, especially if the organisms are present in an infective dose.

Interestingly, only Gram-negative organisms were detected in samples taken after routine ICU ward cleaning. This suggests routine ICU ward cleaning is able to remove Gram-positive organisms and there is a build-up between cleaning sessions. The source of Gram-positive organisms is likely to be constant skin shedding and hand contact by patients and hospital staff **(Andersson *et al.*, 1999; Lee *et al.*, 2007; Rintala *et al.*, 2008)**. In contrast, Gram-negative organisms remain, particularly from areas linked to biofilms (e.g. sinks).

A possibility for this observation may be due to the action of cleaning, for example the cleaning agent may simply be more effective against Gram-positive organisms. Many disinfectants (which have a bactericidal action) work by causing bacterial cell lysis. The Gram-negative cell wall is surrounded by an outer membrane (which contains lipopolysaccharide and lipoproteins) which is not present in Gram-positive organisms **(Mims *et al.*, 1998c)**. The outer membrane can provide Gram-negative organisms with a greater intrinsic resistance against the action of bactericidal cleaning agents. Gram-positive organisms therefore are more permeable and susceptible to the action of biocides because the cell wall is not protected by an outer membrane. This could in part account for why Gram-negative organisms remain after routine ICU ward cleaning.

Observations during the whole of the present study showed that cleaning of hospital sinks consisted of pouring disinfectant into the plughole, therefore, the cleaning action may not be vigorous enough to remove cells (e.g. *Acinetobacter* spp. and *Burkholderia* spp.) known to persist in biofilms **(Davies *et al.*, 1998; Costerton *et al.*, 1999; Conway *et al.*, 2002)**. Burfoot *et al.* (2009) demonstrated the need for pressure washing of stainless steel surfaces (in food production) to remove a biofilm of *P. aeruginosa*. The use of cold water for less than 60 s did not reduce the number of organisms however an increase in cleaning time did reduce the microbial load. Oulahal *et al.* (2004) used ultrasound in combination with chelating agents to remove *E. coli* or *S. aureus* biofilms. They concluded that the effectiveness in removing biofilms can depend on the type of microflora present. This indicates that different cleaning methods can affect Gram-positive and negative organisms in biofilms differently. The majority of detergents in use to remove surface contamination are not generally tested against microbes in biofilms **(Rayner *et al.*, 2004)**. Therefore the hospital cleaning agents may not be effective against organisms known to persist in biofilms. This may in part explain why during this present study only Gram-negative organisms known to persist in biofilms were detected on occasions after routine ICU ward cleaning.

Fungi could be isolated before and after routine ICU ward cleaning. Gniadek and Macura (2007) reported a lower number of fungal CFUs in the evening compared to morning sampling. Since samples during this present study were only taken in the morning there is no data to compare morning and evening. However routine ICU ward cleaning did reduce the number of fungal CFUs isolated. The observation by Gniadek and Macura may also be due to routine cleaning as this was not accounted for during their study. During this present study, there is a build-up of fungi (non-*albicans Candida* spp.) in the ICU environment between cleaning sessions. This suggests either fungal growth in the ICU environment or a constant re-introduction of fungal species, potentially from commensal fungi on patient, visitor or hospital staff skin **(Sullivan *et al.*, 1996; Hobson, 2003)**.

Since the emergence of MRSA and ESBL-producing organisms, several cleaning/disinfection procedures have been applied to remove these persistant organisms after outbreaks **(Wilcox *et al.*, 2003; Naiemi *et al.*, 2005; Dancer *et al.*, 2009)**. During this study target gene-carrying organisms (bla_{TEM} and *mec*A) could only be isolated on occasions before routine ICU ward cleaning. However, the antibiotic resistance determinant (*mec*A or bla_{TEM}) could be detected by PCR. This suggests that the cleaning regime kills/destroys the organism and the DNA containing the antibiotic resistance determinant remains (i.e. after cell lysis).

There is no evidence from the data presented in this chapter to suggest a permanent reservoir of antibiotic resistance on the wards. Previous cultural studies have, however, shown that TEM ESBL-producing organisms can persist in the ICU environment **(Naas *et al.*, 2002; Mammina *et al.*, 2007)**. In this present study bla_{TEM} genes persisted only in the ICU ward sink plughole, where they remained for three consecutive sampling periods over approximately 72 hours.

From data presented in previous chapters (Chapter 4 and 6) there was variability in the frequency of eubacterial DNA detection and fungal isolation, which may have been due to routine ICU ward cleaning. Data presented in this chapter indicates that routine ICU ward cleaning could account for the variability previously observed from hard surfaces; however cleaning did not affect the frequency of detection of some DGGE bands assigned as opportunistic organisms (*Burkholderia* spp. and *Stenotrophomonas* spp.).

Overall the data in this chapter indicates that the routine ICU ward cleaning regime had a substantial and statistically significant effect on the frequency of detection of eubacterial DNA. Cleaning reduced the number of fungal colonies, although fungi could be isolated before and after cleaning. The detection of antibiotic resistance determinants was of a low frequency and target gene-carrying organisms could only be isolated before cleaning, indicating no permanent persistence of antibiotic resistant organisms. Although DGGE bands assigned to opportunistic organisms (i.e. *Burkholderia* spp., *Acinetobacter* spp. and *Stenotrophomonas* spp.) were detected after routine ICU ward cleaning from sites of low patient contact, improvement in the cleaning regime may be beneficial.

8. Discussion

This study used cultural and molecular approaches to establish the microbial ecology of the ICU and HDU environment. The presence of important antibiotic resistance determinants was also assessed. Currently, there is great controversy about the role the ward environment plays in HAIs. Research is based heavily on the acquisition of HAIs, transmission between patients **(Shiomori *et al.*, 2002; French *et al.*, 2004; Oztoprak *et al.*, 2007; Howie and Ridley, 2008; Khan *et al.*, 2009)** and the identification of outbreak sources within the ward environment **(Neely *et al.*, 1999; Bures *et al.*, 2000; Naas *et al.*, 2002; Hartmann *et al.*, 2004)**. However, little is known about the microbial ecology of hospital wards, especially the ICU and HDU environments during periods of normal operation.

Throughout this present study the use of molecular techniques was essential to establish the microbial ecology of the ICU and HDU environments, particularly from sites where bacteria could not be cultured. Bacteria were infrequently cultured from a variety of ICU environmental sites (e.g. plugholes) however the detection of eubacterial DNA implies the presence of microorganisms. The use of PCR-DGGE enabled bacterial species, diversity and communities to be identified. Antibiotic resistant organisms could only be cultured before routine ICU ward cleaning, however, the use of molecular techniques (PCR) enabled the detection of antibiotic resistance determinants in the absence of gene-carrying organisms.

The data presented in this study therefore imply that the ICU and HDU environments were not acting as reservoirs of MRSA or ESBL-possessing organisms (detailed in Chapter 5). However, the data show non-*albicans Candida* species (*C. parapsilosis*, *C. tropicalis*, *C. glabrata*) (detailed in Chapter 6) and opportunistic Gram-negative species (*Stenotrophomonas* spp., *Burkholderia* spp., *Acinetobacter* spp.) (detailed in Chapter 4

and 7) were present. CNS and *Micrococcus* spp. were also frequently isolated from the ICU and HDU environments (detailed in Chapter 4).

The ICU sinks, floors and patient chairs were identified as environmental sites potentially acting as reservoirs of bacteria, fungi and antibiotic resistance determinants. The hospital sinks (interior surface of pipe below sink plughole) yielded the highest bacterial detection, yeast isolation and antibiotic resistance determinant detection. Routine ICU ward cleaning was also not effective against Gram-negative opportunistic species in the hospital sinks. Therefore, from this study the hospital sinks were the site of greatest environmental and hence potential clinical significance.

There was a widespread distribution of *Burkholderia* spp., *Stenotrophomonas* spp. (as detailed in Section 4.3.1 and 4.3.3 Chapter 4) and bla_{TEM} genes in the ICU environment (as detailed in Section 5.3.1 and 5.3.4 Chapter 5). The widespread distribution of *Burkholderia* spp. and *Stenotrophomonas* spp. during this present study is consistent with the hospital sink being the source/reservoir of general contamination with these organisms, although there are other explanations for this widespread distribution. Despite *Burkholderia* spp. not being isolated on *B. cepacia* complex specific media and the fact that bla_{TEM} gene-carrying organisms could only be isolated before routine ICU ward cleaning during this present study, there is evidence from previous studies that hospital sinks can act as sources of widespread contamination onto other sites, as detailed below.

Even though the majority of sites in the ICU are not areas of patient contact, hospital staff can come into contact (generally hand contact) with contaminated surfaces. Bhalla *et al.* (2004) did not provide evidence of patient-to-patient transmission; however they did show that contaminated environmental sites can significantly add to the contamination of hospital staff hands, which are the most recognised vehicle of nosocomial pathogen transmission. Hota *et al.* (2009) also demonstrated that,

dependent upon the ward design, it is possible that splash back from sinks can result in organisms (e.g. *P. aeruginosa*) travelling up to 1 m which can lead to the contamination of nearby medical equipment and devices. Another study showed that although the source of the *P. aeruginosa* outbreak was not defined, there was environmental contamination of the plughole **(Lango *et al.*, 2007)**.

Besides organism transmission from sinks onto medical equipment, it has previously been reported that after hand washing in a contaminated sink (*P. aeruginosa* or *B. cepacia*) positive hand cultures for *P.aeruginosa* and *B. cepacia* are observed **(Doring *et al.*, 1996)**. Other studies have also demonstrated contamination of hospital staff hands with *P. aeruginosa* from backsplash and aerosols during hand washing **(Kohn, 1970; Brown *et al.*, 1977; Noone *et al.*, 1983; Doring *et al.*, 1993)**. One study at a US teaching hospital isolated high CFUs of pathogenic bacteria (*P. aeruginosa*, *Stenotrophomonas maltophia* and *Serratia marscens*) from the sink exit section of drains (plughole) **(Brooke, 2008)**. The same study also indicated the potential for back splash to occur transferring pathogens onto hospital staff hands. This confirms that organisms in hospital sinks can be transmitted onto hospital staff hands or medical equipment, leading to the potential for pathogen transmission within the ward.

There is therefore a need to break the chain of transmission, for example, effectively cleaning hospital sinks would remove potential reservoirs. However, cleaning studies have previously identified the potential for re-distribution of organisms during cleaning and the effect of exposure time. One Welsh ICU study confirmed that the susceptibility of organisms to cleaning agents (e.g. sodium dichloroisocyanurate – NaDCC) is dependent upon exposure time **(Williams *et al.*, 2009a)**. For example, for NaDCC to kill MRSA it requires an exposure time $\geq$2 min, therefore the effectiveness of cleaning is not only affected by the cleaning agent but also method application **(Williams *et al.*, 2009a)**. Mops used during cleaning have been associated with picking up pathogenic bacteria and potentially redistributing them on the floor **(Dharan *et al.*, 1999)**.

Disinfectant wipes are commonly used to clean other hard surfaces, however, if the wipes are reused this can also lead to the redistribution of organisms **(Williams *et al.*, 2009b)**. Since this present study does not permit conclusions about the source of widespread *Burkholderia* spp. and *Stenotrophomonas* spp. in the ICU environment, it is not possible to assess the potential role of cleaning in the re-distribution of organisms in the ICU. Hence in spite of the fact cleaning appears to reduce the number of organisms on the ward, the possibility that it plays a role in the re-distribution of organisms around the ICU environment can not be excluded.

From the data obtained during this present study, it is unknown if the microbial build-up between cleaning sessions is due to environmental organism growth or re-introduction of the same organisms either within the ward or from the external environment. Future work would be necessary to investigate the time period for maximum microbial build-up between cleaning sessions (e.g. by taking samples every hour between sessions).

A possible explanation for the microbial build-up of Gram-negative organisms in sinks may be because the organisms detected during this present study are commonly associated with potable water (drinking water) and their persistence in biofilms **(Davies *et al.*, 1998; Costerton *et al.*, 1999; Conway *et al.*, 2002; Oulahal *et al.*, 2004; Burfoot *et al.*, 2009)**. *Burkholderia* spp. occupy a vast number of ecological niches including water and the hospital environment **(Zanetti *et al.*, 2000; Coenye and Vandamme, 2003; Stoyanova *et al.*, 2007)**. *Acinetobacter* spp. can be commensals on human skin and are often identified in water or moist sites. *Acinetobacter* spp. are severely problematic due to their ability to survive in dry environments also **(Baumann, 1968; Berlau *et al.*, 1999; Paterson, 2006)**. *Stenotrophomonas* spp. has also been found to survive in bottled water and output water from microfiltered water dispensers (leading directly to tap water), consistent with its survival primarily in moist environments **(Wilkinson and Kerr, 1998; Gales *et al.*, 2001; Sacchetti *et al.*, 2009)**.

Therefore the presence of these organisms in moist environments e.g. hospital sinks, is not uncommon.

In addition, the organisms that persist during cleaning (*Acinetobacter* spp. and *Stenotrophomonas* spp.) had also been isolated from ICU clinical samples during this present study. One possibility is that hospital staff may have come into contact with an infected patient resulting in the organism being detected in the hospital sink after hand washing. Evidence for this route (patient to staff) has been shown in previous research **(Bauer *et al.*, 1990; Bhalla *et al.*, 2004; French *et al.*, 2004)**.

Organisms present in biofilms are difficult to remove by cleaning. Since the emergence of MRSA and VRE as dangerous antibiotic resistant organisms in the hospital setting, studies have focused on development of cleaning agents to target these persistent Gram-positive organisms **(Wilcox *et al.*, 2003; Denton *et al.*, 2004; Williams *et al.*, 2009a; Williams *et al.*, 2009b)**. This may in part explain why Gram-negative organisms could be detected in hospital sinks after routine ICU ward cleaning during this present study. Evidence to support this is shown from previous outbreak studies. In these previous studies, the sinks were not specifically identified as the source of infection, but the outbreak could only be halted/stopped after the ward sinks had either been extensively cleaned or removed **(Bukholm *et al.*, 2002; Naas *et al.*, 2002; Wilks *et al.*, 2006; Kotsanas *et al.*, 2008)**.

It is widely acknowledged that sinks have a high bacterial density and diversity **(Davies *et al.*, 1998; Costerton *et al.*, 1999; Conway *et al.*, 2002)**, which could lead to gene transfer. Despite a lack of culturable bacteria, the hospital sinks in this present study yielded antibiotic resistance determinants at low frequency. Cleaning agents commonly result in cell death leading to cell lysis and liberation of DNA so the gene can be detected in the absence of the organism before DNA degradation. However, organisms that are still present after cleaning may be able to take up this naked DNA

by transformation to acquire antibiotic resistance (as suggested by Tenover, 2006). Previous studies have shown the ability for lateral gene transfer of bla_{TEM} **(Elwell *et al.*, 1977; Weill *et al.*, 2004; Lachmayr *et al.*, 2009)**. There is a high probability of bla_{TEM} gene transfer due to their association with plasmids and transposons **(Livermore, 1995; Naiemi *et al.*, 2005; Manzur *et al.*, 2007)**. Therefore hospital sinks are a potential site for gene transfer, where multiple Gram-negative organisms are present. It is also possible for wild type bla_{TEM} genes to mutate to an ESBL **(Wu *et al.*, 1994; Paterson & Bonomo, 2005; Hammond *et al.*, 2005)** which is of clinical significance particularly from the evidence discussed, of transmission from hospital sinks onto staff hands and medical equipment.

In comparing the effect of cleaning on the detection of eubacterial DNA from the RHH and NGH ICU environments there was no statistical difference despite the NGH ICU being cleaned three times a day as opposed to twice at the RHH ICU. However, if the number of cleaning sessions were reduced at the NGH ICU this could potentially have an impact on the detection of bacteria and fungi. As mentioned, the NGH ICU has a higher patient intake and a greater number of hospital staff compared to the RHH ICU. Therefore, the extra cleaning session at the NGH may be required to prevent increased microbial build-up in the ICU environment from internal or external sources. Further investigation would be needed to decide whether cleaning sessions could be reduced or increased at either hospital ICU.

From the data presented in this study it can be concluded, that the routine ICU ward cleaning regime was only effective on hard surfaces. ICU ward sinks are a potential cause for concern as they yielded opportunistic Gram-negative species, non-*albicans* *Candida* spp. and antibiotic resistance determinants before and after routine ICU ward cleaning. Targeted additional cleaning could further improve infection control, to ensure the removal of pathogenic organisms (e.g. *S. maltophilia*, *Acinetobacter* spp. and *Burkholderia* spp.) from the ICU environment. Alternatively, indicator organisms

e.g. *Acinetobacter* spp. in sinks and CNS on hand-touch sites could be used to indicate the build-up of microbes in the ward environment and the potential benefit of extra cleaning.

Future work: -

A number of tetracycline resistant colonies were cultured but the *tet* gene was not identified, further work would be to identify which other *tet* genes were present from the colonies identified in the ICU environment, using a macro-array.

Burkholderia spp.-like sequences were matched from DGGE bands from the sites of greatest eubacterial DNA detection (ICU/HDU patient chair, ICU floor and ICU ward sink plugholes), future work could be done to try and observe if these sequences are present in other sites of the ICU environment.

The question still remains as to whether the environment becomes contaminated from the patient or vice versa. Further sampling and close monitoring of patient intake followed by typing and sequencing of environmental and clinical isolates may enable this question to be answered in part.

9. References

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07 February 2008

Dr Tom Smith
Senior Lecturer
Sheffield Hallam University
BMRC, Faculty of Health & Wellbeing
Sheffield Hallam University
City Campus
Sheffield
S1 1WB

Dear Dr Smith

Full title of study: **Microbial Ecology of the Intensive Care Unit**
REC reference number: **08/H1310/2**

The Research Ethics Committee reviewed the above application at the meeting held on the 31 January 2008. Thank you for attending to discuss the study.

Discussion

Three members of the Research Team attended the meeting i.e. yourself, Dr K Stanley and Ms G Kay. It was observed there were no major ethical concerns in relation to this study and it was a useful piece of research that was well supported.

You were asked to clarify the relationship between the data you were hoping to collect from the floors, walls, surfaces etc and the access to patients' clinical data as well as the timeframe involved. It was explained that the clinical data you would be using was from samples taken from the patient on a routine basis and the team would be particularly interested in samples that were obtained at the same time as they were sampling. Arrangements had been made with the Clinical Microbiologist at the hospital that samples could be obtained in order that the micro-organisms could be stored if necessary. The clinical data would be available to the research team one or two days after the samples had been taken.

It was confirmed that arrangements were in place for the relevant Consultants to be informed of any findings in order that they could take whatever action they felt was necessary and it was acknowledged that patient care should always take priority over the research. The Infection Control Team would also be notified of any findings.

The team acknowledged the possibility that the behaviour of staff on the Unit, who were being observed over quite a long period of time, may change (i.e. the Hawthorne effect) but it was felt that the chances of that happening were remote and that was the reason why the team were taking the approach in the Unit that this was an ecological study.

It was queried whether there was a quantitative aspect to the research as well as qualitative. It was clarified that the study was essentially qualitative in that the team would be trying to ascertain which organisms were present rather than how many times they appeared.

The team was asked to clarify the timeframe for the sampling and it was explained you would be looking to take samples throughout the eighteen month period of the study. In the first instance you would be taking samples on a weekly basis with regular reviews to ascertain whether more or less samples were required. In addition there would be intensive periods of sampling once a week with regular reviews in which any trends identified could be clarified.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application		21 December 2007
Investigator CV		
Protocol	1	19 December 2007
Covering Letter		20 December 2007
Letter from Sponsor		21 December 2007
Peer Review		19 December 2007
Project Summary for ICU Staff	1	23 May 2007
Key Collaborator's CV - Gary H Mills		
Key Collaborator's CV - Karen Stanley		
Flowchart of Protocol	1	19 December 2007

R&D approval

You should arrange for the R&D office at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final approval from the R&D office before commencing any research procedures.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

08/H1310/2	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

J Brown

Jo Abbott
Chair

This Research Ethics Committee is an advisory committee to Yorkshire and The Humber Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES directorate within The National Patient Safety Agency and Research Ethics Committees in England

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Human Resources Department
4 Claremont Place
Sheffield
S10 2TB

Please ask for: Lynn Winter
Telephone: (0114) 271 2796
Date: 21 March 2007

Private & Confidential
Gemma Kay
Apartment 56
Jet Centro
79 St Marys Road
Sheffield
S2 4AU

Dear Gemma

LETTER OF AUTHORITY

I write to inform you that Authority is granted to enable you to take environmental samples from surfaces in the Intensive Care Unit, at the Royal Hallamshire Hospital one day per week from 09 March 2007 to 21 December 2009.

During this period you will be under the supervision of Dr Gary Mills, Research Lead for Intensive Care, Anaesthesia and Theatres.

In accordance with normal procedure this authority is subject to satisfactory medical fitness.

With the exception of small valuables handed to the Authority for safekeeping, the Trust accepts no responsibility for damage to, or loss of, personal property, you are therefore recommended to take out an insurance policy to cover your personal property.

During this placement you may have access to confidential information concerning the hospital and its patients. You must, therefore, observe the following guidelines:

No information shall be disclosed to any third party in respect of any patient.
No information shall be disclosed to any third party in respect of any employee.
No information shall be disclosed to any third party in respect of the hospital, its statistics and its finances.

If you intend to terminate this placement before the stated date, please inform me as soon as possible.

Please note that you will be unable to attend for your visit if you do not have a copy of this letter on the day of your visit to the department.

Finally, I would like to welcome you to the Sheffield Teaching Hospitals NHS Trust and I hope that the period spent with us will prove interesting and beneficial.

Yours sincerely
L. Winter
Lynn Winter
HR Assistant

Please sign both copies of this letter and return one copy to the Human Resources Departmen

I have read and understood your letter dated 21 March 2007 and I undertake to abide by the contents.

Name Gemma Kay

Date

Signature GKay

Cc Gary Mills Gary M. Mills.

Ref: STH14947/AD

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

04 March 2008

Dr Gary Mills
Intensive Care Unit
R Floor
RHH

Dear Dr Mills

Authorisation of project

STH ref: STH14947
Study title: Molecular Microbial Ecology of the Intensive Care Unit

Chief Investigator (Local Contact): Dr Gary Mills, STH
Principal Investigator (Student): Ms Gemma Kay

Sponsor: Sheffield Hallam University
Funder: Sheffield Hallam University

The Research Department has received the required documentation for the study as listed below:

1.	**Sponsorship IMP studies (non-commercial)** **Sponsorship responsibilities between institutions** **Responsibilities of investigators** **Monitoring Arrangements**	NA NA NA NA
2.	**STH registration document: completed and signed**	STH Finance Form, Dr G Mills, 20 February 08
3.	**Evidence of favourable scientific review**	Sheffield Hallam University, 17 December 07
4.	**Protocol – final version**	Version 1, December 07
5.	**Participant Information sheet – final version**	NA
6.	**Consent form – final version**	NA
7.	**Signed letters of indemnity**	NA
8.	**ARSAC / IRMER certificate**	NA
9.	**Evidence of hosting approval from STH directorate**	STH Finance Form, Dr M Richmond, 20 February 08
10.	**Evidence of approval from STH Data Protection Officer**	STH Finance Form, Mr P Wilson, 27 February 08
11.	**Letter of approval from REC**	South Yorkshire REC, 08/H1310/2, 07 February 08
12.	**Proof of locality approval**	NA

13. Clinical Trial Authorisation from MHRA	NA
14. Honorary Contract	Gemma Kay, Letter of Authority, 09 March 07
15. Associated documents	NA
16. Signed financial agreement/contract	STH Finance Form, Mrs L Fraser, 26 February 08

The project has been reviewed by the Research Department and authorised by the Director of R&D on behalf of STH NHS Foundation Trust to begin.

Yours sincerely

Professor S Heller
Director of R&D, Sheffield Teaching Hospitals NHS Foundation Trust
Telephone +44 (0) 114 2265934
Fax +44 (0) 114 2265937

cc. Gemma Kay, SHU

RHH ICU and HDU swab samples for DNA extraction and microbe enumeration (after culturing) (continued over page)

Sample site	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Bedside (ICU)	RHH-1 #2	RHH-2 #2	RHH-3 #2	RHH-4 #2	RHH-7 #2	RHH-8 #2	RHH-9 #2	RHH-10 #2	RHH-11 #2	RHH-12 #2	RHH-13 #2	RHH-14 #2
Computer keyboard (ICU)	RHH-1 #8	RHH-2 #8	RHH-3 #8	RHH-4 #8	RHH-7 #8	RHH-8 #8	RHH-9 #8	RHH-10 #8	RHH-11 #8	RHH-12 #8	RHH-13 #8	RHH-14 #8
Fan (ICU)	RHH-1 #12	RHH-2 #12	RHH-3 #12	RHH-4 #12	RHH-7 #12	RHH-8 #12	RHH-9 #12	RHH-10 #12	RHH-11 #12	RHH-12 #12	RHH-13 #12	RHH-14 #12
Floor (ICU)	RHH-1 #1/7	RHH-2 #1/7	RHH-3 #1/7	RHH-4 #1/7	RHH-7 #1/7	RHH-8 #1/7	RHH-9 #1/7	RHH-10 #1/7	RHH-11 #1/7	RHH-12 #1/7	RHH-13 #1/7	RHH-14 #1/7
HDU computer keyboard	RHH-1 #15	RHH-2 #15	RHH-3 #15	RHH-4 #15	RHH-7 #15	RHH-8 #15	RHH-9 #15	RHH-10 #15	RHH-11 #15	RHH-12 #15	RHH-13 #15	RHH-14 #15
HDU computer stand	RHH-1 #17	RHH-2 #17	RHH-3 #17	RHH-4 #17	RHH-7 #17	RHH-8 #17	RHH-9 #17	RHH-10 #17	RHH-11 #17	RHH-12 #17	RHH-13 #17	RHH-14 #17
HDU patient chair	RHH-1 #18/19/ 20	RHH-2 #18/19/ 20	RHH-3 #18/19/ 20	RHH-4 #18/19/ 20	RHH-7 #18/19/ 20	RHH-8 #18/19/ 20	RHH-9 #18/19/ 20	RHH-10 #18/19/ 20	RHH-11 #18/19/ 20	RHH-12 #18/19/ 20	RHH-13 #18/19/ 20	RHH-14 #18/19/ 20
HDU picture	RHH-1 #16	RHH-2 #16	RHH-3 #16	RHH-4 #16	RHH-7 #16	RHH-8 #16	RHH-9 #16	RHH-10 #16	RHH-11 #16	RHH-12 #16	RHH-13 #16	RHH-14 #16
HDU staff chair	RHH-1 #21	RHH-2 #21	RHH-3 #21	RHH-4 #21	RHH-7 #21	RHH-8 #21	RHH-9 #21	RHH-10 #21	RHH-11 #21	RHH-12 #21	RHH-13 #21	RHH-14 #21

Sample site	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	aSHV Sample session 12
Picture (ICU)	RHH-1 #11	RHH-2 #11	RHH-3 #11	RHH-4 #11	RHH-7 #11	RHH-8 #11	RHH-9 #11	RHH-10 #11	RHH-11 #11	RHH-12 #11	RHH-13 #11	RHH-14 #11
Plughole (ICU ward sink)	RHH-1 #5/6	RHH-2 #5/6	RHH-3 #5/6	RHH-4 #5/6	RHH-7 #5/6	RHH-8 #5/6	RHH-9 #5/6	RHH-10 #5/6	RHH-11 #5/6	RHH-12 #5/6	RHH-13 #5/6	RHH-14 #5/6
Tap (ICU)	RHH-1 #9/10	RHH-2 #9/10	RHH-3 #9/10	RHH-4 #9/10	RHH-7 #9/10	RHH-8 #9/10	RHH-9 #9/10	RHH-10 #9/10	RHH-11 #9/10	RHH-12 #9/10	RHH-13 #9/10	RHH-14 #9/10
Trolley (ICU)	RHH-1 #4	RHH-2 #4	RHH-3 #4	RHH-4 #4	RHH-7 #4	RHH-8 #4	RHH-9 #4	RHH-10 #4	RHH-11 #4	RHH-12 #4	RHH-13 #4	RHH-14 #4
Sluice room sink plughole	RHH-1 #13/14	RHH-2 #13/14	RHH-3 #13/14	RHH-4 #13/14	RHH-7 #13/14	RHH-8 #13/14	RHH-9 #13/14	RHH-10 #13/14	RHH-11 #13/14	RHH-12 #13/14	RHH-13 #13/14	RHH-14 #13/14
Window ledge (ICU)	RHH-1 #3	RHH-2 #3	RHH-3 #3	RHH-4 #3	RHH-7 #3	RHH-8 #3	RHH-9 #3	RHH-10 #3	RHH-11 #3	RHH-12 #3	RHH-13 #3	RHH-14 #3

NGH ICU and HDU swab samples for DNA extraction and microbe enumeration (after culturing) (continued over page)

Sample site	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Computer keyboard (ICU)	NGH-2 #15	NGH-3 #15	NGH-4 #15	NGH-5 #15	NGH-6 #15	NGH-7 #15	NGH-10 #15	NGH-11 #15	NGH-12 #15	NGH-13 #15	NGH-14 #15	NGH-15 #15
Door handle (ICU)	NGH-2 #17	NGH-3 #17	NGH-4 #17	NGH-5 #17	NGH-6 #17	NGH-7 #17	NGH-10 #17	NGH-11 #17	NGH-12 #17	NGH-13 #17	NGH-14 #17	NGH-15 #17
Floor – isolation room (ICU)	NGH-2 #6/7	NGH-3 #6/7	NGH-4 #6/7	NGH-5 #6/7	NGH-6 #6/7	NGH-7 #6/7	NGH-10 #6/7	NGH-11 #6/7	NGH-12 #6/7	NGH-13 #6/7	NGH-14 #6/7	NGH-15 #6
Floor – ward (ICU)	NGH-2 #18/19	NGH-3 #18/19	NGH-4 #18/19	NGH-5 #18/19	NGH-6 #18/19	NGH-7 #18/19	NGH-10 #18/19	NGH-11 #18/19	NGH-12 #18/19	NGH-13 #18/19	NGH-14 #18/19	NGH-15 #18/19
HDU computer keyboard	NGH-2 #4	NGH-3 #4	NGH-4 #4	NGH-5 #4	NGH-6 #4	NGH-7 #4	NGH-10 #4	NGH-11 #4	NGH-12 #4	NGH-13 #4	NGH-14 #4	NGH-15 #4
HDU door switch	NGH-2 #1	NGH-3 #1	NGH-4 #1	NGH-5 #1	NGH-6 #1	NGH-7 #1	NGH-10 #1	NGH-11 #1	NGH-12 #1	NGH-13 #1	NGH-14 #1	NGH-15 #1
HDU floor	NGH-2 #3	NGH-3 #3	NGH-4 #3	NGH-5 #3	NGH-6 #3	NGH-7 #3	NGH-10 #3	NGH-11 #3	NGH-12 #3	NGH-13 #3	NGH-14 #3	NGH-15 #3
HDU plughole	NGH-2 #2	NGH-3 #2	NGH-4 #2	NGH-5 #2	NGH-6 #2	NGH-7 #2	NGH-10 #2	NGH-11 #2	NGH-12 #2	NGH-13 #2	NGH-14 #2	NGH-15 #2

Sample site	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Machine handle (ICU)	NGH-2 #13/14	NGH-3 #13/14	NGH-4 #13/14	NGH-5 #13/14	NGH-6 #13/14	NGH-7 #13/14	NGH-10 #13/14	NGH-11 #13/14	NGH-12 #13/14	NGH-13 #13/14	NGH-14 #13/14	NGH-15 #13/14
Patient chair (ICU)	NGH-2 #9/10/ 11/12	NGH-3 #9/10/ 11/12	NGH-4 #9/10/ 11/12	NGH-5 #9/10/ 11/12	NGH-6 #9/10/ 11/12	NGH-7 #9/10/ 11/12	NGH-10 #9/10/ 11/12	NGH-11 #9/10/ 11/12	NGH-12 #9/10/ 11/12	NGH-13 #9/10/ 11/12	NGH-14 #9/10/ 11/12	NGH-15 #9/10/ 11/12
Plughole (ICU ward sink)	NGH-2 #5/8	NGH-2 #5/8	NGH-4 #5/8	NGH-5 #5/8	NGH-6 #5/8	NGH-7 #5/8	NGH-10 #5/8	NGH-11 #5/8	NGH-12 #5/8	NGH-13 #5/8	NGH-14 #5/8	NGH-15 #5/8
Sluice room sink plughole	NGH-2 #20/21	NGH-3 #20/21	NGH-4 #20/21	NGH-5 #20/21	NGH-6 #20/21	NGH-7 #20/21	NGH-10 #20/21	NGH-11 #20/21	NGH-12 #20/21	NGH-13 #20/21	NGH-14 #20/21	NGH-15 #20/21
Window ledge (ICU)	NGH-2 #16	NGH-3 #16	NGH-4 #16	NGH-5 #16	NGH-6 #16	NGH-7 #16	NGH-10 #16	NGH-11 #16	NGH-12 #16	NGH-13 #16	NGH-14 #16	NGH-15 #16

RHH ICU and HDU contact plate samples for microbe enumeration after culturing

Sample site[1]	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Chair back (HDU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14
Chair base (HDU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14
Curtain (ICU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14

[1] Each contact plate sample was labelled with the sample site and code provided

NGH ICU contact plate samples for microbe enumeration after culturing

Sample site[1]	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Chair back (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Chair base (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Curtain (ICU)	NGH-2	NGH-3	NGH-4	ِ GH-5	omonas	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15

[1] Each contact plate sample was labelled with the sample site and code provided

RHH ICU and HDU dipslide samples for microbe enumeration after culturing

Sample site[1]	12/05/08 Sample session 1	02/06/08 Sample session 2	30/06/08 Sample session 3	14/07/08 Sample session 4	15/09/08 Sample session 5	29/09/08 Sample session 6	13/10/08 Sample session 7	03/11/08 Sample session 8	17/11/08 Sample session 9	01/12/08 Sample session 10	08/12/08 Sample session 11	15/12/08 Sample session 12
Door handle (ICU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14
Handwash bottle (ICU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14
Handwash bottle (HDU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14
Light switch (ICU)	RHH-1	RHH-2	RHH-3	RHH-4	RHH-7	RHH-8	RHH-9	RHH-10	RHH-11	RHH-12	RHH-13	RHH-14

[1] Each dipslide sample was labelled with the sample site and code provided

NGH ICU dipslide samples for microbe enumeration after culturing

Sample site[1]	11/03/08 Sample session 1	08/04/08 Sample session 2	15/05/08 Sample session 3	03/06/08 Sample session 4	01/07/08 Sample session 5	15/07/08 Sample session 6	16/09/08 Sample session 7	30/09/08 Sample session 8	14/10/08 Sample session 9	04/11/08 Sample session 10	18/11/08 Sample session 11	09/12/08 Sample session 12
Blind switch (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Handwash bottle corridor (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Handwash bottle isolation room (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Inner window switch (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Intercom button (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15
Light switch (ICU)	NGH-2	NGH-3	NGH-4	NGH-5	NGH-6	NGH-7	NGH-10	NGH-11	NGH-12	NGH-13	NGH-14	NGH-15

[1] Each dipslide sample was labelled with the sample site and code provided

ABgene	ABgene Ltd
	ABgene House
	Blenheim Road
	Epsom
	Surrey
	KT19 9AP
Bio-Rad	Bio-Rad Laboratories Ltd
	Bio-Rad House
	Maxted Road
	Hemel Hempstead
	Hertfordshire
	HP2 7DX
Cherwell Laboratories	Cherwell Laboratories Ltd
	7 & 8 Launton Business Centre
	Murdock Road
	Bicester
	OX26 4XB
E&O Laboratories	E&O Laboratories
	Burnhouse
	Bonnybridge
	Scotland
	FK4 2HH
Eppendorf	Eppendorf UK Limited
	Endurance House
	Chivers Way

	Histon
	Cambridge
	CB24 9ZR
Eurofins	Eurofins Genetic Services Ltd
	318 Worple Road
	Raynes Park
	London
	SW20 8QU
Fermentas	Fermentas GMBH
	Opelstrasse 9
	D-68789
	St Leon-Rot
Gallenkamp	Weiss-Gallenkamp
	Units 37-38
	The Technology Centre
	Epinal Way
	Loughborough
	LE11 3GE
Heraeus	Mandel Scientific Company Inc
	2 Admiral Place
	Guelph
	Ontario
	Canada
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Invitrogen	Invitrogen 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF
New England Biolabs	New England Biolabs 75-77 Knowl Piece Wilbury Way Hitchin Herts SG4 0TY
Qiagen Ltd	Qiagen Ltd Qiagen House Fleming Way Crawley West Sussex RH10 9NQ
Roche	Roche Products Limited 6 Falcon Way Shire Park Hexagon Place Welwyn Garden City Hertfordshire AL7 1TW

Sigma	Sigma-Aldrich The Old Brickyard New Road Gillingham Dorset SP8 4XT
Stuart Scientific	Rhys International Ltd Unit 41 Halliwell Business Park Rossini Street Bolton Greater Manchester BL1 8DL
Thermo Fisher Scientific	Bishop Meadow Road Loughborough Leicestershire LE11 5RG
UVP	Ultra-Violet Products Ltd Unit 1 Trinity Hall Farm Estate Nuffield Road Cambridge CB4 1T