Understanding the intersection between the hospital and patient microbiome

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ABSTRACT

The microbiota of the built environment has profound implications especially within healthcare. Identifying the movement of microbes within the hospital environment not only remains central for infection control practices but also has major influences on our understanding and management of hospital acquired infections. Despite this, our awareness of microbial reservoirs and their interaction with patients in hospitals remains poor with research mainly limited to outbreak settings. As we move away from traditional culture-based methods, the ability to investigate some of these questions has increased through utilisation of culture-independent techniques.

The overarching aim of this thesis is to understand the complex relationship that exists between microorganisms that colonise a hospital environment and humans occupying the same environment. In this thesis, microbial tracking, and antimicrobial resistance gene (AMR) transmission is explored through utilisation of culture-independent methods specifically by 1) investigating the effects of hospitalisation on inpatient microbiota, 2) examining the characteristics of the hospital microbiome in relation to changes in patient occupancy and space utilisation and 3) evaluating the extent of antimicrobial resistance gene transmission in an open-plan hospital ward.

The characteristics of oropharyngeal (OP) microbiota in hospital inpatients without pneumonia is currently unknown. We explored the degree to which the OP microbiology in these patients compared to healthy controls through a cross-sectional study *(Chapter 2)* which demonstrated a reduction in diversity and abundance of commensal taxa in inpatients, potentially contributing to an increased risk of hospital-acquired pneumonia in these patients.

The presence and movement of microbes is largely influenced by human occupancy and space utilisation. A longitudinal study was conducted between two hospital sites exploring the changes within a hospital microbiome during the transition from an established hospital to a brand-new hospital *(Chapter 3)*. In this study, occupancy was found to be a major determinant of bacterial dispersion within the hospital environment. Bacterial load and microbiota composition were unchanged between the old and new facility at similar occupancy levels despite differing building age and architecture.

Finally, the extent of AMR gene transmission within an open-plan ward was investigated *(Chapter 4)*. Although preliminary results identified significant relationships between resistance genes and inter-patient distance, the majority of these determinants were found to be associated with the cohorting of patients according to a specific medical condition (tuberculosis) rather than wider inter-patient AMR gene transmission within the ward. This suggests that in lower-middle income countries such as Myanmar, transmission within the hospital is a relatively minor contributor to AMR dispersion and focus of AMR control should be at a population level.

Together, the results of this thesis demonstrate the clinical value of culture-independent methods in assessing microbial movement including resistance genes dispersion within the hospital environment and its interaction with the human population. These findings represent an important step towards developing a precision medicine approach to infection control practices based on the microbial characteristics of a healthcare facility.

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DECLARATION OF AUTHENCITY

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
BC	Bray-Curtis
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centre for Disease Control and Prevention
CF	Cystic fibrosis
DALY	Disability-adjusted life year
DISTLM	DISTance based Linear Models
ED	Emergency department
FDR	False discovery rate
GDP	Gross domestic product
HAIs	Hospital acquired infections
HAP	Hospital acquired pneumonia
HBA	Horse blood agar
HCW	Healthcare worker
HIC	High-income countries
ICU	Intensive care unit
IQR	Interquartile range
LMIC	Low- and middle-income countries
LRTI	Lower respiratory tract infection
MBL	Metallo-β-lactamases
MDRTB	Multi-drug resistant TB
MRSA	Methicillin resistant *Staphylococcus aureus*
NMDS	Non-metric multidimensional scaling

LIST OF ABBREVIATIONS (continued)

OP	Oropharyngeal
OT	Operating theatres
PCoA	Principal coordinates analysis
PERMDISP	Homogeneity of multivariate dispersions
PERMANOVA	Permutational multivariate analysis of variance
(q)PCR	(Quantitative) polymerase chain reaction
RAH	Royal Adelaide Hospital
SAHMRI	South Australian Health and Medical Research Institute
SS	Sum of squares
SNVs	Single-nucleotide variants
TB	Tuberculosis
TE	Tris-EDTA
URT	Upper respiratory tract
UV	Ultraviolet
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

This chapter comprises the literature review portion of the thesis. Concepts within this review were adapted into the three major studies within this thesis.

1.1 INTRODUCTION

Hospitals facilitate several processes within the modern healthcare system, including the provision of inpatient care and the centralisation of specialist expertise and facilities. Consequently, hospitals necessarily draw together large numbers of people who are vulnerable to infection or who carry transmissible infectious agents. Despite this, hospitals are traditionally defined as clean environments. This is largely achieved by strict cleaning procedures within the hospital and adherence to infection control measures by staff members. However, we accept that humans will continue to shed organisms into the environment (1) and that bacteria will continue to exist within the hospital environment (2).

1.2 THE HOSPITAL ENVIRONMENT AS A RESERVOIR

A clean hospital is important to patient safety. Despite this, the evidence suggests there is transmission within the hospital environment of both organisms and antimicrobial resistance genes (3, 4). Within the hospital environment, microbial dispersal can occur through several different mechanisms. Contact transmission, for example through touching, is the route associated with the majority of person-to-person transmission (5). The hospital environment can also act as an intermediary in pathogen dispersal. This can take the form of short-term microbial reservoirs, such as aerosolised droplets suspended within the air column, or longer-term colonisation of hospital fomites or contaminated medical equipment and devices (6).

The presence of bacteria has been demonstrated on keyboards (7, 8), medical stethoscopes, bed rails (2), lift buttons (9) and hospital worker's white coats (10), using both culture-based and culture-independent methods. Using the broth enrichment method, Eckstein et al correlated the presence of vancomycin-resistant *Enterococcus* (VRE) on hospital room surfaces such as bedrails, toilets, and call buttons with a VRE colonised or infected occupant (11). Analysis based on bacterial genome sequencing has demonstrated reservoirs contribute to pathogen transmission. *Mycobacterium abscessus* can be transmitted between patients with cystic fibrosis via fomites in shared treatment rooms (12). The colonisation of hospital sinks and faucets by biofilm-forming gram-negative bacteria, such as *Pseudomonas aeruginosa* can result in infections in patients (13).

Environmental microbial activity has been demonstrated in high acuity environments such as intensive care units. In a matched faecal and built environment time series study in a neonatal intensive care unit (ICU) it was demonstrated, with the use of culture-independent methods, that infant gut microbial taxa were widely disseminated in the environment. Sinks, feeding and intubation tubes and healthcare provider hands were most colonised (14). Recent data suggests patients are at higher risk of acquiring multi-resistant gram-positive and gram-negative organisms when accommodated in an area previously occupied by a patient colonised or infected by these organisms (15-17).

The role of healthcare workers (HCW) in microbial transmission within the hospital environment is likely understated. There is evidence that *Staphylococcus aureus*, *Clostridioides difficile* and VRE can persist in the environment for a prolonged period and can transiently colonise the hands of HCW leading to patient transmission events (18, 19). A study examining the frequency of bacterial pathogens on HCW hands after contact with hospital environment surfaces found that 53% of the study participants hand imprints cultured one or more pathogens after touching the hospital environmental surface (20).

1.3 PATHWAYS OF MICROBIAL TRANSMISSION WITHIN THE HOSPITAL ENVIRONMENT

Such findings highlight the importance of targeting and potentially reducing environmental reservoirs, as well as the need to consider the risk of placing vulnerable individuals into shared spaces with those potentially carrying infective agents (21). To develop effective infection control strategies, it is essential to understand the possible routes of transmission of pathogens, including the environmental intermediaries.

The differences in transmissibility between infective agents reflect both their mode of infection and their ability to persist within the environment. Enveloped viral particles are particularly vulnerable to desiccation as a result of their acellular nature and are commonly transmitted through aerosolised droplets or contaminated water sources (22). In contrast, non-enveloped viruses, such as norovirus, survive in harsh environmental conditions, and can survive on environmental surfaces for up to two weeks (23). Some bacterial and fungal pathogens can produce highly resilient spores that cannot be desiccated with heat, ultraviolet light or surfactants used in cleaning (24, 25). The most notable spore-forming bacterium within the hospital environment is *Clostridioides difficile* which can remain in its endospore state for weeks to months (26). Non-spore forming clinically important organisms including methicillin resistant *Staphylococcus aureus* (MRSA) and VRE can be detected on dry surfaces for more than a year (27).

In recent times, attempts have been made to understand the dispersal of specific groups of pathogens. Bridges et al endeavoured to describe influenza transmission within the healthcare environment (28). Their findings suggested that in addition to droplet spread, the virus could be transmitted from non-porous surfaces to hands of HCW and patients for up to 24 hours after deposition. Patients with cystic fibrosis (CF) are at risk of recurrent bacterial respiratory infections. Wood et al investigated the extent of airborne dissemination of common non-*Pseudomonas aeruginosa* pathogens generated through coughing by patients with CF. They

concluded that gram-negative bacteria and *Staphylococcus aureus* can travel up to four metres and remain viable within droplet nuclei in the air for up to 45 minutes (29).

More recently, metallo-β-lactamases (MBLs) have been increasingly reported in non-fermenting gram-negative bacteria and Enterobacterales. The ability of MBLs to hydrolyse nearly all β-lactam agents, including the carbapenems, results in extremely limited treatment options. Multiple studies have investigated the molecular epidemiology and risk factors of MBL infections (30-33). One such study conducted in Brazil demonstrated a clonal predominant genotype among their MBL-producing *Pseudomonas aeruginosa* isolates. This suggests cross-transmission of high level resistance among their isolates emphasising the importance infection control strategies (34).

Investigating specific groups of pathogens and resistance genes which exist within the hospital environment limits our ability to understand the overall microbial community movement and by extension the risk of dispersal and transmission of microorganisms.

Instead, understanding the process of ongoing pathogen dispersal requires an approach that captures all microbes and antimicrobial resistance (AMR) genes within the patient microbiome, regardless of the bacterial species that carry them or the antibiotics to which they confer resistance.

1.4 UTILISATION OF CULTURE INDEPENDENT METHODS

While the need to better understand the pathways and mediators of microbial dispersal within the hospital environment is clear, generating the data presents challenges. Historically, the detection of common hospital pathogens has relied on culture-based microbiology deployed in a targeted fashion. This is relatively low-cost and utilises well-established techniques. The strengths of such an approach include the detection of only viable and therefore potentially infective microbes. Culture-based microbiology is useful when assessing potential routes of

transmission of specific microbes. There are however several disadvantages of culture-based methods. This approach is insufficient to differentiate transmitted microbes from unrelated but phenotypically similar resident microbes. This is an important component in identifying transmission events. Many human-associated microbes are not identifiable with standard culture techniques, limiting the scope of such an approach (35).

The first era of molecular analysis began in the 1980s with the development of polymerase chain reaction (PCR). PCR has the ability to amplify a specific segment of DNA to generate multiple copies of a particular DNA sequence (36). Molecular analysis has progressed with further interest in unculturable organisms leading to the next era of analysis termed metagenomics. Metagenomic analysis typically includes two sequencing strategies: 16S rRNA amplicon sequencing (hereafter 16S sequencing) or shotgun-based metagenomics.

Each analytic method offers a different degree of information. 16S sequencing provides information on bacterial abundance to the genus level, while metagenomics allows bacterial species identification and description of the whole microbial community function (37). Shotgun metagenomic sequencing involves extracting DNA from all cells in a community and shearing it into smaller fragments which are then sequenced independently. Functional metagenomics involves isolating DNA from microbial communities to study the functions of encoded proteins (38). Such approaches can be used in combination with prior culture-based isolation of microbes to differentiate subpopulations of community organisms and identify transmission events (39). However, they can also be applied directly to clinical or environmental samples in a culture-independent manner.

The biggest impact of culture-independent investigations within the health setting has been in the area of the human microbiome. The concept of microbiome refers to the microorganisms and their collective genomes living in their surrounding environment including metabolites (40). Over the last two decades, studies investigating the human microbiome, including the initial Human Microbiome Project (2007)(41) have utilised culture-independent approaches to

understand the impact of disease and interventions on the human microbiome. New approaches to the study of the respiratory microbiota have not only increased our knowledge of the healthy lungs (42) but also lung changes in chronic conditions such as chronic obstructive pulmonary diseases (43,44), bronchiectasis (45) and interstitial lung disease (46), influence of lifestyle factors such as smoking on respiratory microbiota (47) as well as effects of lung transplantation and immunosuppression (48).

The gut microbiota has been an area of interest ranging from the early development of gut microbiota in infancy (49), composition of healthy gut microbiota (50) to causes of gut dysbiosis (51). Gut microbiota has been linked with gastrointestinal conditions such as inflammatory bowel disease (52), irritable bowel syndrome (53), and systemic metabolic diseases (54). Therapeutic manipulation of the gut microbiome through faecal microbiota transplantation has been extensively investigated as a treatment option for *Clostridiodes difficile* infection (55).

1.5 RELATIONSHIP BETWEEN THE BUILT ENVIRONMENT AND HUMANS

1.5.1 Sharing of microbial cloud between environment and humans

Apart from human microbiome studies these technologies have been employed to characterise the microbial communities of the "built" environment (e.g., buildings, transportation systems), and to determine how humans interact with the external microbiome (1, 56). By studying the Hong Kong metro system, Kang et al used metagenomics analysis to investigate the microbiome within public transit communities. Swabs from palms of frequent users of public transportation revealed a diurnal flux of microbial transmission as well as cross-border line-specific changes connecting Hong Kong and Shenzhen in mainland China. Specific line changes were associated with increased rates of antimicrobial resistant gene transmission,

specifically genes encoding vancomycin and tetracycline resistance raising the potential health hazards for citizens (57).

Meadow et al employed sequence-based methods to demonstrate that individuals occupying a confined space emit a distinct personal microbial cloud that can persist after they leave the space (1). By utilising 16S sequencing, air samples from identical, adjacent chambers during and without occupancy demonstrated that most occupants could be identified by their airborne bacterial emissions and settled particles within 1.5-4 hours.

Apart from understanding microbial dispersal, genomic analysis can also be utilised to characterise the reservoir of antibiotic resistance genes (resistomes) in the environment. A study was conducted looking at two low-income resource-limited settings in Latin America: a rural village in El Salvador and a slum in Lima, Peru. Environmental samples and faecal samples from the communities were analysed using 16S sequencing, functional metagenomics, and whole metagenomics shotgun sequencing, comparing the phylogenetic relationships, and associated microbial resistomes. The study demonstrated large networks of antimicrobial resistant genes were shared between microbial communities of humans, animals and the environment facilitated by horizontal gene transfer and spread of bacterial hosts across communities (58). Key non-healthcare studies (within the last 5 years) investigating the relationship between different environments and humans are summarised in Table 1.

These important studies provide insight into the complex interactions between humans and their environment. They contribute to the understanding of the movement of microorganisms, distribution of resistomes and their potential for dissemination across different habitats.

Table 1. Characteristics of key recent non healthcare studies (within the last 5 years) investigating the relationship between different environments and humans

STUDY (AUTHOR, YEAR, LOCATION)	STUDY DESIGN	METHODS	SAMPLING SITES	RESULTS
Sharma et al., United States of America 2019 (59)	Longitudinal, prospective	16S rRNA amplicon sequencing	US air force cadets and samples from dormitory rooms	1. Cohabitation was significantly associated with increased skin microbiota similarity between individuals. 2. Cohabitation did not significantly influence the gut microbiota. 3. Following a departure from the occupied space, the skin microbiota, showed a significant reduction in similarity relative to the building
Gohli et al., Norway 2019 (60)	Longitudinal, prospective	16S rRNA amplicon sequencing	Air and surface samples from subway stations	1. Significant differences between the air and surface bacterial communities, and across seasons. 2. Air samples had significantly higher within-sample diversity. 3. Seasonal and temperature variations with air diversity measures observed.
Mahneart et al., Austria 2019 (61)	Cross-sectional	Shotgun metagenomics	Environmental samples from different built environments	1. Increased confinement and cleaning are associated with a loss of microbial diversity and a shift from Gram-positive bacteria to Gram-negative bacteria. 2. Microbiome of highly maintained built environments have a different resistome pattern when compared to other built environments 3. Loss of microbial diversity correlates with an increase in resistance.
Fresia et al., Uruguay 2019 (62)	Cross-sectional	Shotgun metagenomics	Sewage and beach area	1. Sewage and beach environments have different bacterial communities. 2. Higher prevalence and more diverse antibiotic-resistant genes in sewage samples. 3. Most genes represented carbapenemases and extended spectrum beta-lactamases genes seen in local hospital infections.
Singh et al., United States of America 2018 (63)	Cross-sectional	Shotgun metagenomics	International space stations	1. Increase in AMR and virulence genes of human pathogens. 2. Microbial compositions of international space stations with earth analogues revealed that the space stations environmental surfaces were different in microbial composition.

Kang Kang et al., Hong Kong 2018 (57)	Longitudinal, prospective	Shotgun metagenomics	Skin surfaces of passengers on metro rails	1. Time is the primary determinant of metro microbiome and resistome composition 2. Human commensals and clinical AMR genes are of higher relative abundance in evening samples
O'Hara et al., United States of America 2017 (64)	Cross-sectional	Shotgun metagenomics	Ambulance indoor surfaces	1. Most taxa found in ambulance surfaces were associated with human genera. 3. Widespread evidence of antimicrobial resistance markers detected.
Mayer et al., United States of America 2016 (65)	Longitudinal, prospective	16S rRNA amplicon sequencing	Inflatable lunar/Mars analogous habitat surfaces	1. Significant differences in community structure of samples before human occupation of the habitat and after occupation. 2. *Actinobacteria* and *Firmicutes* were shown to increase over the time.
Pehrsson et al., United States of America 2016 (58)	Longitudinal, prospective	Shotgun metagenomics	Faecal and environmental samples from Latin America	1. Large network of antimicrobial resistance gene sharing between microbial communities of human, animal, and environmental origin
Ross et al., Canada 2015 (66)	Longitudinal, prospective	16S rRNA amplicon sequencing	University campus door handles	1. Dominant phyla were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes.* 2. High inter-handle variability with several individual building entrances harbouring distinct microbial communities that were consistent over time.
Meadow et al., United States of America 2015 (1)	Longitudinal, prospective	16S rRNA amplicon sequencing	Airborne samples from a customised chamber	1. Occupants could be detected by their airborne bacterial emissions. 2. Bacterial clouds from the occupants were statistically distinct, allowing the identification of some individual occupants. 3. An occupied space is microbially distinct from an unoccupied one, demonstrating individuals release their own personalized microbial cloud.

1.5.2 Influence of hospitalisation on patient microbiota

The characteristics of a "healthy" indoor microbiome is not defined and modification of features such as building ventilation, cleaning strategies and utilisation of appropriate building materials which might impact the built environment microbiota in ways to promote better health remains unknown (67). The importance of understanding the indoor microbial influence on occupants is especially important in the hospital setting where there is high turn-over of patient occupancy.

Despite studies demonstrating transmission within the hospital setting (9, 13, 27), studies focusing on hospitalisation as a key driver of alteration of patient microbiota are limited. Ewan and colleagues undertook a longitudinal assessment of oropharyngeal (OP) microbiota composition in hospitalised older patients (mean age, 83 years) with lower limb fracture. They reported that upper respiratory microbiology at admission related to factors such as frailty and comorbidity burden (68). Significant changes in OP microbiota composition were not identified over a 14-day period after hospitalisation, suggesting that these were pre-existing traits.

A few studies have investigated the impact of hospitalisation on gut microbiota in the ICU setting and in immunosuppressed patients. Faecal microbiota of ICU patients was found to have extremely low levels of bacterial diversity (69). "The microbiota of hospitalized patients was dominated by *Enterococcus, Staphylococcus* and Enterobacterales unlike their healthy counterparts. These pathobionts are potential causes of hospital acquired infections (HAI)"(70). Temporal faecal microbiota changes have been evaluated in patients undergoing allogenic hematopoietic stem cell transplant. Intestinal domination by members of the genus *Enterococcus* has been shown and was associated with an increased risk of VRE bacteraemia. Proteobacteria domination was associated with an increased risk of gram-negative bacteraemia (71). Apart from hospitalisation, factors such as age, comorbid conditions,

medical interventions, critical illness and length of stay can impact the faecal microbiota (72) but these potential confounders were not explored.

In Chapter 3, we sought to explore changes in the microbiome associated with hospitalisation by assessing the oropharyngeal microbiota of hospitalised patients compared to healthy controls. Inpatients with clinical or radiological changes to suggest a lower respiratory infection were excluded. Confounding factors such as age, gender and antibiotic use were taken into consideration during study analysis. The influence of occupancy on a hospital-built environment was examined and the findings are summarised below.

1.5.3 Influence of occupancy on a hospital-built environment

There is limited understanding of microbial dispersal, microbial movement and by extension, the risk of pathogen transmission within the hospital environment. One approach to broaden our knowledge is to understand the relationship between hospital space utilisation and the environmental microbiome as it corresponds to patient and staff traffic. Limited studies employing culture-independent approaches have attempted to track microbial movements and antimicrobial resistance gene dispersion within the hospital setting. Evaluation of microbial movements in a new hospital is an uncommon opportunity. Lax and colleagues were fortunate to do this when a brand-new hospital opened in Chicago. They performed a year-long survey exploring the bacterial diversity associated with humans and built surfaces by utilising 16S rRNA amplicon sequencing. Their study was the first study to attempt microbial tracking in a new hospital and showed that bacterial communities on patients and room surfaces became increasingly similar over the course of a patient's stay. Clinical factors such as chemotherapy and antibiotic exposure which historically were thought to contribute, were not significant (3).

In order to inform infection control policies, it is essential to investigate patterns of bacterial colonisation and resistome dispersion within the hospital setting. Longitudinal genomic analysis in a tertiary hospital revealed distinct ecological niches of antibiotic resistance genes within the built environment thereby supporting human influences which corresponds to

spatiotemporal patterns. The persistent nature of these microbes which can survive within specific environments for a prolonged period increases the risk of opportunistic infection of a patient, highlighting the importance of identifying such reservoirs within the hospital environment as a potential target for infection control measures (4).

Chapter 4 explores the characteristics of the hospital microbiome and the temporal changes that occur with occupancy. This was assessed by collecting environmental samples from multiple sites in a brand-new hospital. Sample collection commenced just prior to occupancy and continued for a period of one year. Environmental samples were also collected from an established hospital that was being decommissioned. Samples were collected during occupancy and post-closure. A summary of key recent healthcare studies (within the last 5 years) investigating the relationship between the hospital-built environment and patients are summarised in Table 2. Understanding the bi-directional relationship between patients, staff members and the hospital-built environment is key to understanding hospital-acquired infections. This relationship and the role of hospital infrastructure in antimicrobial resistance gene transmission will be explored.

Table 2. Characteristics of key recent healthcare studies (within the last 5 years) investigating the relationship between hospital built environment and patients using molecular approaches.

STUDY (AUTHOR, YEAR, LOCATION)	STUDY DESIGN	METHODS	SAMPLING SITES	RESULTS
Chng et al, Singapore 2020 (4)	Longitudinal, prospective	Shotgun metagenomics	Environmental samples from a hospital	1. Distinct ecological niches within the hospital environment identified. 2. Multidrug-resistant strains of microbes can persist on surfaces for a prolonged period.
Chopyk et al., United States of America 2020 (73)	Longitudinal, prospective	16S rRNA amplicon sequencing	Environmental samples from a hospital	1. Samples collected before ICU closure had a greater diversity than samples collected after closure. 2. Significant differences in microbiota compositions at renovations with predominance of environmental bacteria and human-associated bacteria after re-opening and before closure.
ElRakaiby et al., Egypt 2019 (74)	Cross-sectional	16S rRNA amplicon sequencing and quantitative PCR	Environmental samples from a hospital	1. Bacterial communities on high touch samples were richer and more diverse than those detected on bed sheets. 2. Resistance screening indicated an expansion of a mobile beta-lactamase-encoding gene (*bla*$_{TEM}$),
Ewan et al., United Kingdom 2018 (67)	Longitudinal, prospective	16S rRNA amplicon sequencing	Throat swabs from inpatients	1. Microbial community structure was related to frailty and comorbidities rather than length of hospital stay. 2. Incidence of respiratory pathogens detection were not associated with time in hospital.
Brooks et al., United States of America 2018 (75)	Longitudinal multi-centre, prospective	16S rRNA amplicon sequencing and droplet digital PCR	Infant faecal, environmental swabs and air samples from a hospital	1. Despite regular cleaning of hospital surfaces, bacterial biomass was detectable at varying densities. 2. Occupancy a main driver of suspended biological particles within the neonatal ICU. 3. Each room demonstrated a unique microbial fingerprint.

Lax et al., United States of America 2017 (3)	Longitudinal, prospective	16SrRNA amplicon sequencing & shotgun metagenomics	Patient, staff and environmental samples from a hospital	1. Bacteria in patient rooms resembled the skin microbiota of the patient occupying the room. 2. Bacterial communities on patients and room surfaces became increasingly similar over the course of a patient's stay. 3. Genes conferring antimicrobial resistance were consistently more abundant on room surfaces than on the skin of the patients.
Chen et al., Taiwan 2017 (76)	Cross-sectional, multi-centre	16SrRNA amplicon sequencing	Environmental samples from hospitals	1. The microbial composition in a naturally ventilated building were different from an air conditioner-ventilated building 2. Core microbiota shared by all the areas included *Acinetobacter*, *Enterobacter*, *Pseudomonas*, and *Staphylococcus* which can be regarded as healthcare-associated pathogens.
Shin et al., United States of America 2015 (77)	Cross-sectional, multi-centre	16SrRNA amplicon sequencing	Environmental samples from hospitals	1. Dust from operating theatres (OT), collected right after a C-section procedure, contains deposits of human skin bacteria. 2. OT microbiota is the first environment for C-section newborns, and OT microbes might be seeding the microbiome in these babies.

1.6 THE ROLE OF HOSPITAL MICROBIAL ECOLOGY IN PREVENTING HOSPITAL ACQUIRED INFECTIONS.

1.6.1 Hospital acquired infections

Early in 2004, a carbapenem-resistant *Pseudomonas aeruginosa* isolate carrying the blaIMP-4 gene was recovered from a blood culture taken from a patient in an Australian ICU (78). This strain was responsible for a subsequent outbreak that affected 16 patients, six of whom died. The majority of patients that acquired infection received care in the same ICU as the index patient. This is a powerful illustration of the high mortality rate associated with outbreaks of HAIs with limited therapeutic options.

HAIs, defined as infections not present or incubating at the time of patient admission to hospital (79), are increasingly common (80). The World Health Organization has estimated that HAIs occur in 7% of patients in high income countries (HICs) and 10% of patients in low- and middle-income countries (LMICs) (81). HAIs are associated with an increase in length of hospital stay of between 5 and 29.5 days (80). Recent estimates from the Centre for Disease Control and Prevention (CDC) suggest that HAIs in the US currently cost the healthcare system between US$ 28-45 billion every year (82). In Australia, the prevalence of inpatients with HAIs ranges from 5.7%- 17.0% compromising largely of surgical site infection, pneumonia and urinary tract infection (83). Greater access to hospitals within LMICs, and an aging population with a high requirement for medical care in HICs (84), will contribute to rising rates of HAIs. This is a significant concern given the impact on length of stay, morbidity and mortality, and economic burden (85).

There are two broad types of HAIs: those arising from the acquisition of pathogens from the healthcare environment, and those caused by a patient's endogenous microbiota (autologous). The majority of HAIs are likely to be autologous in nature. They result from the direct introduction of microbes that normally colonise the skin and mucosal surfaces of the buccal and nasal cavities through physical disruption (central lines, surgical sites, catheters,

intubations for ventilation), or from the expansion of populations of opportunistic pathogens following antibiotic exposure (78-81, 84). In addition to antibiotic exposure other factors that may cause alteration in the microbiota of hospitalised patients include physiological stress, disrupted nutritional status, medications, and medical interventions (71). HAIs can result from the acquisition of pathogens. This is particularly common for certain viral pathogens, with well documented hospital outbreaks caused by norovirus (86), influenza (87), and respiratory syncytial virus (88). Bacterial pathogens, such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumanii* (89), and *Clostridiodes difficile* (90) have been implicated in hospital outbreaks resulting from patient-to-patient transmission.

1.6.2 The challenges of antimicrobial resistance

The surge of antimicrobial resistance (AMR) plays a crucial role within the hospital environment and threatens the function of antibiotics as a therapeutic modality. The development rate of new antimicrobials is currently unable to keep up with the spread of AMR (91). Hospitals are an important reservoir of multi-drug resistant organisms (3, 4). Despite this, studies focussing on the role of the hospital environment in AMR transmission are limited.

Chng and colleagues swabbed multiple surfaces in a tertiary hospital in Singapore and identified two distinctive taxonomic profiles through shotgun metagenomics sequencing consistent with taxa associated with human skin within high touch areas and taxa associated with water as well as sanitation from areas such as the sink. The number of antimicrobial resistance genes within the healthcare environment was greater than those identified in high touch urban environments (4). Another study evaluating microbial movements in a new hospital attempted to map the presence of AMR genes over a one-year period. Their findings demonstrated that genes conferring antimicrobial resistance were consistently more abundant on room surfaces than on the skin of patients. They showed an increased surface accumulation of AMR genes with time (3). Studies have also demonstrated that patients on

antimicrobial therapy have a greater risk of AMR gene dispersion to their surroundings. Importantly, colonised patients receiving antibiotic therapy were heavier dispersers to the surroundings compared to colonised patients not receiving antibiotics (92, 93).

Current hospital AMR surveillance typically employs a combination of targeted molecular and culture-based approaches. These focus on a narrow pathogen spectrum and concentrate on the antibiotic resistance deemed to be of greatest clinical importance (94-96). This potentially can miss transmission events, which may be infrequent, involve non-target species, or relate to resistance to antibiotics not commonly used in the hospital setting. Greater appreciation of the process of AMR dispersal can be achieved with shotgun metagenomics which involves the untargeted sequencing of all microbial genomes present in a sample.

1.6.3 The role of hospital infrastructure in AMR transmission

The most widely used model of inpatient care is the “ward” system, in which patients are co-housed within a common space, typically according to principal morbidity. Such an approach, which dates as far back as the 1st century AD (97), allows the focusing of specialist care within the hospital population, and facilitates efficient patient surveillance and treatment. While wards have remained central to the design of many hospitals, "Nightingale wards", in which an open area accommodates twenty or more beds, have gradually been superseded by multi-bed wards, in which an area is subdivided into bays that accommodate four to six patients (97).

More recently, modern hospital design has moved away from the ward model and towards one in which patients are placed in single rooms. This transition arguably represents the most substantial change in hospital design in the modern era. Single room models offer increased privacy and confidentiality, greater opportunity for family involvement in care (98), reduced rates of falls due to less need for patient movement, and lower rates of medication error (99, 100).

These benefits are set against higher construction costs, reduced patient visibility to staff, and the impact of isolation on psychological wellbeing (101). However, potentially one of the highest impacts is likely to be in relation to HAIs. Amongst infectious diseases physicians and infection control health professionals, the view has generally been taken that single room hospitals are likely to reduce transmission risks by limiting patient-to-patient contact (102). There is however, limited objective data to support this view.

Most studies have provided no evidence of either direct benefit or harm from single room accommodation in terms of health-related outcomes (103-105). However, the presence of single rooms potentially contributes to reduced infection rates through improved cleanliness, changes in clinician behaviour (106, 107), improved ventilation (108) and easier patient isolation during an outbreak situation (109). Most data is from the intensive care setting. The only dedicated single room study assessing relation to HAIs was conducted by McDonald and colleagues. They investigated a new single room facility located in Canada for three years post-occupancy. Their study demonstrated a decrease in MRSA and VRE colonisation rates and a reduction in VRE infections likely related to the provision of ensuite facilities. MRSA infection rates were unchanged (110). As more hospitals in HICs move towards having a predominant single room facility, a lack of empirical evidence hampers our understanding of the potential impact on infectious disease control.

Despite single-bed hospital rooms becoming increasingly common in high-income countries (HIC), low income countries continue to rely on large communal multi-bed "Nightingale" type wards for inpatient care where a combination of risk factors, including physical proximity, high antibiotic exposure, disruption of commensal microbiota, and increased bacterial dispersion (111), increase the likelihood of AMR dispersal.

Chapter 5 explores the extent of AMR transmission in an open plan hospital ward in a lower middle-income setting. This study was a collaborative study conducted within a tertiary healthcare facility in Yangon, Myanmar. The aim of the study was to investigate the extent of AMR transmission in an open plan ward. This cross-sectional study was assessed by collecting rectal swabs from all patients in a single ward and performing shotgun metagenomics as well as quantitative PCRs on resistant genes to determine AMR transmission.

1.7 RESEARCH QUESTIONS

1.7.1 Thesis rationale

The bacteria, viruses and fungi that colonise the indoor environment have the potential to interact with the human microbiome. Studies have shown that humans leave their microbial signature behind when they vacate an occupied environment. These findings are important within the hospital setting which generally is considered a “clean” environment. Despite prior studies highlighting transmission of pathogens and outbreaks involving AMR genes within the hospital environment, little is known about the overall hospital microbiome and the factors which influence microbial dispersal. This thesis aims to bridge these knowledge gaps.

1.7.2 Overarching aim of this thesis

The overarching aim of this thesis is to better understand the hospital microbiota in terms of microbial tracking and antimicrobial resistant gene transmission. This will be achieved with the use of culture-independent techniques.

1.7.3 Research process

Several studies were conducted to address the aim of this thesis. The research projects presented in this thesis were undertaken in both Adelaide, South Australia, Australia and Yangon, Myanmar. The projects were broadly divided into three related studies over the course of the candidature.

1.7.4 Summary of projects

Project 1: A cross-sectional study assessing the impact of hospitalisation on OP microbiota in the absence of acute respiratory illness in comparison to a healthy cohort (Adelaide, South Australia, Australia).

Project 2: A longitudinal observational study investigating the hospital-built environment microbiota with the opening and closure of the same hospital (Adelaide, South Australia, Australia).

Project 3: A cross-sectional study assessing the extent of AMR transmission in an open-plan ward in a lower-middle income setting hospital (Yangon, Myanmar).

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CHAPTER 2: HOSPITAL INPATIENTS DISPLAY PNEUMONIA-ASSOCIATED CHANGES IN OROPHARYNGEAL MICROBIOTA.

PREFACE

Hospital acquired pneumonia (HAP) is one of the most common hospital acquired infection (HAI) and accounts for >20% of HAIs in both USA and Europe (1,2). Infection with endogenous organisms is the most common pathogenesis (1). The oropharynx acts as a reservoir for respiratory pathogens which are the common culprits of lower respiratory tract infection. However, little is known about the dynamics of the OP microbiota with hospital stay.

This chapter details the results of a study investigating the effects of hospitalisation on OP microbiota. The aim of this study was to assess whether hospitalisation has an impact on OP microbiota in the absence of acute respiratory illness in comparison to a healthy cohort. The detailed study protocol is included in Appendix 2.

The results of this investigation have been publicly presented at the 29th European Congress of Clinical Microbiology & Infectious Diseases in April 2019 (poster presentation) as well as the Australian Health and Medical Research conference in June 2019 (oral presentation).

2.1 INTRODUCTION

Hospital acquired pneumonia (HAP) is the second most common hospital acquired infection and is associated with substantial morbidity and mortality (1). In the US, HAP accounts for 21.8% of all healthcare associated infections (HAI) and is associated with considerable clinical and economic consequences, including substantial extension of inpatient stay (2), an increase in average costs of hospital care of up to 75% (2), and associated mortality rates of 10-14% (3, 4). The requirement for broad-spectrum antibiotics in the treatment of HAP is likely to contribute to increasing levels of antibiotic resistance in common bacterial pathogens (5).

Admission to hospital is associated with a significant increase in lower respiratory tract infection (LRTI) risk (1), increasing by 0.3% with each day of hospitalisation (6). The nature of this association is complex and is likely to reflect factors that contribute to risk of hospitalisation (e.g., age, severity of illness, co-morbidity, pre-existing lung conditions, smoking) and factors associated with inpatient clinical care (e.g., mechanical ventilation) (1, 7).

Increasingly, it is recognised that resident upper respiratory tract (URT) microbiology exerts a considerable influence on LRTI susceptibility. Common respiratory pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* are often detectable in the oropharynx of healthy individuals (8, 9). However, in those with increased susceptibility to respiratory infection, including infants and the elderly, carriage is associated with an increased risk of LRTI (9, 10).

It has been proposed that increases in oropharyngeal (OP) levels of such pathobionts precedes the establishment of acute lower airway infection (10, 11). In such a schema, the commensal OP microbiota plays an important role in suppressing pathogen overgrowth through a phenomenon termed “competitive exclusion” (12-15), and by acting as a regulator of local immunity (14). In contrast, factors that disrupt the OP commensal microbiology, including respiratory viral infection (16), exposure to cigarette smoke (17), and antibiotics (18, 19), reduce these protective effects, and have been shown to be associated with a considerable

increase in LRTI risk (10, 20-22). The extent to which changes in the OP microbiome might mediate the effects of recognised HAP risk factors is therefore an area of growing interest, having the potential to provide additional opportunities for prognostic insight and preventative interventions (23).

Little is known about the OP microbiota characteristics of hospital inpatients who are not critically ill or intubated, and do not have current LRTI. The degree to which URT microbiology in these patients exhibits traits common with the altered URT microbiota characteristic of patients with current HAP is also poorly understood. A study using culture-based methods reported an increased prevalence of gram-negative bacilli in the oropharynx of patients receiving critical care (24). However, this work was undertaken prior to the development of sequencing-based approaches that allow in-depth characterisation of the URT microbiota.

This study hypothesised that, in the absence of acute respiratory infection, hospitalised patients would exhibit altered OP microbiota when compared to healthy controls from within the hospital environment. This study also hypothesised that inpatient OP microbiota would exhibit traits previously reported to be associated with increased risk of pneumonia.

2.2 METHODOLOGY

2.2.1 Study population

Ethical approval for this study was obtained from the Central Adelaide Local Health Network (HREC/17/RAH/207) and Southern Adelaide Local Health Network (413.16–HREC/16/SAC/408). OP swabs were collected from 55 adult inpatients within the three general medical wards of a South Australian tertiary hospital. In addition, swabs were collected from 79 healthy controls, of which 30 were medical students, using the same protocol as the inpatients and in accordance with the clinical guidelines of the local health network.

All inpatients able to provide consent were approached for inclusion. Inpatients with a clinical or radiological diagnosis of LRTI (see Supplementary Methods, Appendix 2) and control subjects with self-reported acute respiratory infection were excluded due to high antibiotic

burden. After obtaining consent, demographic and health-related data were collected, including recent antibiotic exposure. Immunocompromised status (defined as clinically proven immunodeficiency, active use of immunosuppressive therapy or immunomodulatory medication, or the use of more than 10 mg prednisone or equivalent each day for the past three months) was also recorded. Data on other antimicrobials (antivirals and antifungals) were not collected.

2.2.2 DNA extraction and 16S rRNA gene amplicon sequencing

DNA extraction from OP swabs was performed using a methodology described previously (18), with minor adjustments. A detailed extraction protocol is provided in the Supplement (Appendix 2).

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from OP swabs using the modified universal bacterial primer pairs 515F and 806R (25). Amplicons were cleaned, indexed, and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, California). Sequence read data is deposited in the NCBI Sequence Read Archive (Accession number: PRJNA530653). 16S rRNA sequence data were processed with QIIME2 (version 2018-2), using DADA2 inbuilt software for sequence modelling and correcting against SILVA 132 database (26, 27). After sequence processing and removal of spurious and contaminant reads, a median read depth of 9,677 (quartile 1 and quartile 3, 7,765 and 12,017) was achieved. Sequence data were subsampled to a uniform depth of 3,000 reads, based on rarefaction curve asymptotes. Detailed sequencing and data processing protocols are provided in the Supplement (Appendix 2).

2.2.3 Diversity measurements and statistical analyses

Two α-diversity indices were employed: taxon richness and Faith's phylogenetic diversity. β-diversity (inter-sample variance) were determined using PRIMER-E (version 7; PRIMER-E, Plymouth, UK). Bray-Curtis similarity scores were determined from square root transformed relative abundances of taxa. Non-metric multidimensional scaling (NMDS), principal coordinates analysis (PCoA) and distance based linear modelling algorithm were performed

using PRIMER-E. Between group comparisons were performed by permutational multivariate analysis of variance (PERMANOVA) and homogeneity of multivariate dispersions (PERMDISP) using PRIMER-E.

In order to adjust for differences in age and gender between the two groups, we used the residual method in which the relative abundance of each bacterial taxon was regressed on age and gender, and the resulting residuals were used for PERMANOVA analysis. Given the difference in the proportion of male subjects in the controls and inpatients (36.0% vs 58.0%) we also stratified the analysis according to gender (males: 30 inpatients, 27 controls; females: 23 inpatients, 48 controls). Continuous data were tested for normality using the D'Agostino-Pearson omnibus and Shapiro-Wilk normality test. Mann-Whitney U test and unpaired t-test were used for numerical comparisons, the Chi-squared test was used for categorical data, and the Spearman test was used for correlations (GraphPad Prism, version 8.2.1; GraphPad Software, La Jolla, California, USA). Multivariate linear regression was performed with Faith's phylogenetic diversity and distance to centroid based on weighted UniFrac distances, as two dependent variables reflecting aspects of diversity (Stata software, version 15.0; Statacorp, Texas, USA). The exposure variable of interest was the hospital status (patients versus controls) and age, gender and recent antibiotic use were included as covariates.

Variation in microbiota composition at the genus-level was determined statistically using the Mann-Whitney test and a corrected False Discovery Rate (FDR) p-value cut-off <0.05 used for inclusion (R software, version 3.5.1, Vienna, Austria).

2.3 RESULTS

2.3.1 Study population and clinical characteristics

One hundred and thirty-four subjects were recruited, representing 55 inpatients and 79 healthy controls. The leading reasons for admission were non-respiratory infection (n=25), cardiovascular events (n=12), falls (n=5), or poor diabetic control (n=5). As this was a ward-based study, none of the inpatients were exposed to prescribed chlorhexidine mouthwash.

Six subjects were excluded due to persistent low read depth when 16S rRNA gene amplicon sequencing was applied to DNA extracts from their OP swab (two inpatients, four controls). Characteristics of the remaining 128 subjects (53 hospitalised patients and 75 controls) are shown in Table 1. Compared to the inpatient cohort, controls were younger, had a lower male: female ratio, and lower recent antibiotic exposure ($p < 0.005$). Median number of days into inpatient admission was 4 days (IQR:3.5 days) during sample collection.

Table 1: Demographics and clinical parameters of healthy adults and inpatients

Variable	Healthy	Inpatients	p value	Chi-square values
No of samples (% of total)	75(58.59 %)	53(41.41%)		
Age-median (IQR)years[1]	25(22-27)	68(44.5-78)	<0.005*	
Gender[2]			<0.02*	4.87
Male	27(36%)	31(58.49%)		
Female	48(64%)	25(41.51%)		
Recent antibiotic use (4 weeks in inpatients and 3 months in healthy)[2,3]			<0.005*	14.68
Antibiotics	12(16%)	25(47.17%)		
No antibiotics	63(84%)	28(52.83%)		
Route of antibiotics			<0.001*	12.48
Oral antibiotics	12(100%)	8(32%)		
Intravenous antibiotics	0(0%)	17(68%)		
Chronic respiratory condition[4]	n/a	4(7.55%)		
Immunosuppressed medication/status[5]	n/a	2(3.77%)		
Multiple co-morbidities[6]	n/a			
Co-morbidity index 0-4		36(67.92%)		
Co-morbidity index 5-8		17(32.08%)		
Number of days into admission (days) –median (IQR)	n/a	4(3.50)		

Significant effects ($p < 0.05$) are indicated with an asterisk (*).

IQR: interquartile range

n/a: not available/not applicable

1. Mann-Whitney U test
2. X2- test for categorical data
3. Antibiotic use differed in both groups (4 weeks in inpatients and 3 months in healthy population)
4. Chronic respiratory conditions compromised of chronic obstructive pulmonary disease, pulmonary fibrosis, and pulmonary hypertension
5. Defined as clinical suspected or proven immunodeficiency, use of immunosuppressive therapy or immunomodulating medication in the past 3 months, or the use of more than 10mg prednisolone or equivalent each day for the past 3 months
6. Charlson co-morbidity index used to assess impact of co-morbidities. Higher score indicates higher 10-year predictive mortality rate

2.3.2 OP microbiota characteristics

Across both inpatient and control groups, OP microbiota composition was broadly consistent with that reported in previous sequencing-based studies (8, 28). A total of 224 sequence variants were detected, representing 13 bacterial phyla. The majority of detected taxa belonged to the phylum Firmicutes (59.6%), followed by Bacteroidetes (12.1%), Proteobacteria (9.5%), Actinobacteria (9.0%) and Fusobacteria (6.2%). Of all the sequence variants, the relative abundance of *Neisseria*, *Haemophilus*, *Fusobacterium*, *Streptococcus*, *Prevotella* and *Veillonella* contributed most substantially to microbiota differences within the study population (Figure 1).

Hospitalisation status contributed the most to differences in microbiota composition (9.25%, $p<0.001$), followed by age (5%, $p<0.001$), and antibiotic exposure (1.88%, $p=0.02$) (Figure 2). No significant relationship was identified between gender and microbiota composition (0.78%, $p=0.40$). When age- and gender-adjusted values for bacteria relative abundance were used, significant differences in microbiota composition and dispersion between hospitalised patients and healthy controls remained (PERMANOVA $p<0.001$, pseudo-$F=9.335$; PERMDISP $p<0.001$, $F=15.31$).

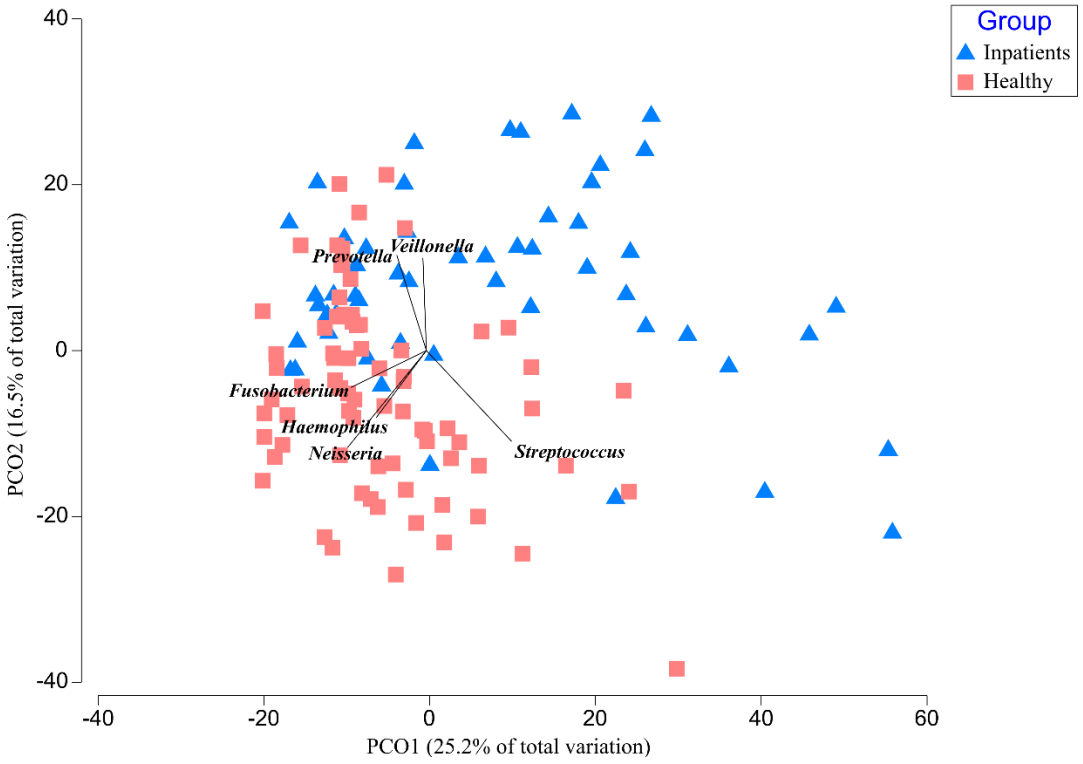

Figure 1: Principal coordinates analysis bi-plot according to taxa. The length of the lines approximates the variances of the variables. *Prevotella* and *Veillonella* were predominant organisms seen within the inpatient group.

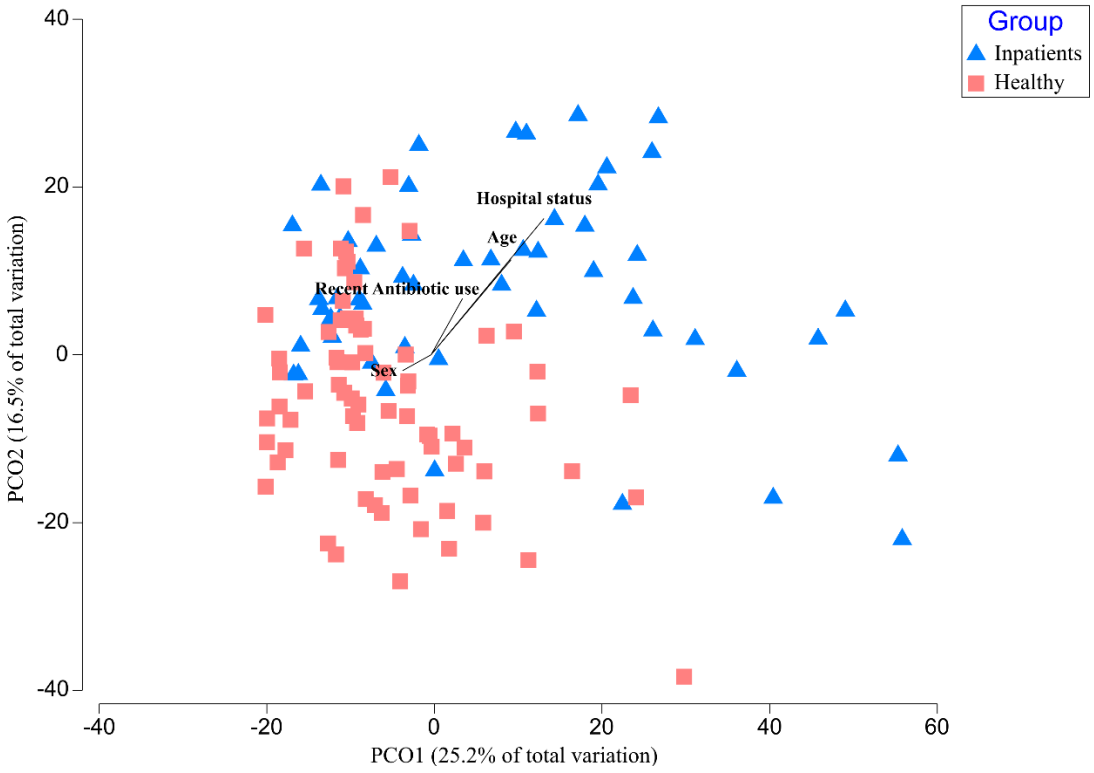

Figure 2. Principal coordinates analysis bi-plot according to variables. The length of the lines approximates the variances of the variables. Hospital status, age and antibiotic exposure were the variables which contributed to differences in microbiota composition between both groups.

2.3.3 Comparison of inpatients and healthy controls OP microbiota composition

Taxon richness and Faith's phylogenetic diversity were significantly lower in inpatients compared to healthy controls ($p<0.050$, Figure 3A-B). However, no significant differences in α-diversity were observed between groups when controlling for antibiotic exposure (Figure S1, Appendix 2). α-diversity scores were also not significantly different between inpatients when grouped according to length of stay (<3 days vs ≥3 days) or burden of co-morbidity (Charlson co-morbidity index, 0-4 vs. 5-8) (Figure S2A-B, Appendix 2).

The relative abundance of *Rothia, Veillonella, Lactobacillus, Atopobium* and *Bifidobacterium* was significantly higher for the inpatient group compared to healthy controls, while the relative abundance of *Gemella, Neisseria, Haemophilus, Actinobacillus and Fusobacterium* was significantly reduced (FDR-corrected p<0.050; Figure 4). Despite contributing substantially to overall microbiota dispersion, the relative abundance of *Streptococcus* and *Prevotella* did not differ significantly between inpatients and healthy controls.

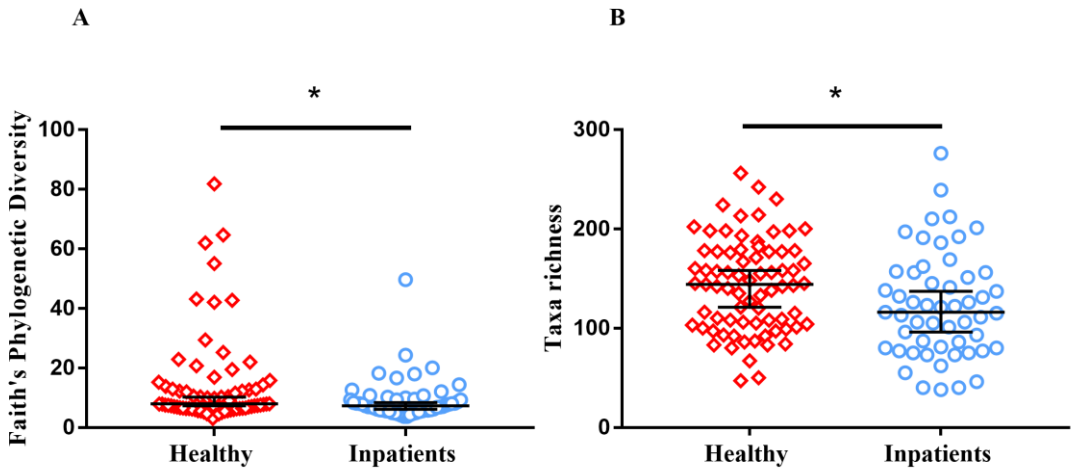

Figure 3A. Faith's phylogenetic diversity is significantly lower in adult inpatients; **B.** Taxa richness is significantly lower in adult inpatients. *p<0.05. Bars show medians +/- 95% CIs.

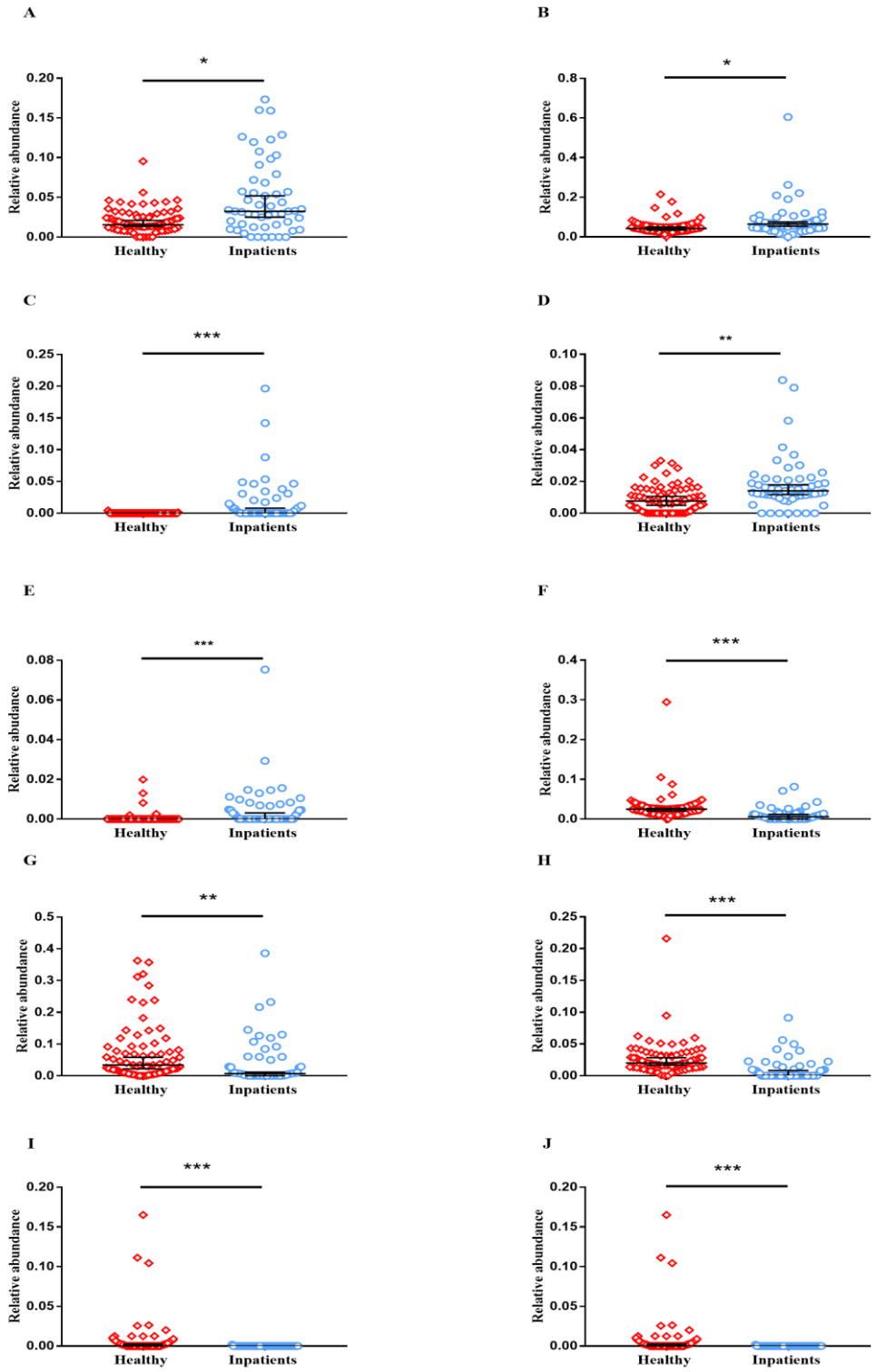

Figure 4. Patterns in prevalence of taxa in adult inpatients and healthy cohort. Relative abundance is depicted per individual for **A**. *Rothia*, **B.** *Veillonella*, **C.** *Lactobacillus*, **D.** *Atopobium*, **E.** *Bifidobacterium*, **F.** *Gemella*, **G.** *Neisseria*, **H.** *Haemophilus*, **I.** *Actinobacillus* and **J.** *Fusobacterium*. *p<0.05; **p<0.01; ***p<0.001.

2.3.4 Analysis of potential confounders of intergroup differences in OP microbiota composition

While hospitalisation was the strongest predictor of microbiota composition, significant differences were also observed in antibiotic exposure, age, and gender between the two cohorts, and recent antibiotic exposure also contributed significantly to microbiota variation after adjusting for inpatient or control status (PERMANOVA p=0.014, pseudo-F=2.927). When subjects were stratified according to recent antibiotic exposure, differences in OP microbiota between inpatients and controls were observed only amongst subjects with no recent antibiotic exposure (PERMANOVA p<0.001, pseudo-F=5.736; Table S1, Appendix 2). Several taxa that differed in relative abundance between the inpatient and control groups were also consistently observed to be altered between non-exposed subgroups. Amongst subjects not exposed to antibiotics, the relative abundance of *Rothia, Lactobacillus, Atopobium, Bifidobacterium, Scardovia, Stenotrophomonas, Cryptobacterium, Kingella, Anaerococcus, Acinetobacter, Corynebacterium* and *Finegoldia* were significantly higher in inpatients, while the relative abundance of *Gemella, Neisseria, Haemophilus, Actinobacillus, Fusobacterium, Peptococcus*, and *Porphyromonas* was significantly higher in healthy controls (FDR p<0.05, Figure S3A-B, Appendix 2).

Differences in microbiota composition between male and female subjects remained significant in gender-stratified analysis (PERMANOVA: male, p=0.001, pseudo-F= 5.396; female, p=0.004, pseudo-F=3.891). Microbiota dispersion remained significant in males, but not females (PERMDISP: male, p<0.001, F=11.766; female, p=0.073, F=3.729) (Figure S4, Appendix 2).

Finally, in multivariate analyses that included all subjects, hospitalisation status significantly predicted Faith's phylogenetic diversity (p=0.013, Rate Ratio: 0.62, 95% CI (0.42, 0.9)) while age, gender and antibiotic use did not (p=0.100, p=0.060, and p=0.471, respectively) (Table S2, Appendix 2). No variable significantly predicted microbiota dispersion (age p=0.440, gender p=0.847, antibiotic use p=0.213 and hospital status p=0.121) (Table S2, Appendix 2).

2.4 DISCUSSION

HAP is a common contributor to morbidity and mortality, with substantial implications for length of stay, treatment costs, and requirement for antibiotics (1, 2, 29). Understanding the mechanisms through which LRTI develops, the associated risk factors, and the mediation of these risks is essential if rates are to be reduced, and clinical outcomes improved. There is growing recognition that, in addition to representing reservoirs of common LRTI-associated microbes, the URT microbial populations can influence the risk of pathobiont overgrowth and translocation to the lungs. Indeed, the impact of factors such as prior antibiotic exposure, age, viral infection, and smoking, on the OP microbiota might explain, in part, their association with LRTI risk.

Ewan and colleagues previously undertook a longitudinal assessment of OP microbiota composition in hospitalised older patients (mean age, 83 years) with lower limb fracture, reporting upper respiratory microbiology at admission to be related to factors such as frailty and comorbidity (28). Significant changes in OP microbiota composition were not identified in this population over a 14-day period post hospitalisation, suggesting that these were pre-existing traits.

Upper respiratory microbiology has also been investigated in individuals with current pneumonia. de Steenhuijsen Piters and colleagues compared OP microbiota of elderly and young adult patients with CAP to healthy controls (8). While it is not possible to determine the extent to which microbiota characteristics preceded, or resulted from, infection and associated clinical care, this work did identify potentially predictive OP microbial markers of pneumonia risk.

The OP microbiota in patients diagnosed with LRTI was characterised by reduced bacterial diversity, an increased predominance of the *Lactobacillus, Rothia* and *Streptococcus,* and a reduced relative abundance of *Prevotella, Veillonella* and *Leptotrichia.* Notably, these traits were common across young adults and elderly patients (8).

Previous studies of relevance have focused predominantly on ventilated patients, with and without pneumonia, in the intensive care setting (30, 31). However, there is a paucity of studies looking at the OP microbiota changes in patients who are not ventilated or critically ill, despite this population being at increased risk in developing HAP. By using culture-independent methods to perform a cross-sectional OP microbiota analysis in non-intubated adult patients in a general medical ward, we identified the relative abundance of *Rothia, Veillonella* and *Lactobacillus* to be significantly different in hospitalised individuals compared to healthy controls. These genera, although not commonly associated with HAP, have been implicated in pneumonia (8), especially in immunocompromised hosts (32, 33). However, such changes in microbiota composition might also influence susceptibility to pathogen overgrowth in the oropharynx. Indeed, the traits we observed in hospitalised patients were consistent with those reported by de Steenhuijsen Piters and colleagues, where an over-representation of *Lactobacillus* and *Rothia* was associated with pneumonia (8). In contrast to our findings, they reported predominance of *Veillonella*, *Prevotella* and *Leptotrichia* associated with health, whereas health was associated with a greater relative abundance of the genera *Gemella*, *Neisseria, Haemophilus, Actinobacillus* and *Fusobacteria* here.

We observed OP microbiota diversity to be significantly reduced in hospitalised individuals. Low OP microbiota diversity is associated with factors that predispose to LRTI, including antibiotic exposure, age, viral infection (18-19, 28, 34), and a reduced resistance to pathogen overgrowth (35). However, we did not observe a significant association between diversity metrics and antibiotic exposure, length of stay, age, and pre-existing co-morbidities, suggesting differences represent the cumulative effect of many factors.

Analysis of age- and gender-adjusted taxon relative abundance revealed differences between hospitalised and non-hospitalised subjects. Gender is significantly correlated with types and levels of morbidity (36), smoking (37), rates of hospitalisation (38), and HAP (39). Analysis of gender-stratified subgroups showed significant differences in microbiota composition between hospitalised and healthy male subjects, but not between hospitalised and healthy female subjects. Age has been associated with microbiota composition at other anatomical sites, particularly the intestinal tract (40), but little is known about this relationship in the respiratory tract. Despite our attempts to control for age and gender, other unobserved confounders that are related to these two factors may partially explain the intergroup differences. A larger observational cohort study would better address the possibility of residual confounding and reverse causation.

An unexpected finding was the absence of a relationship between morbidity (Charlson index) or length of stay and microbiota composition in inpatients. Length of stay (30) and severity of illness (4, 30) have been reported as influences on ICU patient URT microbiology. In this study, we did not observe a significant relationship between OP microbiota characteristics and age, severity of illness, or length of hospital stay. The heterogeneity of our hospitalised population in regard to illness severity and co-morbidities, compared to the severely ill population in the ICU setting, could potentially account for this.

This study had several limitations. First, analysis was cross-sectional, and the direction of causality cannot be established with certainty. The extent to which the microbiota composition changed during hospitalisation, or whether any of the subjects without LRTI subsequently went on to develop lung infections, was not assessed. Second, there were differences between the baseline characteristics of the healthy and hospitalised cohorts. To address this, we performed multivariate analysis, age-matched subgroup comparisons and gender-stratified comparisons. However, residual confounding may still exist and a larger cohort study using a more homogenous, age-matched population would better determine an unbiased estimate of the effect of hospitalisation.

Third, we did not attempt to identify in detail other factors that could potentially influence microbiota composition such as pre-existing nutritional status, medications (excluding antibiotics) and diet. Although the effect of antibiotic exposure was assessed, this was done as a dichotomous variable. The duration of antibiotic exposure, route of administration, class of antibiotics and other antimicrobials were not assessed in detail. Fourth, assessments were based on genus-level taxon characterisation. With genera such as *Haemophilus, Streptococcus* and *Staphylococcus* including both pathogens and commensal species, the clinical implications of changes in their relative abundance in the oropharynx will require further clarification at a species-level as genus-level changes were not observed in our cohort.

In summary, we report significant differences between the OP microbiota in hospitalised individuals and non-hospitalised healthy counterparts. We identified inpatients as having lower OP diversity and richness and reduced relative abundance of commensal bacteria. *Rothia*, *Veillonella* and *Lactobacillus* were found to be significantly more abundant in these individuals. OP microbiota differences between inpatients and non-hospitalised healthy controls are likely to arise due to the influence of multiple factors, both pre-existing, and relating to hospital care. The clinical potential of the OP microbiota traits reported here to identify hospitalised patients at increased LRTI risk warrants further investigation. Future longitudinal studies investigating the temporal relationship between URT microbiome and hospitalisation while controlling for confounders such as antimicrobial use, diet and co-morbidities could identify specific taxa that predisposes to HAP. Analysis of the OP could be used to identify patients with high risk of LRTI acquisition who might benefit from early intervention with antimicrobial therapy.

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CHAPTER 3: THE EFFECT OF OCCUPANCY AND SPACE UTILISATION ON THE HOSPITAL MICROBIOME

PREFACE

Healthcare related infection remains a major problem despite scientific and technical advances in the field of health. Recognition of the importance of environmental microbial reservoirs and their potential contribution to healthcare related infections is reflected in the role of hygiene in hospital management and clinical practice. However, our understanding of how transmission pathways contribute to pathogen spread remains poor. Many of the currently employed infection control measures have minimal evidence.

A major gap in the literature is understanding the type and number of bacteria detectable in healthcare environments, and how this relates to space use and occupancy. The manuscript in chapter 3 details the results of a longitudinal study examining the characteristics of the hospital microbiome in relation to changes in patient occupancy, facility design and space use. The study utilised the closure of a major tertiary hospital and the move to a newly built 800-bed facility resulting in the transfer of patients and staff. The detailed study protocol is included in Appendix 3.

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How occupancy and space-use shape the hospital microbiome: changes in environmental microbiology during transition from a major tertiary hospital to a newly built 800-bed facility

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Key words: Environmental microbiome, hospital occupancy, 16S sequencing

Article summary

We report that occupancy and usage of inpatient and common areas within nosocomial settings are major determinants of hospital microbiome. These findings provide an evidence base for the design of effective infection control strategies.

3.1 ABSTRACT

Background While infection control is critical to safe hospital care, little empirical data underpins many measures currently. How detectable bacteria in nosocomial environments relate to space usage and occupancy represents a major knowledge gap. To address this, we exploited a unique opportunity; the closure of a major tertiary hospital and the transfer of patients and staff to a newly built 800-bed facility.

Methods Environmental swabs were collected from common and inpatient areas in the old and new Royal Adelaide Hospitals during a 12-month period. Microbiota characteristics were determined by 16S rRNA gene amplicon sequencing and quantitative (q)PCR. Targeted assays were used to detect Methicillin-resistant *Staphylococcus aureus* (MRSA) and *vanB*-positive Vancomycin-Resistant Enterococci (VRE).

Results The transition to full occupancy in the new facility was associated with a significant increase in environmental bacterial load (inpatient areas, 3 months p=0.001; common areas, 6 months p=0.039) and a significant change in microbiota composition (baseline-12 months post-occupancy, PERMANOVA p=0.002). These changes were characterised by an increase in human microbiota-associated taxa, including *Acinetobacter* and *Veillonella*. Conversely, closure of the existing facility was associated with a significant decrease in bacterial load (p=0.040), but not microbiota composition. Bacterial load in the old and new hospitals did not differ significantly at equivalent occupancy. Detection of MRSA did not differ significantly between new and old sites.

Conclusion Occupancy is a major determinant of bacterial dispersion within hospital environments. Steady-state bacterial levels and microbiota composition provide a basis for assessment of infection control measures, including space usage and cleaning strategies.

3.2 INTRODUCTION

The microbial populations associated with the human body are in a state of constant flux. During our daily lives, we shed countless microbes from our body into the surrounding environment (1). At the same time, microbes that we come into contact with can be taken up and incorporated into our microbiota (2,3). This process of microbes moving between the human body and the external environment is one route by which clinically significant microbes can spread within human populations (1,4). Importantly, such movement does not rely on direct interpersonal contact, or even that individuals are present within an environment at the same time (1).

The transmission of microbes via external reservoirs is particularly relevant in the context of hospital environments. Hospitals serve large populations of individuals that are both more likely to be susceptible to infection and are more likely to be shedders of pathogenic microbes (5,6). The importance of reducing the risk of pathogen spread via the environment is reflected in the central role of hygiene in hospital management and clinical practice. However, our understanding of how these transmission pathways contribute to pathogen spread remains surprisingly poor.

Important knowledge gaps reduce our ability to manage the movement of microbes within hospitals, and by extension, reduce pathogen transmission. Central to this is the need to better understand relationships between hospital space utilisation and the characteristics of the environmental microbiome. Our ability to do this has been greatly increased by the development of culture-independent methods, including high-throughput sequencing technologies. Such approaches have been applied successfully to survey dispersion of microbes and antibiotic resistance traits within the hospital environment (7,8), revealing complex temporal relationships between patient and environmental microbiomes (7) and correspondence between resistance gene distribution and spatiotemporal human influences (8).

Despite this, investigating the relationships between the hospital microbiome and the human-associated microbiota presents considerable challenges. Interactions between hospitals, their staff and patient populations, and their infection control practices, are unique to individual facilities. However, by taking advantage of a rare opportunity - the closure of a large tertiary hospital and the transfer of the staff and patient catchment to a newly built 800-bed facility, we were able to relate the hospital microbiome to occupancy and space utilisation. Specifically, we examined both the changes that followed the departure of patient and staff populations, and those that occurred during the population of the new facility.

Our study was conducted at two tertiary teaching hospitals, the "old" Royal Adelaide Hospital (oRAH) and the "new RAH" (nRAH), both located in metropolitan Adelaide, South Australia (the southern, central state of mainland Australia). Established in 1840, the oRAH was the largest tertiary public hospital in South Australia, providing complex medical, surgical, emergency, mental health care and diagnostics to an estimated 85,000 inpatients and 400,000 outpatients annually and servicing a population of 1.68 million people (9). The decision was made to replace the oRAH with a new hospital with construction beginning on the new site in 2011. The six-year construction and AU$2.44 billion expenditure resulted in an all single-room facility with the largest fully automated microbiology laboratory in the southern hemisphere and state-of-the-art hi-tech equipment, replacing the nearly 180-year-old single room and multi-bed bay format oRAH. On the 4th of September 2017, the clinical activities of the RAH began a transition from the old site to the new site approximately 2.0 km away. We used this unique opportunity to test the hypothesis that the characteristics of the hospital microbiome reflect the changing occupancy and use of hospital facilities.

3.3 METHODOLOGY

3.3.1 Study design and sample collection

Environmental swabs were obtained from multiple sites (common areas and inpatient areas) at the nRAH beginning on 31st August 2017 (prior to hospital opening but following a terminal commissioning clean). Environmental swabs were obtained from similar locations at the oRAH during occupancy on 31st August 2017 and six weeks post-closure (common areas only). Common areas were defined as non-clinical and clinical areas with high foot-traffic of 100 or more visitors per day. Swabs were collected at each time point from four separate common area sites (nRAH and oRAH), five inpatient rooms from different clinical teams (nRAH) and two separate bays (oRAH: six beds from medical and surgical bays, four shared bathrooms, Table 1).

Common area sampling points were identical between the oRAH and nRAH. Inpatient areas within the oRAH included multi-bed bays and shared bathroom facilities, while nRAH inpatient areas were individual rooms with attached bathrooms. Hospital cleaning and infection control practices were similar at both sites. Common areas with high foot-traffic and all inpatient room floors were cleaned daily with detergent (REVEAL heavy duty floor cleaner, an ammonium based cleaner). High touch surfaces were cleaned with a microfibre cloth. Inpatient rooms occupied by patients with multi-resistant organisms (MRO) and all bathroom areas were cleaned daily with sodium hypochlorite (0.1%, 1000ppm).

Samples were collected using sterile swabs pre-moistened with sterile (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia). Environmental sampling from the common areas and inpatient room areas were sampled by the study investigator using a standardised template. Study investigator sampled the sites at similar times during the sampling intervals. Regular cleaning of both the common areas and inpatient areas typically occurred in the morning, therefore sampling for this study was performed at the end of the day for consistency to minimise its impact on detection of bacterial load. A circular metallic template (diameter: 0.22m; area: 0.038m^2) was used for floor swabs. Bed rail and lift buttons swabbed

areas of approximately 0.004m^2 and 0.0007m^2, respectively. Environmental swabs were stored in sterile TE buffer at -80°C until DNA extraction. The study was approved by the Central Adelaide Local Health Network (HREC/17/RAH/207).

Table 1: Summary of sample collection sites

A. oRAH

Areas	Occupied	Unoccupied (6 weeks later)
Common areas	Includes hospital foyer, outpatient clinical area, emergency department (ED) triage area and common lift buttons (n=8)	Includes hospital foyer, outpatient clinical area, emergency department (ED) triage area and common lift buttons (n=8)
Room areas	Includes general medical ward and general surgical ward (bedrail, bedroom floor and toilet flush) (n=16)	No access to wards

B. nRAH

Areas	3 monthly time points
Common areas	Includes hospital foyer, outpatient clinical area, emergency department (ED) triage area and common lift buttons (n=40)
Room areas	Includes five selected rooms from haematology, general medical, general surgical, intensive care unit (ICU) and acute medical unit wards (bedrail, bedroom floor and toilet flush). Only bedrail and floor swabs collected from ICU (n=70)

3.3.2 16S rRNA gene amplicon sequencing and bacterial load quantitation

DNA extraction from environmental swabs, and amplification of the V4 hypervariable region of the bacterial 16S rRNA gene (hereafter, 16S sequencing), was performed using published methods (10,11). Sequence read data was deposited in the EMBL European Nucleotide Archive (Accession number: PRJEB40938). Sequence data were processed with QIIME2 (version 2018-2), using the DADA2 inbuilt software for sequence modelling, and taxonomic assignment was performed against the SILVA reference database (release 132) (12,13). Sequence data were subsampled to a uniform depth of 1700 reads, based on rarefaction curve asymptotes.

3.3.3 Diversity measurements and statistical analyses

β-diversity (inter-sample variance) and Nonmetric multidimensional scaling (NMDS) was performed using PRIMER-E (version 7; PRIMER-E, Plymouth, UK). Between group comparisons were performed by permutational multivariate analysis of variance (PERMANOVA) and homogeneity of multivariate dispersions (PERMDISP) using PRIMER-E. Bray-Curtis similarity scores were determined from square root transformed relative abundances of taxa.

Mann-Whitney U test and Wilcoxon signed rank test matched-pairs were used for non-parametric and parametric comparison of continuous data, respectively (GraphPad Prism, version 8.2.1; GraphPad Software, California, USA). Outliers were identified for unoccupied hospital swabs due to minimal foot traffic and therefore values represented by these swabs were regarded as potential confounders. Outliers were calculated using the 1.5 IQR (interquartile range) rule. Variation in microbiota composition at the genus-level was determined statistically using the Mann-Whitney test and a p-value cut-off <0.05 used for inclusion (R software, version 3.5.1, Vienna, Austria).

3.3.4 Quantitation of bacterial load and resistance gene carriage.

A quantitative PCR (qPCR) assay targeting the 16S rRNA gene was used to assess total bacterial load (14). Levels of the *vanB* gene within swabs with *Enterococcus* species were assessed using a SYBR green assay (this study), and the presence of *Staphylococcus aureus* was determined using a *nuc* gene Taqman assay (Table S1, Appendix 3) (15). Methicillin resistant *S. aureus* (MRSA) was detected using a modified multiplex PCR at South Australia (SA) Pathology as previously described (16).

3.4 RESULTS

Analysis was performed on 110 swabs (common areas, n= 40; inpatient rooms, n=70) from the nRAH during the first 12 months of operation, and 32 swabs (common areas, n= 16; inpatient areas, n=16) from the oRAH over a 6-week period spanning closure. All swabs were subjected to 16S sequencing, with four excluded due to persistent low read depth (nRAH common areas, n=3; RAH inpatient area, n=1).

3.4.1 Environmental microbiota dynamics following facility opening

Bacterial load within common areas increased significantly during the first six months after hospital opening (Wilcoxon test, p=0.039, Figure 1A), before plateauing after six months. Swabs from inpatient rooms showed a similar but more pronounced change, achieving statistical significance by month three (p=0.001, Figure 1B). Bacterial load plateaued at a significantly higher level in common areas compared to inpatient rooms (Mann-Whitney test, $p<0.001$, common areas median [IQR]: 2.44×10^5 cells/swab [2.42×10^5]; inpatient areas: median [IQR]:1.10×10^5 cells/swab [2.17×10^5]).

Overall, environmental samples were characterised by a predominance of bacterial taxa from the Firmicutes (24.9%), Proteobacteria (24.8%), Actinobacteria (17.0%) and Bacteroidetes (15.3%) phyla.

Microbiota composition in common areas did not differ significantly between pre-occupancy and one-year post-opening (PERMANOVA p=0.685, pseudo-F=0.57) (Figure 2A). However, microbiota composition did change significantly in inpatient rooms (PERMANOVA p=0.002 pseudo-F=3.16, Figure 2B). Microbiota dispersion did not change significantly in either group (PERMDISP: common, p=0.973, F=0.002; room, p=0.513, F=0.48).

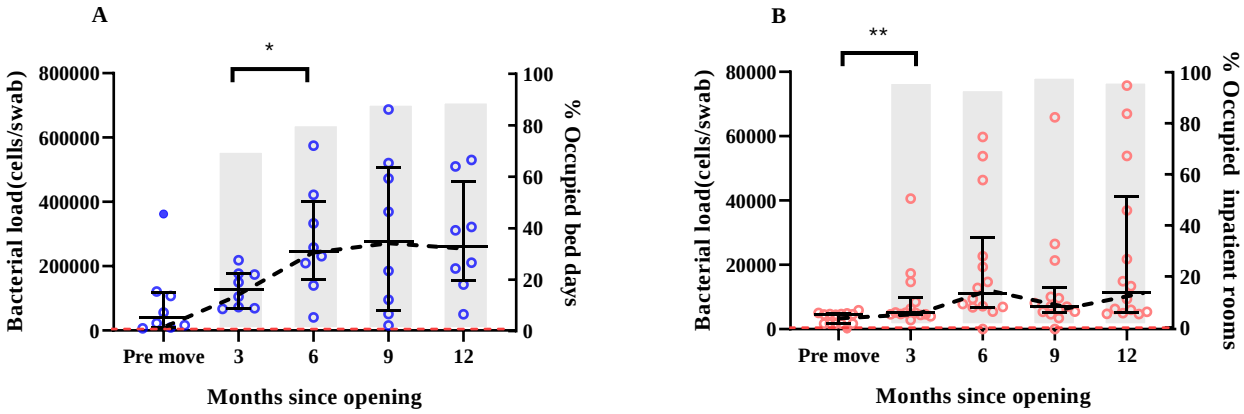

Figure 1: Relationship between bacterial load and occupancy in (A) common areas and (B) inpatient rooms. (A) Left y-axis: bacterial load (cells/swab) represented by open circles; Right y-axis: Overall percentage of hospital occupied bed days/total bed available represented by bars. Closed circles represent an outlier which was not included for statistical analyses. (B) Left y-axis: bacterial load (cells/swab) represented by open circles; Right y-axis: Percentage of occupied inpatient rooms which was assessed during the study period represented by bars. Detection threshold represented by dotted red line. Middle and error bars denote median and interquartile ranges. Values represented on left sided y-axis used for statistical analysis. Wilcoxon matched-pairs signed rank test *p<0.05; **p<0.01.

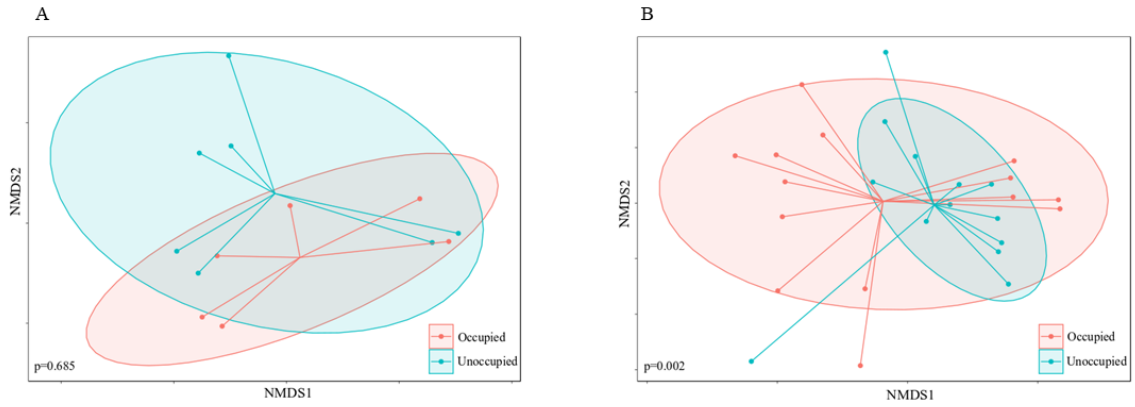

Figure 2: Nonmetric multidimensional scaling (NMDS) ordination plot according to pre-occupancy and 1-year post-occupancy in (A) common areas (B) inpatient rooms of the nRAH based on Bray-Curtis (BC) distance. Unoccupied: Pre-opening of hospital; Occupied: 1-year post hospital opening. Statistical analysis to determine the significance of microbiota composition differences between both groups were performed using the PERMANOVA test.

The relationship between occupancy and microbiome composition was explored further by comparing samples collected at successive time points. During hospital opening (0-3 months), the change in microbiota composition within the common areas was reflected by a median Bray-Curtis dissimilarity index (BC) of 42.38 (IQR: 13.74) (a higher score indicating lower similarity). This change increased significantly in the following three months (3-6 months: median BC score [IQR]: 46.84 [16.79], p=0.040, Figure S1A, Appendix 3). In contrast, the greatest change in microbiota composition in inpatient areas occurred between 0-3 months (median BC score [IQR]: 42.75 [12]) and was significantly greater than the change at 3-6 months (BC score: 40.41 [12.7], p<0.001, Figure S1B, Appendix 3).

The microbial composition of the common areas across all time points significantly differed to those of the inpatient room areas (PERMANOVA p≤0.001, pseudo-F=8.39, Figure S2, Appendix 3). Additionally, microbiota composition varied more between inpatient rooms compared to common areas (PERMDISP p=0.009; distance to centroid, inpatient area=0.23, common areas=0.19) (Figure S2, Appendix 3). Since microbiota composition did not change significantly in common areas between pre-occupancy and one-year post-occupancy, changes in specific taxa were not explored. However, such changes were assessed in inpatient areas. An increase in the abundance of skin- and gut-related bacterial taxa was observed at one year, particularly unclassified members of the Lachnospiraceae family, and members of *Subdoligranulum*, *Acinetobacter*, *Veillonella* and *Lactococcus* (Figure 3).

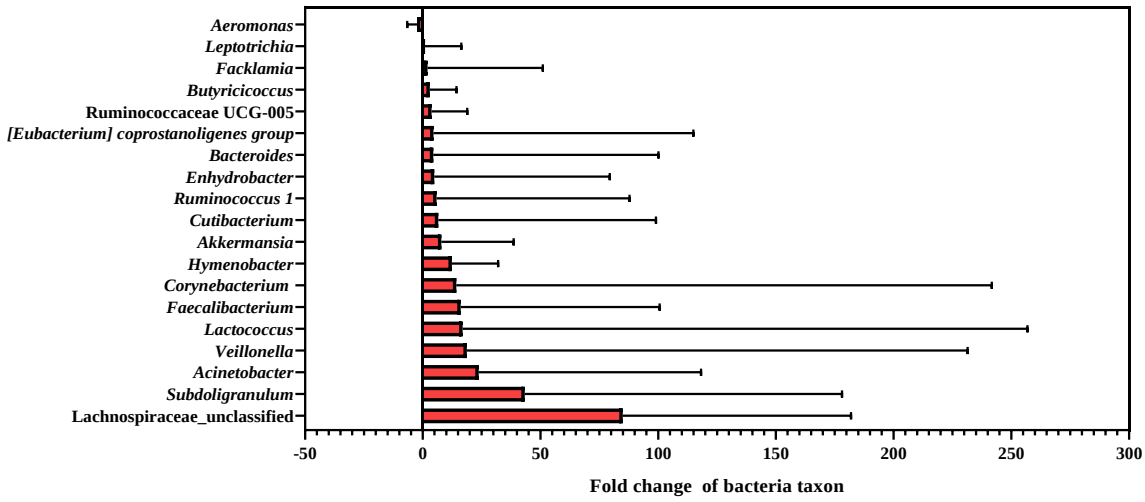

Figure 3: Bacterial taxon with significant changes in the inpatient rooms of the nRAH between pre-occupancy and 1-year post hospital opening. Fold changes are based on measurements absolute abundance (cells/swab). Bars represented median fold changes with interquartile ranges. Positive changes indicate an increase 1-year post-occupancy.

3.4.2 Microbial changes during hospital closure

Bacterial load within the common areas declined significantly following closure (Wilcoxon test p=0.040) (Figure 4), while microbial composition and dispersion did not change significantly (PERMANOVA p=0.149, pseudo-F=1.61, PERMDISP p=0.392, F=1.55). A similar separation of common areas and inpatient rooms to that observed in the new hospital was observed in oRAH samples (PERMANOVA p<0.001, pseudo-F=4.35, PERMDISP p=0.483, F=0.65; Figure S3, Appendix 3).

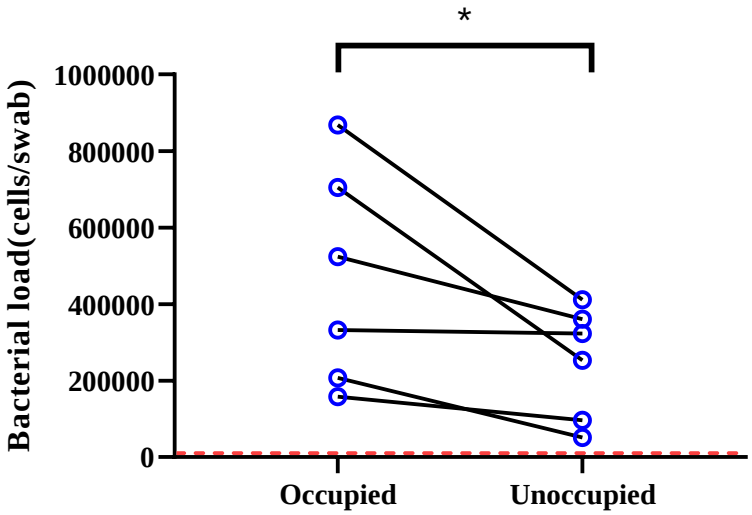

Figure 4: Relationship between bacterial load and occupancy within common areas in oRAH during occupancy and six weeks after the hospital closure (unoccupied). Detection threshold represented by dotted red line. Wilcoxon matched-pairs signed rank test *p<0.05.

3.4.3 Comparison of hospital microbiome between an established and a new facility

When operating at similar levels of occupancy, the hospitals did not differ significantly in the levels of detectable bacteria in common and inpatient areas (Figure 5A & 5B). However, hospital closure and opening resulted in significant changes in bacterial load. Microbiota characteristics (composition and dispersion) were not significantly different between hospitals when fully occupied (Table S2, Appendix 3), despite the differing age and architecture. Microbiota profiles in common areas instead grouped by sample site (e.g., lift buttons, floor, Figure S4, Appendix 3), rather than building (Figure 6A). Microbial structure within the inpatient areas during occupancy was not significantly different between hospitals (Figure 6B, Table S2, Appendix 3).

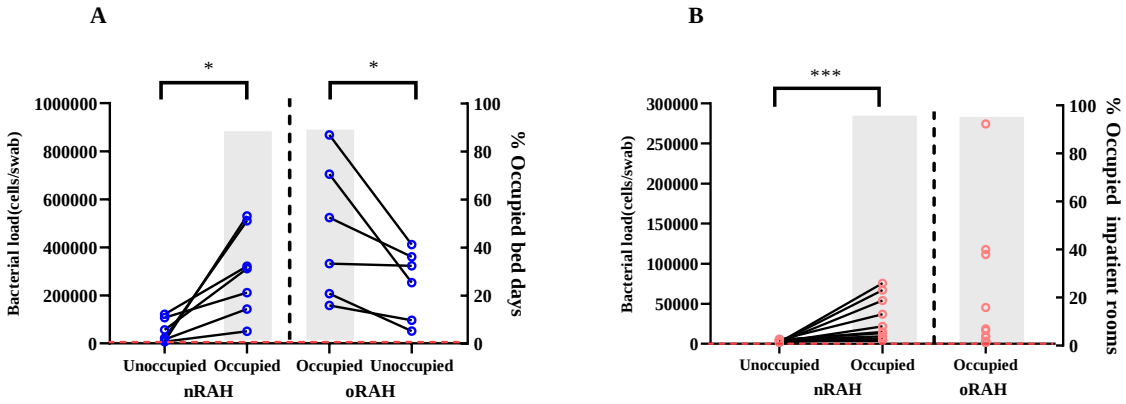

Figure 5: Bacterial load changes in nRAH and oRAH for (A) common areas and (B) inpatient rooms. (A) Left y-axis: bacterial load (cells/swab) represented by open circles; Right y-axis: Overall percentage of hospital occupied bed days/total bed available represented by bars. (B) Left y-axis: bacterial load (cells/swab) represented by open circles; Right y-axis: Percentage of occupied inpatient rooms which was assessed during the study period represented by bars. Detection threshold represented by dotted red line. Values represented on left sided y-axis used for statistical analysis. Wilcoxon matched-pairs signed rank test *p<0.05; ***p<0.001.

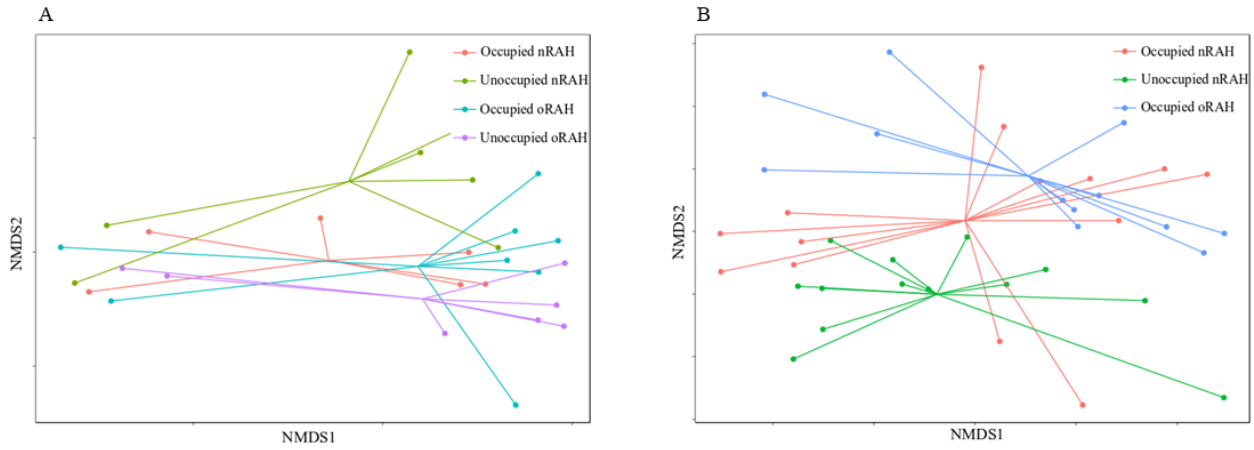

Figure 6: NMDS ordination plot comparing nRAH and oRAH in (A) common areas (B) inpatient areas based on BC distance. nRah: Unoccupied: Pre-opening of hospital; Occupied: 1-year post hospital opening. oRah: Unoccupied: Closure of hospital; Occupied: prior to closure of hospital. Statistical analysis to determine the significance of microbiota composition differences between groups were performed using the PERMANOVA test. (A): Unoccupied nRAH vs Occupied nRAH p=0.677; Unoccupied nRAH vs Occupied oRAH p=0.329; Unoccupied nRAH vs Unoccupied oRAH p=0.332; Occupied nRAH vs Occupied oRAH p=0.241; Occupied nRAH vs Unoccupied oRAH p=0.758; Unoccupied oRAH vs Occupied oRAH p=0.144. (B): Unoccupied nRAH vs Occupied nRAH p=0.001**; Unoccupied nRAH vs Occupied oRAH p<0.001; Occupied nRAH vs Occupied oRAH p=0.138.

3.4.4 Detection of bacteria associated with nosocomial infections

S. aureus was detected in 51 swabs (nRAH: 40 swabs; oRAH:11 swabs) based on the presence of the *nuc* gene. Positive swabs related mainly to the common areas of both hospitals (47.5% new hospital, 50% old hospital, Table S3, Appendix 3). Of these, 29 (nRAH: 26 swabs; oRAH: 3 swabs) were MRSA. Although MRSA was detected more frequently in nRAH (nRAH:14/40; 35% common areas, 12/70; 17.1% inpatient areas, oRAH: 3/16; 18.8% common areas, 0/8 inpatient areas), the difference was not statistically significant (Fischer's exact test p=0.087). *Enterococcus sp.* was identified in 46 swabs (nRAH: 34 swabs; oRAH: 12 swabs, based on 16S sequencing), predominantly from inpatient areas (34.3% new hospital, 50% old hospital, Table S3, Appendix 3). Of these, *vanB* was only detected in one sample (general medical room floor, nRAH).

3.5 DISCUSSION

Despite the serious consequences of pathogen transmission within healthcare settings, current strategies to limit the movement of microbes rely to a considerable extent on received understanding. To date transmission events have not been used to inform hospital cleaning protocols (17). Achieving greater clarity around the bidirectional movement of bacteria in the hospital environment and the people that use it would aid in the development of robust infection control policies. Our study addressed the central question of how the hospital microbiome relates to space occupancy and use.

Historically, investigations of microbial populations within healthcare environments have involved targeted approaches, either based on bacterial culture or molecular assays. These approaches can be effective in detecting specified microbes or groups of microbes. However, their narrow focus provides little opportunity to characterise general microbial dispersion. As demonstrated in a number of recent studies, (7,18,19) 16S sequencing can be used to define the hospital microbiome effectively and relate its characteristics to hospital occupancy and use.

The unique opportunity to compare environmental microbiology in an established hospital and a newly built facility, serving the same population and employing the same staff, allowed us to explore the influence of occupancy on the hospital microbiome. Our investigation was based on the hypothesis, supported by previous studies, (7,8) that the hospital microbiome would reflect facility use, with greater occupancy associated with higher bacterial loads and a greater relative abundance of bacterial taxa common within human microbiota. We further hypothesised that the two different contexts assessed would exhibit opposite trends in microbiome composition.

Bacterial load was seen to increase rapidly in inpatient rooms in the first three months after the new hospital opened, and in the first six months in common areas. Our analysis showed the hospital microbiome shifted significantly in composition following the opening of the new facility within the inpatient areas. This change was driven by an increase in levels of bacterial taxa that are associated with the human microbiome, particularly the skin and faecal microbiota. Similar findings were reported in a previous study by Lax and colleagues, where an increase in the contribution of skin-associated genera, such as *Corynebacterium, Staphylococcus* and *Streptococcus*, was observed following hospital opening (7).

The relationship between how an area of the hospital is used and the characteristic of its environmental microbiome is illustrated by differences observed between inpatient and common areas. Overall microbial composition between common areas and inpatient areas were significantly different in the new and old hospital. When these changes were explored further, significant shifts in microbiota composition were only seen within the inpatient areas. The microbial composition within common areas were consistent throughout the sampling period. Common area samples clustered according to exposure (e.g., high touch and high foot traffic sites) rather than according to time point or hospital. Occupied inpatient areas exhibited a predominance of taxa associated with human skin and faeces, highlighting the close relationship between patient and hospital microbiota.

Reciprocal changes to those observed during hospital opening were evident at the site of the old hospital during decommissioning. At the old hospital, the bacterial load decreased rapidly within the six weeks post-closure. Comparison of the old hospital and new hospital revealed some similarities despite differing building age and infrastructure, and no major differences were identified between the old hospital and new hospitals after one year of occupancy (despite changing from a predominantly six-bed bay layout to all single rooms). These observations suggest that the age of the facility is a lesser influence on the environmental microbiome than the manner in which spaces are used, the characteristics of patient/staff populations, and management variables, such as cleaning practices.

It was notable that while there appears to be an ongoing movement of bacteria from occupants into the hospital environment, levels of bacteria detected did not continue to rise indefinitely. Moreover, when the human source of bacteria was no longer present (post-closure) bacterial levels declined. This suggests a dynamic process of dissemination and clearance. The levels at which environmental bacterial load plateaus differs and is likely to be influenced by space use and occupancy. For example, differences in cleaning approaches and less foot traffic are likely to explain substantially lower levels of bacteria in inpatient spaces compared to common areas. Our analysis was designed to be inclusive of all bacteria present within environmental samples. While antibiotic-resistant pathogens were not a central focus of this study, such microbes are disproportionately important for patient outcomes. We therefore undertook a targeted assessment of MRSA and VRE dispersion, two pathogens that are common causes of severe nosocomial infections. The detection of VRE in our samples (based on PCR detection of *vanB*, the dominant VRE gene in Australia (20,21) was rare (0.9% new hospital, 1/110 swabs). In contrast, MRSA detection rates of 23.6% in the new hospital is comparable with those reported previously (22,23). Overall, our findings are consistent with a previous study of the impact of single-room hospital models, which were associated with low VRE infection and colonisation rates, but stable MRSA infection and colonisation rates (24).

Our study had limitations that should be considered. The focus was solely on environmental samples and samples from staff or patients were not assessed. While demonstrating the similarities between patient and environmental samples could potentially be informative, our aim was to describe the microbial changes of an environment in conjunction with overall hospital occupancy rather than a single patient. While we employed an amplicon-sequencing based approach for this study, a shotgun strategy might have provided greater phylogenetic resolution. Finally, occupancy was defined broadly and included the collective exposures of patients, staff members, cleaning, ventilation, and other factors. We did not investigate the relative contribution of these variables to the characteristics of the environmental microbiome in isolation.

The ability to better understand the composition and dynamics of hospital microbiome using molecular approaches described here provides an opportunity to develop evidence-based and effective infection control practices. In addition to traditional outbreak surveillance approaches, strategies to prevent the spread of pathogens between individuals via the built environment should now draw on our increasing ability to define the totality of microbial populations within human and environmental niches.

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CHAPTER 4: INVESTIGATING THE IMPACT OF AN OPEN-PLAN HOSPITAL WARD ON RESISTOME DISPERSION.

PREFACE

The threat of antimicrobial resistance (AMR) in South-East Asia is extremely concerning. High rates of bacterial infection, limited healthcare resources, and poor regulation of antibiotic use all contribute to this region being identified by the WHO as at highest risk globally. The development of strategies to address this threat has been undermined by a failure to identify principal routes of AMR transmission.

Hospital wards could represent an important venue for AMR spread. Open-plan "Nightingale" model wards that allow ready interaction between patients, remain standard in South-East Asia. However, hospital AMR surveillance, which is largely limited to targeted, culture-based, diagnostic microbiology, is poorly suited to assessing ongoing AMR circulation between patients. In contrast, metagenomic sequencing is a powerful analytical strategy that allows characterisation of the resistome in a single reaction. However, this highly informative technology is unavailable in most LMIC countries.

The manuscript in chapter 4 details the results of a cross-sectional study investigating the extent of AMR transmission in an open-plan hospital ward within a lower middle-income setting. The detailed study protocol is included in Appendix 4.

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Transmission of antimicrobial resistance in an open-plan hospital ward: A cross-sectional metagenomic study of resistome dispersion in a lower middle-income setting.

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Key words: antibiotic resistance; metagenomics; resource-limited settings; healthcare; resistome dispersion

4.1 ABSTRACT

Background Antimicrobial resistance (AMR) represents a profound global health threat. Reducing AMR spread requires the identification of transmission pathways. The extent to which hospital wards represent a venue for substantial AMR transmission in low- and middle-income countries settings is poorly understood.

Methods Rectal swabs were obtained from adult male inpatients in a "Nightingale" model general medicine ward in Yangon, Myanmar. Resistome characteristics were characterised by metagenomic sequencing. AMR gene carriage was related to inter-patient distance (representing inter-patient interaction) using distance-based linear models. Clinical predictors of AMR patterns were identified through univariate and multivariate regression.

Results Resistome similarity showed a weak but significant positive correlation with inter-patient distance (r=0·12, p=0·04). Nineteen AMR determinants contributed significantly to this relationship, including those encoding β-lactamase activity (*OXA-1*, *NDM-7*; adjusted p<0·003), trimethoprim resistance (*dfrA14*, adjusted p=0·0495), and chloramphenicol resistance (*catB3*, adjusted p=0·002). Clinical traits of co-located patients carrying specific AMR genes were not random. Specifically, AMR genes that contributed to distance-resistome relationships (*OXA-1*, *catB3*, *dfrA14*) mapped to tuberculosis patients, who were placed together according to ward policy. In contrast, patients with sepsis were not placed together, and carried AMR genes that were not spatially significant or consistent with shared antibiotic exposure.

Conclusions AMR dispersion patterns primarily reflect the placement of particular patients by their condition, rather than AMR transmission. The proportion of AMR determinants that varied with inter-patient distance was limited, suggesting that nosocomial transmission is a relatively minor contributor to population-level carriage.

4.2 INTRODUCTION

Antimicrobial resistance (AMR) represents a profound health threat (1), particularly in low- and middle-income countries (LMIC) (2). In many regions of South-East Asia, access to antibiotics is widespread but poorly regulated, and their use is often inappropriate (3,4). The off-prescription sale of antibiotics (3), and the unregulated use of critically important agents in animal husbandry (5,6), is further compounded by high rates of infectious diseases, rapid unplanned urbanisation, poor sanitation (7) and inadequate waste management (8). As a consequence, the World Health Organization (WHO) has identified South-East Asia as the global region at greatest risk from AMR (9).

The considerable challenges of addressing AMR are evident in Myanmar, a country of more than 53 million people. Following the end of military rule in 2011, Myanmar was reclassified in 2015 from a "low-income" to "lower-middle income" country (currently, 74th in global gross domestic product (GDP) rankings) (10). However, despite growing national prosperity, a substantial portion of Myanmar's population live in rural settings (69%), with many of those in urban settings dwelling in slums (41%) (11). Expenditure on health remains low, representing 4·7% of GDP, substantially less than global averages (9·9%) (12). Disability-adjusted life year (DALY), is high by global standards (394 per 1000 population, compared with a global average of 328) (13) and life expectancy remains below the global average (M/F: 64/69 versus 70/75) (14).

Myanmar released a National Action Plan for the Containment of Antimicrobial Resistance in 2017 (15), and in 2018, joined the WHO's Global Antimicrobial Resistance Surveillance System (16). Such commitments to coordinated surveillance are critically important. However, effective AMR containment is hampered by poor identification/description of the principal reservoirs of AMR and modes of AMR transmission.

Open plan hospital wards potentially represent an important venue for person-to-person AMR transmission. Despite single-bed hospital rooms becoming increasingly common in high-income countries (HICs), countries such as Myanmar continue to rely on large communal multi-bed "Nightingale" type wards for inpatient care where a combination of risk factors, including physical proximity, high antibiotic exposure, disruption of commensal microbiota, and increased bacterial dispersion (17), increase the likelihood of AMR dispersal.

Current AMR surveillance in hospitals typically employs culture-based approaches that focus on a narrow range of pathogens and on resistance to antibiotic agents that are of greatest clinical importance. Using such an approach, the potential to miss transmission events, which may be infrequent, involve non-target species, or relate to resistance to antibiotics not commonly used in the hospital setting is therefore high. Instead, understanding the process of ongoing AMR dispersal can be better achieved using an approach that captures all AMR genes within the patient microbiome, regardless of the bacterial species that carry them or the antibiotics to which they confer resistance. Shotgun metagenomic sequencing provides such a capacity, however, this genomic technology is usually unavailable in LMIC contexts.

Our aim was to better understand the extent of AMR transmission in an open plan hospital ward in a lower middle-income setting. We hypothesised that the occurrence of ongoing patient-to-patient AMR transmission within an open plan ward would be reflected in a greater similarity of resistome characteristics between patients, the greater their proximity.

4.3 METHODOLOGY

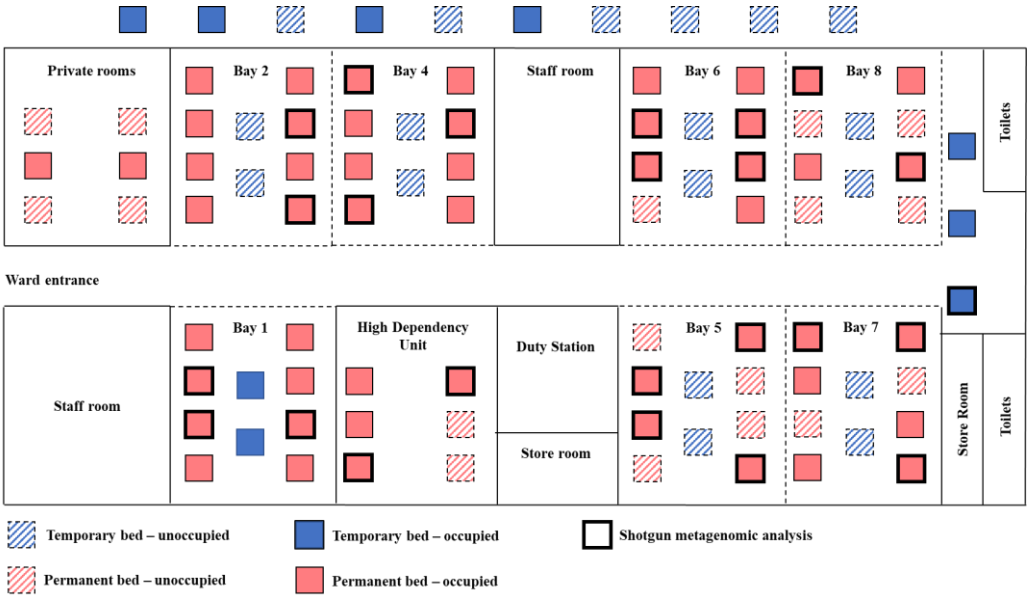
4.3.1 Study population and sample collection

The study was undertaken in an adult male general medical ward at the Insein hospital, a 500-bed public tertiary teaching hospital in Yangon, Myanmar. Hospital infection control practices included the use of hand-sanitiser gel by staff prior to patient care, and the use of gloves and gowns for patients known to have transmissible infections or multi-resistant organisms. Bed rails were cleaned daily with a chlorhexidine-containing germicide (Septol), floors were cleaned daily with detergent, and other ward surfaces twice-weekly.

The hospital follows a policy of cohorting suspected and confirmed patients with TB (bays 7 & 8; Figure 1). Two patients were listed as having TB but were located in bay 4 and the private room respectively. However, both are believed to have had extra-pulmonary TB. Where a patient is confirmed to have MDR-TB, they are moved to beds in the corridor prior to transfer to a dedicated TB hospital. N95 masks are used by medical staff for the routine care of all TB patients. Patients with TB were required to wear surgical masks. There were no dedicated negative pressure rooms in the hospital.

All included patients were provided with study information and consented to participate. Rectal swabs were collected on 19th January 2018 by medical professionals in accordance with hospital guidelines, frozen, and transported on dry ice to the South Australian Health and Medical Research Institute (SAHMRI), Australia where it was stored at -80°C until analysis. Ethics approval for the study was received from Review Committee 1, University of Medicine 2, Yangon (34/ERC-1,12-2017).

(A)



(B)

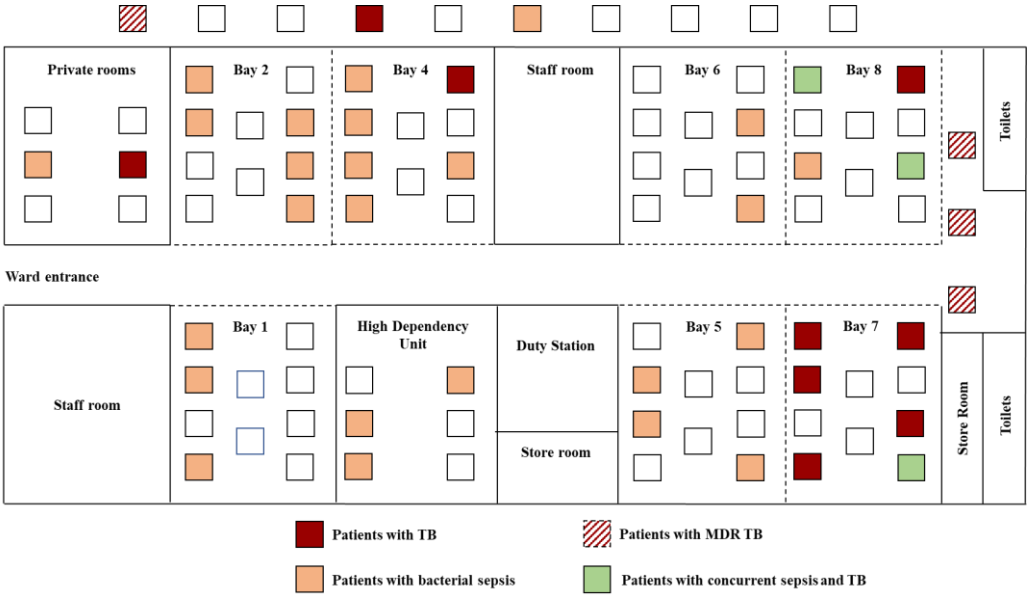


Figure 1: Ward map. Square denotes hospital bed where solid shading = patient occupied and pattern shading = unoccupied. Dotted lines represent bays separated by curtains and solid lines represent bays separated by walls. (A) location of study cohort and metagenomic sub-study (B) patients with sepsis (orange), tuberculosis (TB, maroon), multidrug resistant (MDR) TB (pattern shading, maroon) and sepsis + TB (green).

4.3.2 16S rRNA gene amplicon sequencing

DNA extraction from rectal swabs was performed as described previously (18). The V4 hypervariable region of the bacterial 16S rRNA gene was amplified as previously (19), and sequence read data was deposited in the EMBL European Nucleotide Archive (Accession number: PRJEB39247). 16S rRNA sequence data were processed with QIIME2 (version 2018-2), using DADA2 inbuilt software for sequence modelling and taxonomic assignment was performed against the SILVA reference database (release 132) (20,21). Sequence data were subsampled to a uniform depth of 1900 reads, based on rarefaction curve asymptotes. Core microbiota were determined based on taxon prevalence (detection in more than 50% of individuals) at a median relative abundance of 0·01 or above.

4.3.3 Shotgun metagenomic sequencing

Resistome composition was determined through shotgun metagenomic sequencing. Twenty-four patients were selected based on location and processed using Nextera XT DNA Library Prep Kit (Illumina Inc., CA, USA), and Nextera XT Index kit (Illumina Inc., CA, USA) in accordance with manufacturer instructions. An average of 13,977,146 ± 4,922,297 quality-filtered reads were obtained per sample. Sequence read data was deposited in the EMBL European Nucleotide Archive (Accession number: PRJEB39247). Identification of antibiotic resistance genes was achieved through alignment-based homology searches against the Comprehensive Antibiotic Resistance Database (CARD), as previously described (22).

4.3.4 Diversity measurements and statistical analysis

Two α-diversity indices were employed: taxon richness and Faith's phylogenetic diversity using QIIME2 (version 2018-2). Between group comparisons were performed by permutational multivariate analysis of variance (PERMANOVA) and homogeneity of multivariate dispersions (PERMDISP) using PRIMER 7 (PRIMER-E Ltd, Plymouth, UK).

Bray-Curtis similarity scores were determined from square root transformed relative abundances of taxa. Mann-Whitney U test was used for numerical comparisons (GraphPad Prism, version 8.2.1; GraphPad Software, La Jolla, California, USA) and the Spearman's test was used for correlations (R software, version 3.5.1, Vienna, Austria).

4.3.5 Distance-resistome similarity relationships

Inter-patient distance was scored using a categorical system (Table S1, Appendix 4). A resistome similarity matrix was generated using the Sørensen–Dice index and gene presence/absence data. DISTLM (DISTance based Linear Models; a distance-based regression analysis of univariate or multivariate data in response to predictor variables), using step-wise model selection for the R^2 criterion, was used to analyse the relationship between inter-patient distance and the resistome (PRIMER 7, PRIMER-E Ltd, Plymouth, UK). Correction for multiple testing was performed using the Bonferroni method.

4.3.6 Targeted AMR gene detection by quantitative PCR (qPCR)

Distribution of AMR genes that contributed to a distance-resistome similarity relationship (*NDM-7, OXA-1, dfrA14, catB3, fusB,* and *rmtB*), and three additional genes of high clinical relevance (*CTX-M-14, CMY-2, mcr-1.0*) was assessed by qPCR. Non-redundant representative sequences of each gene from the sequence data were aligned with reference sequences obtained from the CARD using the CLUSTAL OMEGA program (23), and to the NCBI database using the BLASTn module, to confirm specificity of gene annotation (24). Gene carriage was determined by qPCR using SYBR Green assays (Table S2, Appendix 4). Sanger sequencing was used to confirm specificity of amplicon sequences that were more than 150 base pairs (*OXA-1, NDM-7, CTX-M-14, rmtB, dfrA14, fusB and mcr-1.0).*

4.3.7 Assessment of distance-resistome similarity relationships

Potential contributors to distance-resistome similarity relationships were investigated by univariate and multivariate regression using SAS statistical software (SAS University Edition, 2018). Multivariate analysis included common clinical variables that are associated with the development of antibiotic resistance genes; antibiotic use, conditions associated with broad spectrum antibiotic use (TB and sepsis), and length of hospital stay.

4.4 RESULTS

Our study was conducted in an open-plan adult general medicine ward with a total capacity of 95 beds (68 permanent and 27 temporary). The ward was divided into nine bays, of which one was a “private” bay, and one a high dependency unit. Thirteen temporary beds were situated in an adjacent corridor. There were no restrictions placed on patient movement within the ward, although patients varied in their mobility status. Patients within the ward shared the same toileting and showering facilities.

There were 60 resident inpatients (63% of total ward capacity, 88% of permanent ward capacity) the ward on the day of sample collection (Figure 1A). One patient (temporary bed, Bay 1) could not be located and was excluded from the study. Patients were aged between 14 and 77 years and admitted for a range of indications (Table 1). Median length of stay was four days (IQR: 7·25 days). In the two weeks prior to sample collection, 43 patients (72·9%) had received either oral or intravenous antibiotics.

Table 1: Demographics and clinical parameters of subjects.

Variable	Inpatients (n=59)
Age-median (IQR)years	43 (27)
Sex	
Male	59 (100%)
Recent antibiotic use (2 weeks)	
Antibiotics	43 (72.9%)
No antibiotics	16 (27·1%)
Route of antibiotics	
Oral antibiotics	11 (25·6%)
Intravenous antibiotics	32 (74·4%)
Length of stay – median (IQR) days	4 (7·3)
Reason for admission	
Tuberculosis	16 (27·1%)
Liver disease (including alcohol related)	11 (18·6%)
Complications of diabetes mellitus	10 (16·9%)
Sepsis	7 (11·9%)
Hypertension	4 (6·8%)
Malignancy	3 (5·1%)
Ischaemic heart disease	3 (5·1%)
Others	5 (8·5%)
Multiple co-morbidities[a]	
Co-morbidity index 0-4	51(86·4%)
Co-morbidity index 5-8	8(13·6%)

[a] Charlson co-morbidity index used to assess impact of co-morbidities. Higher score indicates higher 10-year predictive mortality rate.

4.4.1 Rectal microbiota composition

All rectal samples were subjected to initial 16S rRNA gene amplicon sequencing (hereafter, 16S sequencing). One subject (permanent ward bed, Bay 1) was excluded from downstream analysis due to persistent low read depth. In total, 224 sequence variants were detected, representing 12 bacterial phyla. Of these, Firmicutes (51·5%), Actinobacteria (17·3%), Proteobacteria (14·2%), Bacteroidetes (11·1%) and Fusobacteria (1·3%) were predominant. Nine "core" bacterial genera were identified, of which seven were taxa associated with faeces (*Finegoldia*, *Anaerococcus*, *Enterococcus*, *Peptoniphilus*, *Escherichia-Shigella*, *Bacteroides*, *Streptococcus*) and two were taxa associated with the perianal region (*Staphylococcus*, *Corynebacterium*) (Figure S1). No relationship was identified between the microbial composition of rectal swabs and the distance between patients (Spearman correlation r= -0·02, p= 0·08, Figure S2, Appendix 4).

4.4.2 Metagenomic characterisation of resistome features

Shotgun metagenomic analysis was performed on 24 representative patients (Figure 1A). In total, 453 *de novo* assembled AMR genes were identified in rectal metagenomes. These included genes encoding multidrug efflux proteins (n=173, 38·2%); and genes conferring resistance to glycopeptides (n=52, 11·4%); beta-lactams (n=37, 8·2%); macrolides (n=33, 7·3%); aminoglycosides (n=28, 6·2%); phenicol (n=27, 6%); tetracyclines (n=20, 4·4%); peptides (n=17, 3·8%); diaminopyrimidine (n=16, 3·5%) and other resistance traits (n=50, 11%) (Table S3, Appendix 4).

In contrast to the resistance genes detected, the principal antibiotics prescribed during the study period were cephalosporins (23·7%) anti-TB medications (rifampicin, ethambutol, pyrazinamide, isoniazid) (20·3%), and fluoroquinolones (16·9%) (Table S4, Appendix 4).

4.4.3 Association between bacterial taxa and AMR genes

The relative abundance of 453 resistance traits and 224 bacterial taxa, detected in the metagenomes of 24 subjects, were assessed by Spearman's correlation. After adjustment for false discovery, 76 significant interactions were identified ($p<0.05$, Table S5, Appendix 4). Apart from glycopeptide resistance genes, the majority of traits that were significantly correlated with bacterial taxa (beta-lactamase resistance, peptide resistance, or encoded efflux pumps) are commonly associated with Gram-negative bacteria. However, the taxa that were associated with these genes were predominantly Gram-positive anaerobic gut bacteria, belonging to the phylum Firmicutes. This observation may be the result of antibiotic use in this cohort. Cephalosporins (including combination cephalosporins) and fluoroquinolones represented 49.1% of total antibiotic use (Table S4, Appendix 4). These broad-spectrum are disproportionately active against Gram-negative bacteria, potentially resulting in a relative selection of Gram-positive anaerobes, and a concomitant selection of resistance determinants in Gram-negative taxa.

4.4.4 Spatial resistome distribution

A resistome similarity matrix was generated based on the detection of resistance determinants within each patient (Figure S3, Appendix 4). Resistome similarity scores showed a weak but statistically significant positive correlation related with spatial distance similarity scores ($r=0{\cdot}12$, $p=0{\cdot}04$; Figure S4, Appendix 4). Exploratory DISTLM analysis identified 19 AMR genes that contributed significantly to the location-resistome relationship. These included genes conferring beta lactam resistance (n=5), glycopeptide resistance (n=3), efflux pump (n=3), peptide antibiotics (n=2), and others (n=6) (Table 2).

The 19 AMR genes identified by DISTLM analysis were ranked by detection frequency. Those within the interquartile range (*OXA-1*, *NDM-7*, *dfrA14*, *catB3*, *fusB*, and *rmtB*) were further assessed by targeted qPCR analysis in the study population as a whole (n=59) (Figure S5, Appendix 4). Three AMR genes which are of high clinical importance, but which did not show a location-distribution relationship (*CTX-M-14*, *CMY-2*, and *mcr-1.0*), were also included.

The results of targeted qPCR-based detection of *fusB* (40·7% of samples) *CMY-2* (31·0%), *NDM-7* (30·5%), *dfrA14* (28·8%), *CTX-M-14* (25·4%), *OXA-1* (24%), *catB3* (22%), *rmtB* (15·3%), and *mcr-1·0* (5·1%) (Table S6, Appendix 4) was consistent with resistome data, with metagenomic detection confirmed in >96% of cases. DISTLM analysis based on qPCR data also confirmed the results of metagenomic analysis, with a significant relationship identified between patient location and AMR gene carriage for *OXA-1*, *NDM-7*, *dfrA14*, *catB3*, *fusB*, *and rmtB* genes. This relationship remained statistically significant after correction for false discovery for *catB3* (p =0·002), *OXA-1* (p =0·003), *NDM-7* (p =0·047), and *dfrA14* (p=0·0495) (Table 3).

Table 2: Resistance genes that contribute to spatial patterns within the sub-population where metagenomic analysis was performed.

Gene	SS (trace)	Pseudo-F	p-value	Explained variance (proportion)	Resistance mechanism
OXA-1	66·812	9·953	0·001	0·322	Beta-lactams resistance
MCR-1·9	56·881	7·916	0·004	0·274	Peptide antibiotic resistance
BRP(MBL)	55·229	7·603	0·005	0·266	Beta-lactams resistance
dfrA14	48·845	6·454	0·006	0·235	Diaminopyrimidine resistance
Omp38	45·105	5·823	0·011	0·217	Beta-lactams resistance
rmtB	42·099	5·336	0·012	0·203	Aminoglycoside resistance
NDM-7	40·965	5·157	0·016	0·197	Beta-lactams resistance
catB3	40·085	5·020	0·014	0·193	Phenicol resistance
vanSG	37·290	4·593	0·024	0·180	Glycopeptide resistance
tsnR	36·330	4·450	0·017	0·175	Peptide antibiotic resistance
mgtA	36·330	4·450	0·015	0·175	Macrolide resistance
mdfA	36·019	4·404	0·023	0·173	Multidrug efflux pump
KpnF	32·752	3·930	0·037	0·158	Multidrug efflux pump
vanN	32·473	3·890	0·041	0·156	Glycopeptide resistance
vanO	31·383	3·736	0·048	0·151	Glycopeptide resistance
CRP	31·383	3·736	0·049	0·151	Multidrug efflux pump
vgaE	31·383	3·736	0·048	0·151	Streptogramin resistance
fusB	31·309	3·726	0·039	0·151	Fusidic acid resistance
CfxA6	30·898	3·668	0·046	0·149	Beta-lactams resistance

Performed using DISTLM test. SS - sum of squares.

Table 3: Resistance genes that contribute to spatial patterns within the study population.

Gene	SS (trace)	Pseudo-F	p-value	FDR adjusted (p)	Explained variance (proportion)
catB3	118·660	5·732	0·0002	0·002	0·091
OXA-1	106·230	5·078	0·0003	0·003	0·081
NDM-7	73·588	3·424	0·0052	0·047	0·057
dfrA14	76·405	3·564	0·0056	0·0495	0·059
fusB	60·368	2·779	0·0163	0·147	0·046
rmtB	52·082	2·382	0·0435	0·392	0·040
CMY-2	38·315	1·733	0·110	0·992	0·030
MCR-1·0	13·053	0·579	0·602	5·417	0·010
CTX-M-14	14·255	0·633	0·690	6·214	0·011

Performed using DISTLM test. SS - sum of squares

4.4.5 Prediction of distance-resistome relationships by clinical variables

When AMR gene carriage was mapped against patient location, it was notable that the distribution of the genes identified as contributing to the distance-resistome relationships were disproportionately prevalent on one side of the ward (Bay 5-8) and in the corridor (Table S7, Appendix 4). Co-dispersion patterns of individual AMR genes were also identified, including a positive relationship between determinants that conferred resistance against antibiotics with Gram-negative activity (Figure 2). While inter-patient transmission could explain such localised clustering, the deliberate co-location of patients with particular conditions (e.g., TB; Figure 1B), and particular antibiotic exposures (e.g., antibiotics with Gram-negative activity) could result in such an effect. We therefore explored whether AMR dispersion might be explained by presence of sepsis, TB, MDR-TB, use of antibiotics or length of stay by using both univariate and multivariate approaches.

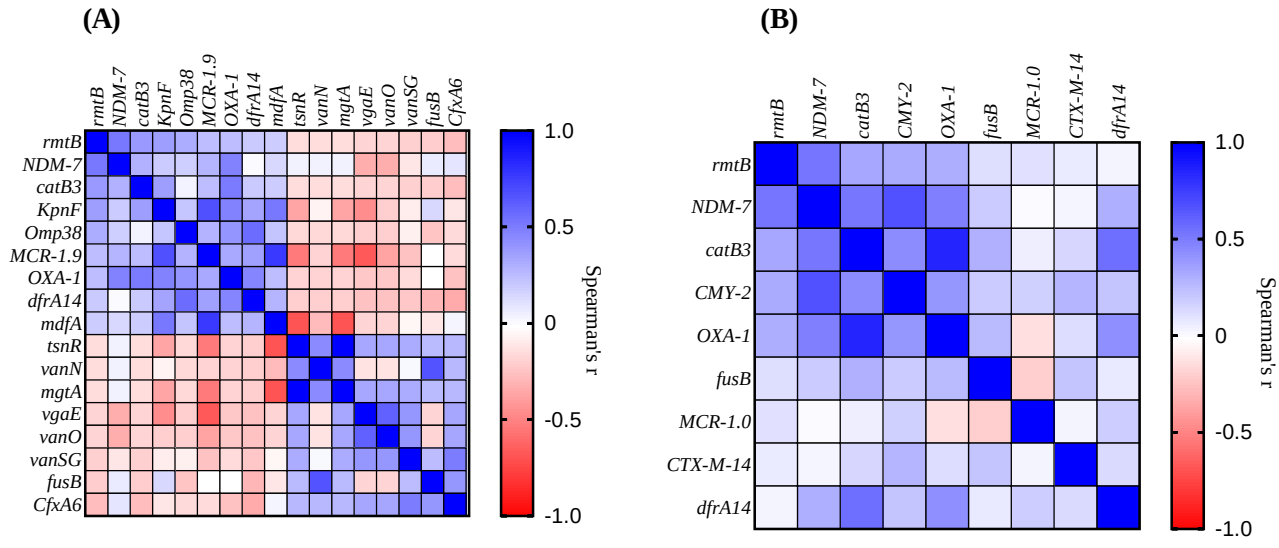


Figure 2: Heatmaps showing correlations between resistance genes that were identified as spatially associated from (A) shotgun metagenomic analysis of 24 individuals included in the sub-study and (B) quantitative PCR analysis of the entire study cohort. Performed using Spearman's correlation on gene presence/absence.

In age-adjusted univariate analysis, TB predicted the carriage of *OXA-1*(p=0·02), *catB3* (p=<0·01), and *dfrA14* (p<0·01); length of stay in hospital predicted carriage of *NDM-7* (p=0·02) and *CMY-2* (p=0·04); and antibiotics use predicted carriage of *CMY-2* (p=0·03) (Table S8). TB still predicted the presence of *OXA-1* (p=0·03), *catB3* (p<0·01), *dfrA14* (p=0·02), and *rmtB* (p=0·03) in multivariate analysis, where age, sepsis and length of hospital stay were adjusted for. Length of stay predicted detection of *NDM-7* (p=0·04) and *CMY-2* (p=0·03) while bacterial sepsis predicted detection of *CMY-2* (p=0·03) (Table S9, Appendix 4).

We identified a number of resistance genes that were associated with patients with TB (*OXA-1, catB3, dfrA14* and *rmtB*). A likely contributor to this relationship was the longer average length of stay of TB patients compared to the wider patient population (median length of stay [IQR]: TB = 7.5 days [15.6]; non-TB = 4 days [6.0]), increasing the likelihood of AMR acquisition. We observed no differences in microbiota diversity or richness between TB and non-TB patients (Faith PD, p=0.58; Observed OTUs, p=0.36), or in overall community composition (PERMANOVA p=0.30, PERMDISP p=0.81). However, levels of Firmicutes were lower and levels of *Escherichia-Shigella* were significantly higher in those with TB (Figure S6, Appendix 4). *Escherichia-Shigella* was significantly correlated with *OXA-1, dfrA14* and *catB3* (Table S10, Appendix 4).

4.5 DISCUSSION

An improved understanding AMR transmission in South-East Asia is essential for the development of effective strategies to limit AMR spread. Selection pressures, reservoirs, and modes and routes of transmission vary considerably between settings. As a consequence, strategies developed in HICs may be inappropriate in LMIC. Despite this, LMIC responses to the threat of AMR spread are largely informed by studies in HIC settings. Local deployment of genomic technologies can provide valuable insight typically unavailable in low-income regions. Investigations of inter-patient AMR transmission usually focus on specific pathogens and resistance phenotypes.

While such an approach can inform infection control strategies, it provides limited insight into the ongoing circulation of AMR determinants within patient populations. In contrast, metagenomic approaches provide several important advantages. First, they are able to identify AMR genes inclusively, without the need for *a priori* target selection. Second, they are able to assess relationships between resistance carriage and patient variables at a resistome level, reducing the impact of individual determinants or bacterial species. Finally, objective identification of individual resistance determinants enables targeted analysis by qPCR. Indeed, metagenomics-based approaches have also been employed successfully in a number of other contexts, including to map opportunistic pathogens and AMR genes in a tertiary hospital environment in Singapore (25), and to determine the non-nosocomial circulation of AMR determinants in low-income communities (26).

In our study population, resistome analysis revealed a diverse range of AMR determinants. A substantial number of these genes contributed to a significant location-resistome relationship, in which their co-carriage in separate individuals varied inversely with inter-patient distance. Such a relationship is consistent with patient-to-patient AMR transmission, assuming that transmission risk increases with increasing patient proximity. Even after correction for false discovery, significant spatial distribution patterns existed for clinically important AMR genes, including *OXA-1* and *NDM-7*.

Importantly, transmission was not the primary driver of patient location-resistome relationship. When we explored the distribution of AMR genes associated with significant spatial distribution, we found that TB predicted carriage of genes that showed significant relationships with inter-patient distance, including *OXA-1*, *catB3*, and *dfrA14*. The contribution of other variables such as antibiotic therapy, sepsis and length of stay were explored and demonstrated that length of stay predicted *NDM-7* and *CMY-2*, and bacterial sepsis predicted *CMY-2*.

Closer examination revealed that placement of TB patients was largely limited to one quarter of the ward. Our findings, therefore, suggest that the observed resistome-location relationships are likely to result largely from patient placement. While our assessment did not demonstrate a wider transmission event within the ward, it is possible that a higher frequency of environmental and direct inter-patient transmission of resistance genes may have contributed to the observed patterns of AMR gene carriage within patients with TB.

Patient length of stay in hospital was closely associated with *NDM-7* and *CMY-2*. These genes both confer broad-spectrum β-lactam resistance typically associated with prolonged antibiotic exposure. The relationship may therefore be due to cumulative exposure to antibiotics provided in hospital. Similarly, an observed association between *CMY-2* and sepsis, in addition to the relationship with length of stay, could reflect prolonged high antibiotic exposure, or the clinical impact of sepsis causes by pathogens that carry *NDM-7* or *CMY-2*.

The relationship between TB and carriage of *OXA-1, catB3, rmtB* and *dfrA14* is likely to reflect the impact of gut microbiota disruption arising as a result of prolonged hospital stay and antibiotic therapy. A previous study has reported the relative abundance of gut Firmicutes to be decreased in those with TB, and the prevalence of Proteobacteria to be increased (27). We observed a reduction in Firmicutes and an increase in *Escherichia-Shigella*, with the latter correlated with *OXA-1, dfrA14* and *catB3.*

Notably, our analysis identified a number of AMR genes that are of considerable clinical concern, including *OXA-1* and *NDM-7*. Carbapenem-resistant organisms are a major health threat, particularly in LMICs (28), and *NDM* and *OXA* genes have emerged as key contributors to carbapenem resistance in multidrug resistant Gram-negative pathogens (29). The fact that patients are likely to have brought bacteria carrying these AMR determinants into the hospital environment within their intestinal microbiota highlights the importance of gaining a better understanding of AMR carriage and transmission within the wider Myanmar population.

Consideration of AMR carriage tends to focus on point-source infection outbreaks in hospitals or within the wider community. However, the resistome associated with the intestinal microbiome is constantly being contributed to and reshaped by antibiotic and non-antibiotic exposures. The acquisition of an AMR-carrying bacterial strain or resistant determinant might precede overt infection by a substantial period, particularly where the determinant is carried within a commensal species prior to migrating into a pathogen population. Such dynamics could explain differences between the types of AMR gene detected in our study population and contemporary antibiotic exposure of patients. Other than multidrug efflux pumps, AMR determinants identified in our study most commonly conferred resistance to glycopeptides, followed by beta-lactams, macrolides, and aminoglycosides. In contrast, the principal antibiotic exposures of patients at the time of the study were cephalosporins, anti-TB medications, and fluoroquinolones. Such a disparity has been reported previously in other resource-limited settings (29) and highlights the role of AMR transmission beyond the hospital setting in defining the patient intestinal resistome.

Prior to metagenomic analysis, we also applied 16S sequencing to rectal swab DNA, both to confirm sample quality and to enable relation of resistome traits to microbial diversity. AMR determinants can move between bacterial populations through horizontal gene transfer. As a result, microbiota and resistome traits are not always strongly aligned, and indeed, intestinal microbiota depletion can result in increased acquisition of resistant bacteria from the environment (30). However, in our study population, microbiota diversity was not significantly correlated with the number of AMR genes carried and was unrelated to patient location.

Our study had limitations that should be considered. We chose not to assess AMR reservoirs such as fomites within the hospital environment, or potential mediators of transmission, such as clinical staff and inter-patient interaction. While potentially informative, it was decided to instead focus first on determining whether evidence of substantial transmission existed. We did not assess whether common resistance determinants in co-located patients were identical, a process that cannot be readily achieved through the metagenomic approach employed. As the

focus of our study was AMR gene detection as a marker of a potentially transmissible trait, we did not assess phenotypic resistance conferred by AMR markers, or which bacterial species carried them. Finally, our study was cross-sectional in nature and involved a single ward within an individual hospital. As such, further longitudinal analysis, performed across multiple settings, would provide additional insight into potential causality in associations between AMR gene distribution and patient variables.

Despite these limitations, our use of a metagenomic strategy to define AMR carriage within hospital inpatients in a resource-limited setting did not identify substantial inter-patient transmission. These findings are consistent with effective infection control, which is critical in reducing risks of nosocomial outbreaks. However, they do highlight the need for wider assessments of AMR carriage and transmission beyond the hospital environment as a basis for establishing evidence-based national AMR prevention and containment strategies. The development of effective strategies to reduce AMR transmission at a population level would likely provide substantial benefit, not least by reducing AMR carriage in patients being admitted to hospital which can then be transmitted within the nosocomial environment. Examples of population-based strategies that could be considered to reduce AMR carriage would include a One Health approach by monitoring antimicrobial use in livestock and domestic animals, reducing over the counter and community antimicrobial prescriptions, implementing surveillance of AMR in agricultural settings and food production as well as improved waste management. Deployment of new technologies, such as metagenomic sequencing, could help to better understand the origin of organisms, and the role of population movement in the spread of AMR. Metagenomic analysis represents a powerful means to generate the data needed to inform such measures by understanding the origin of micro-organisms and the role of population movement in the spread of AMR.

Availability of data and materials

The sequence read data was deposited in the EMBL European Nucleotide Archive (Accession number: PRJEB39247).

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, I have presented studies which have increased our understanding of the hospital microbiome and its interaction with the patient population. The rise of hospital-acquired infections (HAI) in conjunction with limited development of new antibiotics has prompted future research to shift focus to the hospital microbiome as a potential therapeutic target. However, research dedicated to understanding the hospital microbiome remains limited. Prior to this thesis, it was known that there is a temporal relationship not only between the patient microbiota with hospital microbiota (1) but also with resistance gene distribution in the hospital environment (2). Ward layout is also thought to be contributory (3, 4) . However, knowledge gaps still exist in understanding factors that influence microbial movement and pathogen transmission within the hospital.

Through application of sequence-based approaches to environmental and patient samples from different healthcare contexts, my thesis aims to provide new insights into the hospital microbial ecology. This will increase understanding of microbial tracking and resistance gene transmission. Specifically, three interactions were assessed: the influence of hospitalisation on patient microbiota, the influence of occupancy on hospital microbiota and transmission of antibiotic resistance genes within an open plan ward. This was analysed with two cross-sectional studies and a longitudinal study. This thesis provides a deeper understanding of the evolution of the hospital microbiome and how it interacts with patients. The work gives new insights into the role of the hospital infrastructure, specifically ward layout on microbial transmission. The knowledge from these studies and the future directions for this work will be discussed.

5.1 KEY OUTCOMES, SIGNIFICANCE AND LIMITATIONS

A. The relationship between hospital microbiome and patient microbiome

The relationship between the indoor environment and humans living within the environment is complex. Humans are known to leave their personal microbial signature behind once they have vacated an indoor area (5). This relationship is important within the healthcare setting which is traditionally considered a clean environment. Exploring this relationship would give us insight into the movement of microbes between patient and their environment which has profound implications in understanding HAIs.

The influence of hospitalisation on patient microbiota.

In chapter 2, the effects of hospitalisation on oropharyngeal (OP) microbiota of inpatients without lower respiratory tract infection was investigated. It was found that inpatients exhibited significantly reduced OP microbiota diversity compared to healthy controls. Microbiota composition also differed significantly from controls, with inpatients displaying greater dispersion. The relative abundance of commensal taxa was reduced in inpatients, with significant increases in *Rothia, Veillonella* and *Lactobacillus;* microbiota traits which have been observed by others in patients with underlying nosocomial pneumonia (6). These differences were not explained by age, gender, length of stay or antibiotic exposure. The cross-sectional nature of this study limited longitudinal assessment to determine if inpatients with altered OP microbiota subsequently developed pneumonia. Another limitation was the difference in baseline characteristics between the healthy and inpatient cohort which we attempted to address by performing multivariate age-matched and gender-stratified comparisons. Despite the limitations, this study demonstrates the changes observed in the URT of hospitalised patients, highlighting the clinical potential of OP microbiota traits in identifying hospitalised patients who are at risk of healthcare associated pneumonia.

The influence of patient occupancy on hospital microbiota.

In chapter 3, the changes observed in hospital microbiome during the relocation of a hospital from an old site, which was decommissioned, to a newly built facility was investigated. It was found that patient occupancy of the new hospital building was associated with a significant increase in the environmental bacterial load and a significant change in microbiota composition. Within the inpatient areas of the new building there was an increase in relative abundance of human microbiota-associated bacterial taxa. These findings were also reported in a previous study where an increase in the contribution of skin-associated genera was observed following a brand new hospital opening (1). Conversely, the closure and decommissioning of the old hospital site was associated with a decrease in bacterial load in the old building. Interestingly, bacterial load and microbiota composition were not significantly different in both new and old hospitals at similar levels of occupancy. Occupancy was defined broadly in this study and included the collective contribution of patients, staff members, cleaning, ventilation, visitors, and other factors therefore limiting the assessment of each of these variables on the hospital microbiota. The results from this study improve understanding of the composition and dynamic changes in hospital microbiota that occur with occupancy.

B. The influence of ward layout on antimicrobial resistance gene transmission

In chapter 4, the extent to which hospital wards act as a venue for antimicrobial resistance gene transmission in low- and middle-income countries settings was investigated. It was found that overall resistance gene similarity showed a weak but significant positive correlation with inter-patient distance within the ward. Resistance genes which contributed to this relationship included those encoding β-lactamases, trimethoprim resistance, and chloramphenicol resistance.

However, clinical traits of co-located patients carrying specific AMR genes were not random and specifically mapped to patients with tuberculosis who were cohorted. This contrasted with non-cohorted patients with sepsis who carried AMR genes that were not spatially significant. In this study we chose to focus on patients rather than a combination of patients and fomites as the main study aim was to identify an association between patient location and resistance gene transmission. This cross-sectional study was conducted in a single ward which limited our assessment. Further longitudinal analysis, performed across multiple settings, would provide additional insights into potential causality in associations between AMR gene distribution and patient variables. Despite these limitations, through shotgun metagenomic approaches, we did not find any evidence to suggest substantial inter-patient transmission within the ward. These findings suggest that AMR transmission likely occurs outside of the hospital setting.

5.2 IMPLICATIONS FOR CLINICAL PRACTICE

The research presented in this thesis illustrates two important points. First, the value of culture-independent techniques to enhance understanding of the complex microbial ecology of the hospital environment. Second, the importance of recognising the role of the hospital microbiome in understanding microbial movements which in turn forms a basis to develop evidence-based infection control polices. Based on existing literature and results from this thesis, the implication on clinical practice is discussed below.

A. Investigating the changes in human microbiota during hospitalisation could potentially identify those at risk for healthcare acquired infections.

Previous studies have investigated the impact of hospitalisation on gut microbiota of immunosuppressed and critically unwell patients. Findings from these studies demonstrate hospitalised patients generally have reduced faecal diversity with increase abundance of potentially pathogenic taxa such as *Enterococcus*, *Staphylococcus* and Enterobacterales (7-9). HAP is the most common healthcare acquired infection with increased risk burden with increasing length of stay (10). It is known that increased levels of pathobionts in the OP can

precede the development of LRTI (11, 12) The extent to which changes in the OP microbiome mediate the development of HAP is unknown with a single published study failing to identify changes in the OP microbiota associated with hospitalisation (13). The results of this thesis support changes seen within the OP microbiota of inpatients with increased taxa that are potentially associated with LRTI. OP microbiota analysis could be used to identify patients with high risk of LRTI.

B. Occupancy is the key driver in shaping the hospital microbiome.

Identifying the optimal hospital microbial environment is a challenging task. Despite being considered a clean environment, the hospital environment is constantly challenged by a vulnerable population who potentially carry highly transmissible organisms. Recently, there has been an increased interest in understanding the hospital microbiome in an attempt to map dissemination of resistance genes and identify potential reservoirs of pathogenic organisms. However, studies focusing on this remain limited as the number of new hospitals built with adequate molecular technology to investigate this relationship are scarce. Studies examining this, identified complex temporal relationships between patient and environmental microbiomes with bacterial taxa within the environment closely reflecting the microbiota of the patient occupying the room (1). It has been identified that distinct ecological niches of antibiotic resistance genes within the hospital environment correspond to spatiotemporal patient influences (2). Our study examined the effects of relocation of a hospital to a newly built facility in relation to occupancy on the hospital microbiome. We demonstrated minimal differences within the hospital microbiome between an old and new site at similar occupancy levels. The results from this study add another dimension to the understanding of the composition and dynamics of hospital microbiome indicating that occupancy rather than building age or architecture exerts most influence.

C. Ward layout does not affect resistance gene transmission in low-and middle-income setting.

Hospital design has slowly transitioned from a multi-bed bay model to predominantly single room facilities. Among health care professionals, it is a common belief that single room hospitals are likely to reduce transmission of resistant pathogens by limiting the amount of inter-patient direct and indirect contact through shared spaces (14). However, this approach has not been accepted in low-and middle-income countries. In fact, most developing countries continue to rely on large communal multi-bed type wards for inpatient care where a combination of risk factors, including physical proximity, high antibiotic exposure, and increased bacterial dispersion (15), increases the risk of AMR spread. We examined the extent of AMR transmission within an open plan ward in Myanmar and found that AMR dispersion patterns primarily reflect the placement of patients by their medical condition, rather than wider inpatient AMR transmission within the ward. Our study suggests that nosocomial transmission of AMR genes is a relatively minor contributor in countries where AMR transmission is high within the population outside the hospital setting.

5.3 FUTURE DIRECTIONS

Based on the work performed in this thesis, future research directions to explore the role of the hospital microbiome and its implication in infection control practices are presented below.

A. Utilisation of human microbiota traits to predict sepsis

Previous studies have demonstrated changes particularly in the gut microbiota of hospitalised patients. The majority of these studies have suggested an increase in phylogenetic groups that include potential pathogens which can cause HAIs as well as reduced gut bacterial diversity (7-9). However, most of these studies have focussed on critically ill or immunosuppressed patients. Studies looking at the changes within the OP microbiota are limited (13). The study conducted as part of my thesis suggested differences in OP microbiota of hospitalised patients with lower diversity and richness along with an increase of selective taxa which can predispose to hospital acquired pneumonia.

There is a need for well-designed human clinical studies investigate hospital-related dysbiosis by controlling confounders such as antimicrobial use, nutritional status, and pre-morbid conditions. A longitudinal study is needed to investigate if the observed microbiota changes predispose to HAI. Does OP microbiota changes in hospitalised patients precede development of hospital acquired pneumonia?

B. Understanding the bacterial structure within a hospital environment to map microbial tracking and understand dispersion of antimicrobial resistance genes

By understanding the bacterial community structure that exists in a hospital environment, we can identify if a link exists between hospital microbial communities and HAIs. Despite studies demonstrating that hospitals have selective niches which harbour antimicrobial resistance genes (2) and that there is a temporal relationship between the patient and environment microbiome (1), there is still a lack of understanding of how these relationships

and niches correspond to acquisition of HAIs. Through this thesis, the role of occupancy associated with the decommissioning of an old hospital site and the relocation to a newly built facility was explored.

Moving forward, there are several questions that needs to be addressed. As the interactions between patient microbiota and hospital microbiota become clearer, how are we able to mitigate harmful interactions throughout a patient's stay in hospital? Will understanding these interactions result in a change in patient's risk of acquiring a HAI? Infection control practices traditionally are based on expert intellectual opinions as well as previous culture-based studies (16). Can we utilise metagenomic sequencing to provide better evidence for infection control practices?

C. The role of an all single-room hospital compared to a multi-bed bay hospital in reducing antimicrobial resistance gene transmission

Although most modern hospitals in high-income countries are transitioning to a single-room hospital model, the evidence of single-room hospitals in reducing rates of HAI remain scarce (4, 17, 18). One way of investigating this is to understand the level of AMR dispersion within the hospital setting by using culture-independent methods. In this thesis, we attempted to map AMR dispersion in an open plan ward within a tertiary hospital in a LMIC setting and found no evidence of ward layout increasing patients' risk of resistance gene acquisition.

This now requires further investigation and could lead to future change in hospital building design. In countries where AMR genes are dispersed widely beyond the hospital setting, it is unlikely that a change in ward layout alone could prevent transmission events. However, our study focussed on a single ward with a relatively small sample size and a bigger clinical study not just focusing on patient samples but also samples from inanimate objects as well as intermediary hosts such as staff members is warranted.

Similar studies investigating AMR dispersion comparing different ward layouts and single rooms should also be performed in high income countries which potentially might demonstrate different results especially countries with low levels of AMR genes within the community setting.

D. Utilisation of culture-independent techniques in evaluating current hospital practices

Culture-independent techniques have broadly increased our ability to understand bacterial diversity that can be difficult to observe with culture-based methods. Moving ahead, the utilisation of culture-independent techniques could be beneficial in examining current hospital practices and assessing targeted interventions. For instance, good quality data on the effectiveness of hospital environmental cleaning programmes remain limited (19). Through culture-independent techniques, questions relating to the effectiveness of current cleaning methods and application of certain cleaning products particularly in promoting or reducing in AMR genes within the hospital environment could be investigated.

Increasingly affordable high throughput sequencing methods have been used in public health surveillance and outbreak investigations. The most recent example of this is the utilisation of whole genome sequencing (WGS) as a powerful tool in understanding transmission dynamics of SARS-CoV-2 (20, 21). Prior to this, WGS was widely used in outbreaks of bacterial infection (22, 23). Now that WGS has been integrated into outbreak investigation and surveillance, the future might soon see the utilisation of these methods in diagnostic assays resulting in large changes within clinical microbiology.

5.4 CONCLUDING REMARKS

In summary, the results presented in this thesis contribute to the understanding of the complex interaction that exists between the hospital and patient microbiota. The studies in this thesis support the bi-directional influences of humans and built environment within the healthcare setting and investigates the role of ward layout in AMR transmission. This not only provides a foundation for future studies but also allows an insight into the role of metagenomic sequencing in understanding the movement of microbes within a healthcare setting.

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APPENDICES

Appendix 1: Optimisation of methodology

Multiple steps in microbiome analysis including sample collection, sample processing, DNA extraction and PCR amplification of 16S rRNA gene have the potential to introduce bias. Bacterial biomass can significantly influence bacterial 16S rRNA amplicon sequencing profiles; low biomass samples can generate sequencing artefacts. This is especially true for environmental samples where variable amount of bacterial loads are present (1). The study examining the effect of occupancy and space utilisation on the hospital microbiome (*Chapter 3*), involves environmental sampling, therefore careful consideration was given in optimising adequate sampling technique and processing to maximise DNA recovery for 16S rRNA sequencing. These include assessing different swab materials, reducing the risk of sequencing artefacts by incorporating decontamination methods and increasing DNA recovery through optimisation of DNA extraction procedure and utilisation of a higher DNA biomass as a template for sequencing.

Study variables:

- Different swabbing materials
- Optimal DNA extraction method for samples with potentially low bacterial load
    a. Utilisation of UV light to reduce pre-sampling contamination
    b. Utilisation of Phenol Chloroform in DNA extraction methods
    c. Utilisation of vacuum concentrator on samples with low biomass

The aim of these validation studies was to compare various swabbing materials and DNA extraction techniques for microbiota analysis to identify the most effective DNA recovery method for use on environmental surfaces.

Experiment 1: Comparison of different swabbing materials

Swabs are indirect sampling devices made of various materials, including cotton, rayon, or foam, and they may be flocked by design. To determine the swab material that gives the best DNA recovery from swabbing surfaces. Common swabs which have previously been evaluated in environmental studies and readily available within the clinical setting were chosen. These swabs include:

1. Nylon flocked dry swab (COPAN Diagnostics Inc, USA)
2. Wooden sterile cotton tip single ended applicator (Interpath, Australia)
3. Alcohol swab (70% isopropyl alcohol, Medi-Swab, BSN Medical, Victoria)

Methods:

Pure culture plates of *Escherichia coli* and *Staphylococcus aureus* were grown on horse blood agar (Thermo Fischer Scientific, USA). A single colony of each strain was transferred into separate enriched CSF broth (Thermo Fischer Scientific, USA) and incubated at 37°C overnight. The following day 100uL aliquots of the resultant *E. coli* and *S. aureus* broth cultures were randomly pipetted onto ten separate sites of a sterile empty petri plate (100mm ×15mm, Sigma-Aldrich, MO, USA) and allowed to dry in the biosafety cabinet for approximately 60 mins. Each petri dish was swabbed thoroughly with a specific swab, and the process was performed in three technical replicates for each swab. In total, five combinations of swabs and conditions were tested, which comprised of a flocked dry swab, a moist flocked swab (dipped in a 1.5mL Eppendorf tube containing100uL normal saline), a dry wooden sterile cotton tip swab, a moist wooden sterile cotton tip swab (dipped in a 1.5mL Eppendorf tube containing100uL normal saline) in and an alcohol swab. Non-inoculated empty petri plates were used as negative controls for each swab. Additionally, 100uL of the *E. coli* and *S. aureus* broth culture were directly transferred into separate 1.5mL Eppendorf tubes as positive controls.

Following swabbing, each swab was transferred into separate 1.5mL Eppendorf tubes containing 400uL of sterile Tris-EDTA buffer (TE) (10mM Tris, 1mM EDTA) (ROCHE, Victoria, Australia) and stored at -80°C prior to extraction. All swabs were processed using the same DNA extraction method to assess DNA yield. For DNA extraction, swabs were vortexed in TE storage buffer for 30 sec. To maximise DNA recovery, the swab was then transferred into a 3mL syringe that is attached to a 2mL tube and the assemble was placed in a 50mL Falcon tube (company), followed by centrifugation at 3374 x g for 5 min to obtain the remaining solution from the swab. The swab was then discarded, and DNA extraction was performed on the combined TE suspension. For alcohol swabs, the swab was removed prior to centrifugation and DNA extraction was performed on the remaining solution.

TE suspension was heated to 95 °C for 1 minute, and then cooled at 4°C for 2 minutes. Lysozyme and lysostaphin (Sigma-Aldrich, MO, USA) were then added to a final concentration of 2.9 mg/mL and 0.1 mg/mL, respectively, and samples incubated at 37 °C for 1 hr. Proteinase K (Fermentas, ThermoFisher Scientific, Victoria, Australia) and sodium dodecyl sulphate (Sigma-Aldrich, MO, USA) were then added to a final concentration of 1.2 mg/mL and 1.5 %, w/v, respectively.

Following incubation for 1 hr at 50 °C, approximately 0.13 g of washed beads (1:1 of 0.1mm and 1mm silica zirconium beads) was added to the sample, followed by bead beating at 6.5 m/s for 1 min using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, USA). The supernatant was transferred into a 2 mL screw cap microcentrifuge tube and 100 ul of 5M sodium chloride were added and samples vortexed for 60 sec. DNA was precipitated by the addition of 10 M ammonium acetate and 100% ethanol (Sigma Aldrich, MO, USA) in a 1:10 and 1:1 ratio of sample volume, respectively. DNA was then purified using an EZ-10 Spin column in accordance with manufacturer's instructions (Bio Basic, Inc., Ontario, Canada). DNA was eluted in three lots of 50 ul UltraPure DNase/RNase-free distilled water (Gibco,

ThermoFisher Scientific, Victoria, Australia) to a total of 150 uL. The DNA yield was quantitated by using 2 uL of the DNA sample on a Qubit 2.0 Fluorometer (Thermo Fischer Scientific, USA).

Results:

The nylon flocked swab dipped in normal saline prior to swabbing, proved to be the most effective swab in terms of DNA yield when compared to the alcohol swab and wooden cotton tip swab (see figure below).

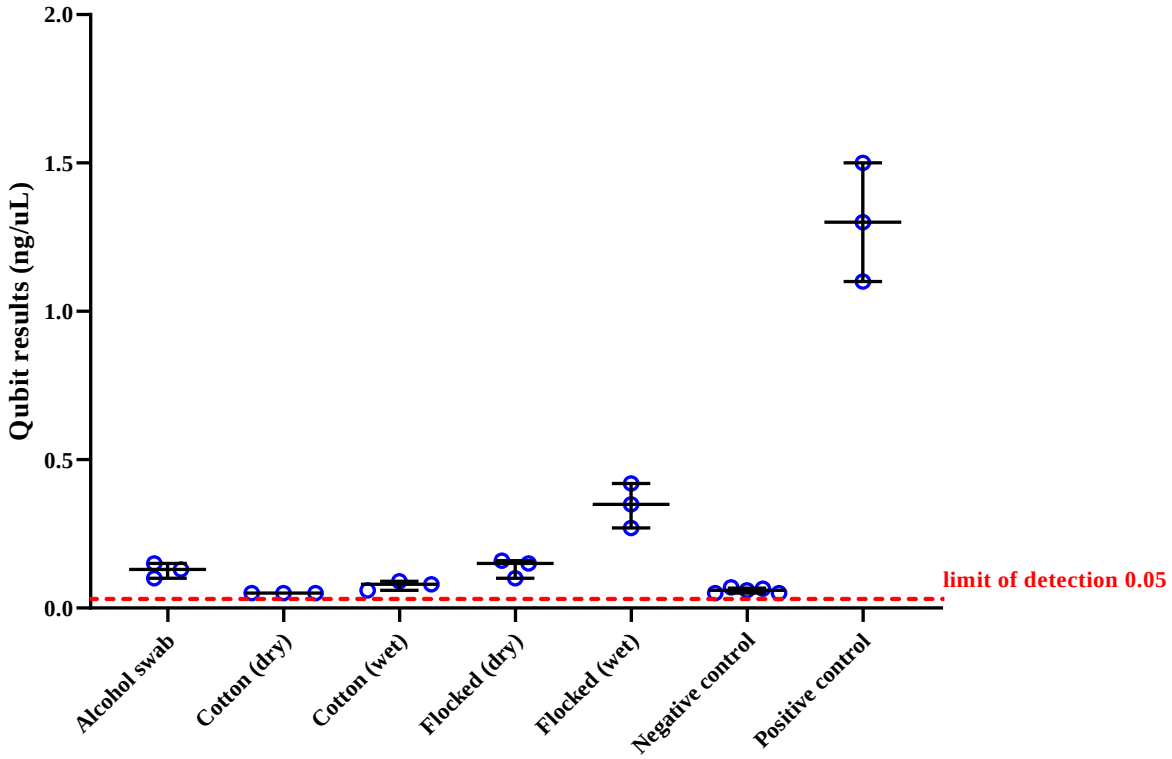

Figure 1: Quantitation of bacterial DNA load recovered from different swab material.

Conclusion:

The nylon flocked swab dipped in normal saline provided the best DNA yield in for the tested swabs and thus was selected for the ongoing studies in this thesis.

Experiment 2: Utilisation of ultraviolet light to reduce pre-sampling contamination

Spurious fragments of nucleic acids such as DNA can be present on sterile surfaces including swabs. Sterilisation methods such as autoclaving is not effective in disintegrating nucleic acids, therefore posing a risk of spurious amplification in the PCR amplification step of 16S rRNA amplicon sequencing. The utilisation of ultraviolet (UV) light is known to the breakdown nucleic acid bonds (2). The amount of inactivation is directly proportional to the UV intensity and duration of exposure (3).

Sample collection mediums such as nylon flocked dry swabs usually go through vigorous sterilisation processes during manufacturing. However, a baseline rate of contamination may still exist, which could potentially impact our study results. We attempted to investigate the utilisation of UV light to reduce potential contamination arising from the swab pre-sampling. A UV light exposure time of 20 minutes was selected for the sterile swabs, consistent with the recommended UV light exposure time of 15-20 minutes for decontamination of laboratory biosafety cabinets (4). This experiment was performed to determine the effects of UV light in reducing spurious amplification arising from the swab prior to sample collection.

Methods:

Twelve nylon flocked dry swabs in a single sterile swab package were exposed to UV light for a total of 20 mins (10 mins on each side). Twelve nylon swabs without UV exposure were used as comparisons.

Pure culture of *Escherichia coli* ATCC25922 was grown on horse blood agar (HBA, bioMerieux, Australia) at 37°C overnight. Colonies were dispersed in 1 mL PBS and diluted to give a suspension of 4 McFarland units (~$1.2x10^9$ CFU/mL). This suspension was then serially diluted 10-fold in PBS to produce suspensions of $1/10^6$,$1/10^8$ and $1/10^{10}$. For each dilution, 100uL suspension was aliquoted onto 8 disposable empty petri plates and allowed to

dry. Additionally, 100uL suspension of the neat *E.coli* was directly transferred into four separate Eppendorf tubes as the positive control. Four Eppendorf tubes with 400uL sterile (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia) was used as the negative control.

All swabs were dipped in TE buffer solution prior to swabbing each petri dish with the diluted E coli colonies. Swabs were transferred into separate Eppendorf tubes containing 400uL of sterile TE buffer. To maximise yield, swabs were vortexed in TE storage buffer for 30 sec and solution recovered from swabs by centrifugation (5 min at of 3374 x g). The swabs were then discarded, and DNA extraction was performed as previously described (Experiment 1). Samples were then stored at -80 °C prior to analysis.

A quantitative PCR (qPCR) assay targeting the 16S rRNA gene was used to assess total bacterial load due to its higher sensitivity. Primer sequences F: 5'-TCCTACGGGAGGCAGCAGT-3' and R: 5'-GGACTACCAGGGTATCTAATCCTGTT-3' were used (5). For SYBR Green qPCR assays, 1 uL of DNA extract, 0.2 μM of each primer, 17.5 uL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and the appropriate volume of water was added to a 35 uL total reaction volume. qPCR was performed on three technical replicates, at 10 uL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Cycling conditions for SYBR Green qPCR assays were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min.

Melt curve analysis was then performed at the following conditions: 95°C for 15 secs, followed by an initial stage temperature of 60°C for 1 min and a final temperature of 95°C for 15 secs, with readings recorded at increments of 0.05°C/s. Standard curves were generated for each qPCR reaction based on serial dilutions of *E.coli* genomic DNA for the 16S rRNA gene.

Results:

The UV exposed nylon flocked dry swab detected similar amounts of bacterial load compared to the non-UV exposed swab across each *E. coli* dilution tested (see figure below).

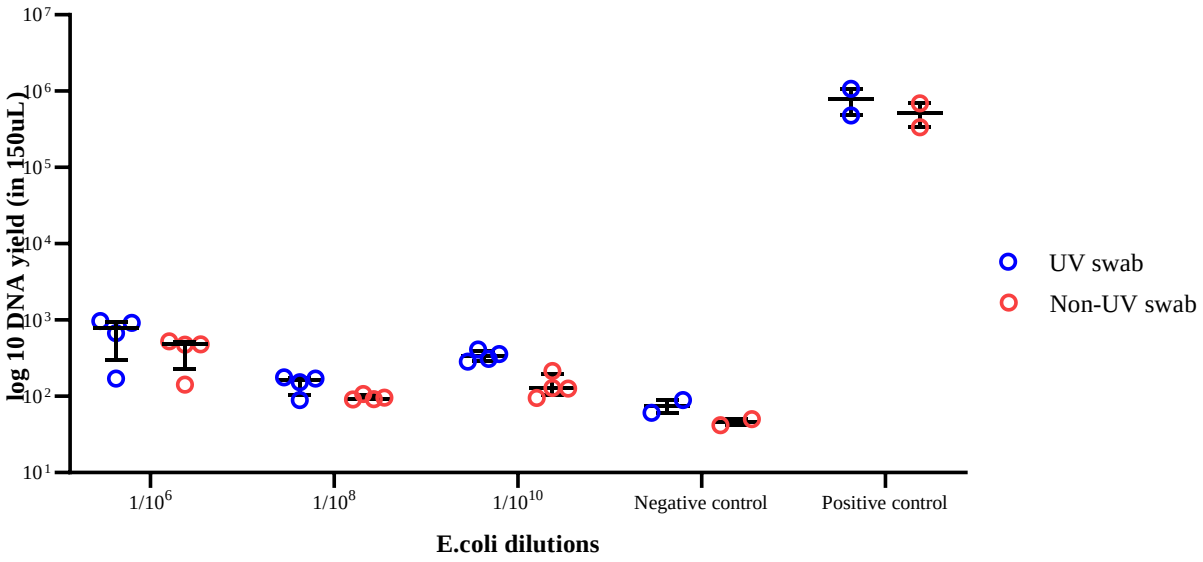

Figure 2: Detection of bacterial load based on utilisation of UV.

Conclusion: The effect of UV light to reduce potential contamination existing on swabs did not result in differences in detection of bacterial load.

Experiment 3: Comparison of Phenol Chloroform DNA extraction with standard DNA extraction method

Optimal DNA extraction methods are not well defined for samples with low bacterial load. Apart from mechanical disruption caused by the beat beating method, studies have also suggested the addition of phenol:chloroform:isoamyl alcohol (25:24:1) (6). When used in conjunction with the beat beating method, phenol:chloroform demonstrated higher DNA yield within samples compared to some commercially available kits (7). Phenol:chloroform is an additional purification step in DNA extraction. The utilisation of phenol:chloroform enables partitioning of the debris into an organic phase, leaving the DNA in the aqueous phase (8). This experiment was performed to determine the effect of phenol:chloroform as an additional purification step in DNA extraction.

Methods:

As described previously, preparations of *Escherichia coli* and *Staphylococcus aureus* diluted to give a suspension of 4 McFarland units (~$1.2x10^9$ CFU/mL) and aliquoted into separate 2mL Eppendorf tubes to be used as controls for future experiments. These samples were thawed and 200ul of each organism was further aliquoted into six Eppendorf tubes. Nylon flocked dry swabs were dipped into twelve Eppendorf tubes. Each swab was then transferred into a separate Eppendorf tube containing a further 200ul of (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia) for a final volume of 400uL. Four further Eppendorf tubes with 400uL TE buffer was used as the negative control.

To maximise yield, swabs were vortexed in TE storage buffer for 30 sec and solution recovered from swabs by centrifugation (5 min at of 3374 x g). The swabs were then discarded, and DNA extraction was performed as previously described (Experiment 1) for half of the samples. The other half of the samples underwent a phenol extraction method. In

between the addition of 100 ul of 5M sodium chloride and DNA precipitation step, 700uL of phenol:chloroform was added to the supernatant and vortexed for 60 sec. The aqueous-organic layers were separated by centrifugation at 13,000 x g for 20 minutes and 600 ul of the aqueous layer was transferred to a new 2 mL microfuge tube prior to the addition of 10 M ammonium acetate and 100% ethanol for DNA precipitation. The rest of the DNA purification were as described (Experiment 1). A qPCR assay targeting the 16S rRNA gene was used to assess total bacterial load using conditions described (Experiment 2).

Results:

Bacterial load was measured across the samples including negative controls. The phenol extraction method proved to be the most efficient by detecting higher amounts of bacterial load in comparison to standard extraction method (see figure below).

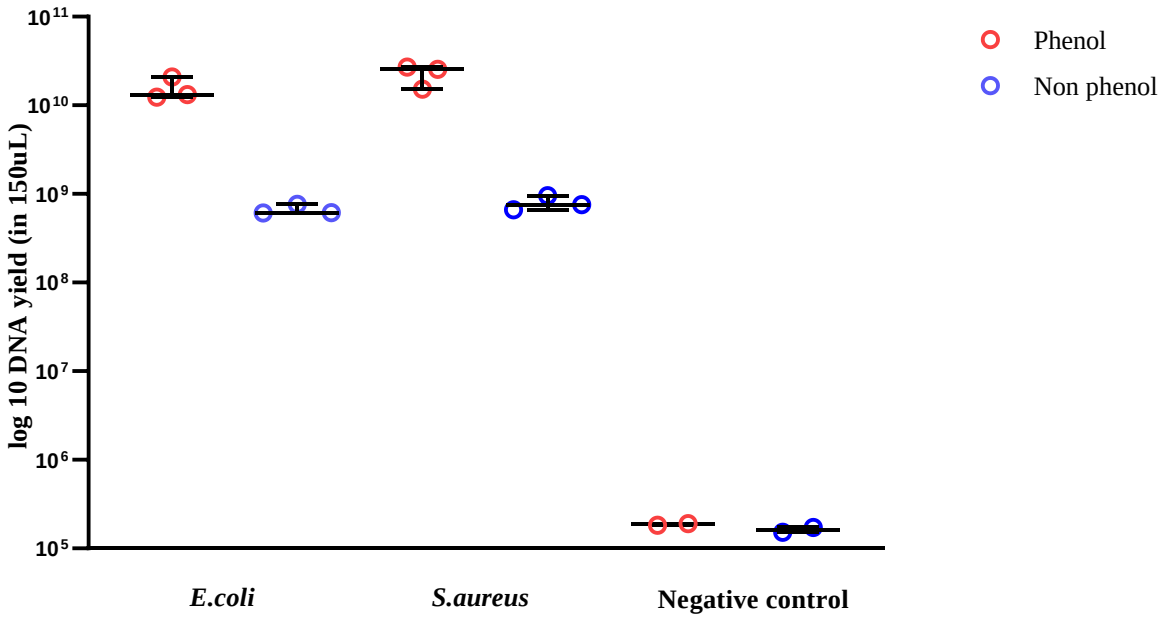

Figure 3: Detection of bacterial load according to different extraction method

Conclusion:

The phenol DNA extraction method resulted in higher extracted DNA yield and was included in the extraction method for ongoing studies.

Experiment 4: Utilisation of vacuum concentrator on samples with low biomass to increase DNA recovery

Increasing DNA recovery from sample is crucial especially when dealing with low biomass samples. A direct method for increasing the DNA concentration of a sample is by reducing the sample volume by using a vacuum concentrator. This experiment was done to validate the effect of vacuum concentration on the DNA concentration of samples with low biomass (environmental samples from hospital surfaces).

Method:

Sixteen swabs were used for environmental samplings. Swabs were taken in duplicates of eight areas (toilet flush, toilet bowl, staff desk *3, door handle *3). Area of swabbing for staff desks were 400cm^2. Swabs were then immediately transferred into 400 µL of sterile Tris-EDTA (TE) buffer (10mM Tris, 1mM EDTA) (ROCHE, Victoria, Australia). To maximise DNA yield, swabs were vortexed in TE storage buffer for 30 sec to dislodge bacterial cells into the suspension solution and then centrifuged at 3374 x g for 5 min to recover the remaining solution. Swabs were then discarded, and DNA extraction performed on TE solution. Four aliquots of 400uL *E. coli* suspension, each containing 100 uL of neat *E. coli* and 300 uL of TE buffer, was used as positive controls. Four aliquots of 400uL TE buffer solution were used as negative controls.

DNA extraction and purification were performed as previously described (Experiment 3) using the phenol extraction method. Ten extracted samples(including controls) were vacuum concentrated until pellet formation using a CHRIST-RVC 2-18 CDplus machine (John Morris Group, Germany). DNA pellets were resuspended in 50 µL UltraPure DNase/RNase-free distilled water. The other ten were used as comparisons after the elutent stage (total 150µL in each sample).

A qPCR assay targeting the 16S rRNA gene was used to assess the DNA concentration in the original and concentrated samples using the method described (Experiment 2) .

Results:

Bacterial load was measured across both methods; one involving a concentration step at the end of DNA extraction and the other without a concentration step. Only paired environmental samples were included for analysis. The samples which were concentrated had significantly higher (p=0.007) DNA levels compared to the non-concentrated samples (Figure 4a). The increase in DNA levels was lower than the predicted DNA levels with the increase in concentration (Figure 4b).

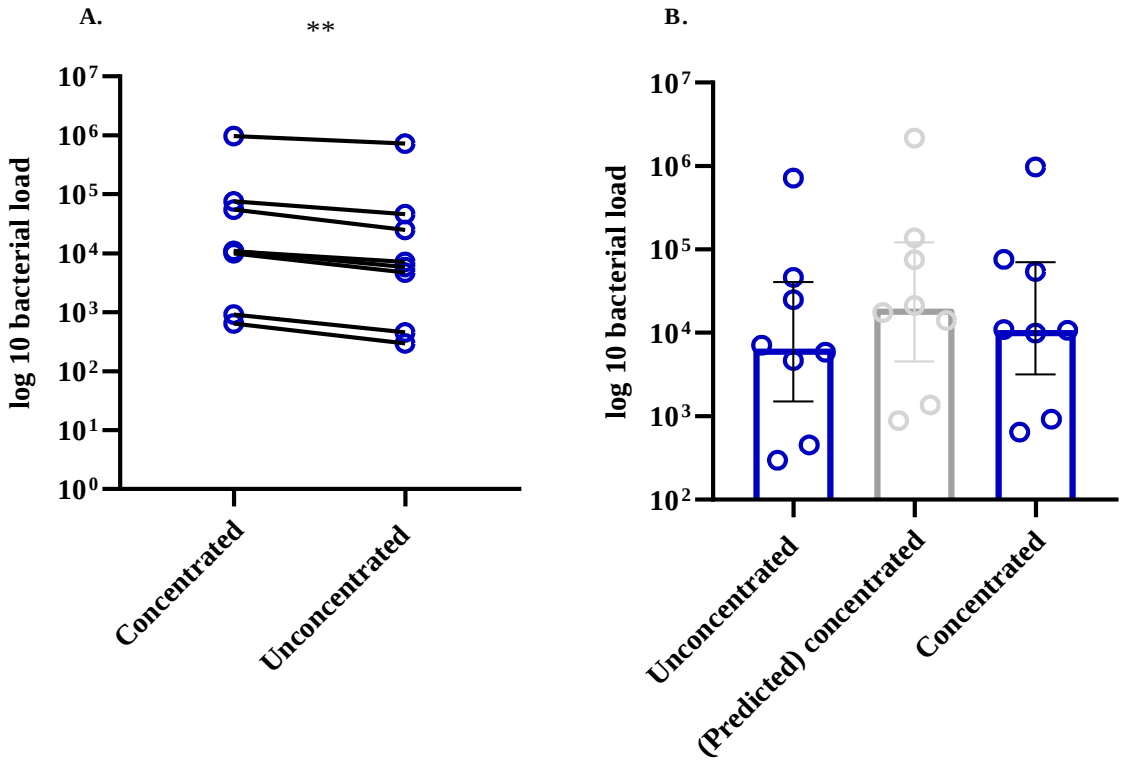

Figure 4: (A) Detection of bacterial load using vacuum concentrator on low biomass samples, (B) comparison of actual and predicted bacterial load from concentrated samples.

Conclusion:

Environmental samples which were concentrated had a higher DNA yield, supporting the addition of this step at the end of DNA extraction prior to further analysis.

Discussion

There is increasing evidence that the hospital built environment contributes to the spread of pathogens. However, there is limited evidence on how best to sample these surfaces and extract DNA from low biomass samples.

Our study demonstrated that the nylon flocked swab provided the best DNA yield compared to the cotton and alcohol swab. Pre-moistened swabs also improved DNA yield by increasing absorption and has been successfully demonstrated in other studies (9, 10). The utilisation of phenol/chloroform not only provides a purification step by removing excess debris and protein but also demonstrated superior results in comparison to the in-house method for DNA recovery.

The use of vacuum concentrator was tested as an additional step to increase DNA concentration and demonstrated higher DNA yield in environmental samples. These four optimisation experiments conducted prior to the commencement of sample collection resulted in a modified in-house protocol using a pre-moistened nylon flocked swab for sample collection, followed by the addition of phenol/chloroform to maximise DNA yield from environmental samples with low biomass in this study. Finally, the volume of extracted DNA samples was reduced by using a vacuum concentrator to increase DNA concentration of samples for 16S rRNA amplicon sequencing.

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Appendix 2: Supplementary Materials from Chapter 2

Supplementary Methods

LRTI Definition

Acute LRTI was defined as a new or progressive infiltrate on chest radiograph or clinical decision made by the treating medical physician. Factors influencing the clinical decision met one or more of the following criteria: fever (temperature ≥38.0°C) or hypothermia (temperature <35.0°C), new cough with or without sputum production, abnormal percussion or auscultation of breath sounds, dyspnoea or tachypnoea, hypoxia, leucocytosis, or leukopenia.

Swab processing

OP swabs were immediately stored in 400 µL of sterile (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia) and frozen at -80°C until DNA extraction. To maximise yield, swabs were vortexed in TE storage buffer for 30 sec and solution recovered from swabs by centrifugation (5 min at of 3374 x g). Swabs were then discarded, and DNA extraction performed on TE solution.

DNA extraction

TE solution containing sample was heated to 95 °C for 1 minute, before being cooled at 4°C for 2 minutes. Lysozyme (ROCHE, ThermoFisher Scientific, Victoria, Australia) and lysostaphin (Sigma-Aldrich, MO, USA) were then added to a final concentration of 2.9 mg/mL and 0.1 mg/mL, respectively, and samples incubated at 37 °C for 1 hr. Proteinase K (Fermentas, ThermoFisher Scientific, Victoria, Australia) and sodium dodecyl sulphate (Sigma-Aldrich, MO, USA) were then added to a final concentration of 1.2 mg/mL and 1.5 %, w/v, respectively. Following incubation for 1 hr at 50 °C, approximately 0.13 g of washed beads (1:1 of 0.1mm and 1mm silica zirconium beads) was added to the sample prior to a cycle of bead beating at 6.5 m/s for 1 min using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, USA). The

supernatant was transferred into a new 2 mL conical screw cap microtube and 100 µl of 5M sodium chloride and 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1; saline buffered at pH8.0; Sigma-Aldrich, MO, USA) were added and samples vortexed for 60 sec. The aqueous-organic layers were separated by centrifugation at 13,000 x g for 20 minutes and 600 µl of the aqueous layer was transferred to a new 2 mL microfuge tube. DNA was precipitated by the addition of 10 M ammonium acetate and 100% ethanol (Sigma Aldrich, MO, USA) in a 1:10 and 1:1 ratio with sample volume, respectively and recovered using an EZ-10 Spin column in accordance with manufacturer's instructions (Bio Basic, Inc., Ontario, Canada). DNA was eluted in 50 µl UltraPure DNase/RNase-free distilled water (Gibco, ThermoFisher Scientific, Victoria, Australia) three times (total 150 µl) and samples were vacuum concentrated until pellet formation using CHRIST-RVC 2-18 CDplus machine (John Morris Group, Germany). DNA pellets were resuspended in 100 µL UltraPure DNase/RNase-free distilled water. Samples were then stored at -80 °C prior to analysis.

16S rRNA gene amplicon sequencing

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from OP swab DNA using modified universal bacterial primer pairs 515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA-3'). with Illumina adapter overhang sequences as indicated by underline. Amplicons were generated, cleaned, indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) with certain modifications. Briefly, an initial PCR reaction contained at least 12.5 ng of DNA, 0.5 µL of forward primer (1 µM), 0.5 µL of reverse primer (1 µM) and 12.5 µL of 2× KAPA HiFi Hotstart ReadyMix (KAPA

Biosystems, Wilmington, MA, USA) in a total volume of 25 μL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 5 min. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The final library was paired-end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v4 on the Illumina MiSeq platform. Sequencing was performed at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute.

Sequence data processing

The Quantitative Insights Into Microbial Ecology (QIIME2-2018.2) software was used to analyse the 16S rRNA sequence generated from paired-end amplicon sequencing using bioinformatics pipeline based on the tutorial available on the QIIME2 website (https://docs.qiime2.org/2018.11/tutorials/moving-pictures). Single-nucleotide variants (SNVs) were assigned to the reads against the SILVA database release 132 (December 2017).

Diversity measurements and statistical analyses

Bray-Curtis similarity scores were calculated based on sample-normalised, square root transformed taxa relative abundance. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were used to visualize clustering of samples based on their similarity matrices. Distance from centroid was calculated as previously described, using PRIMER-e v.7. Permutational multivariate analysis of variance (PERMANOVA) on the beta-diversity matrices was used to test the null hypothesis of no difference amongst the defined groups using PERMANOVA + add-on package for PRIMER.

The overall homogeneity of dispersion between the two groups was also analysed using PERMDISP analysis in PRIMER. Both these tests were computed using unrestricted permutation of raw data with 9,999 random permutations and at a significance level of 0.05.

Supplementary Figures and Tables

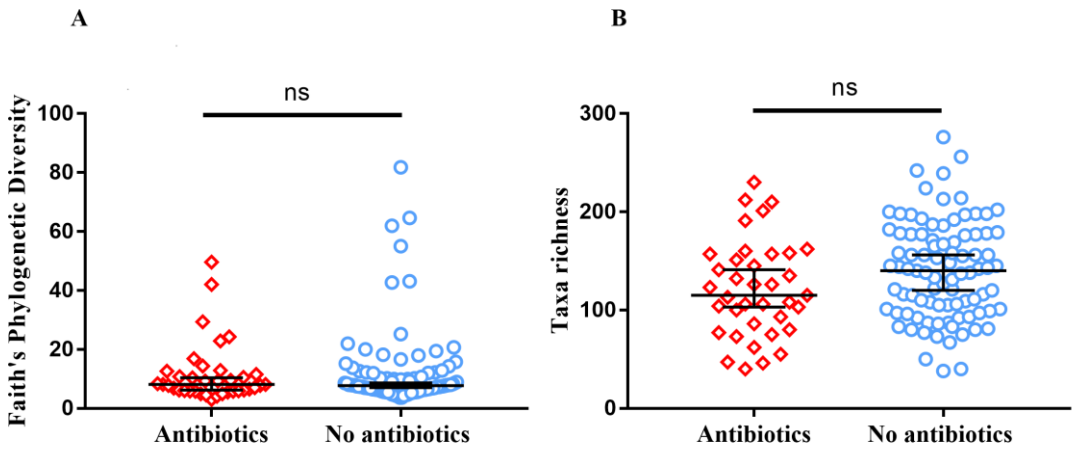


Figure S1. Alpha diversity measures among potential confounders- antibiotics. A. Faith's Phylogenetic Diversity B. Taxa richness. ns, not significant. Bars show medians +/- 95% CIs.

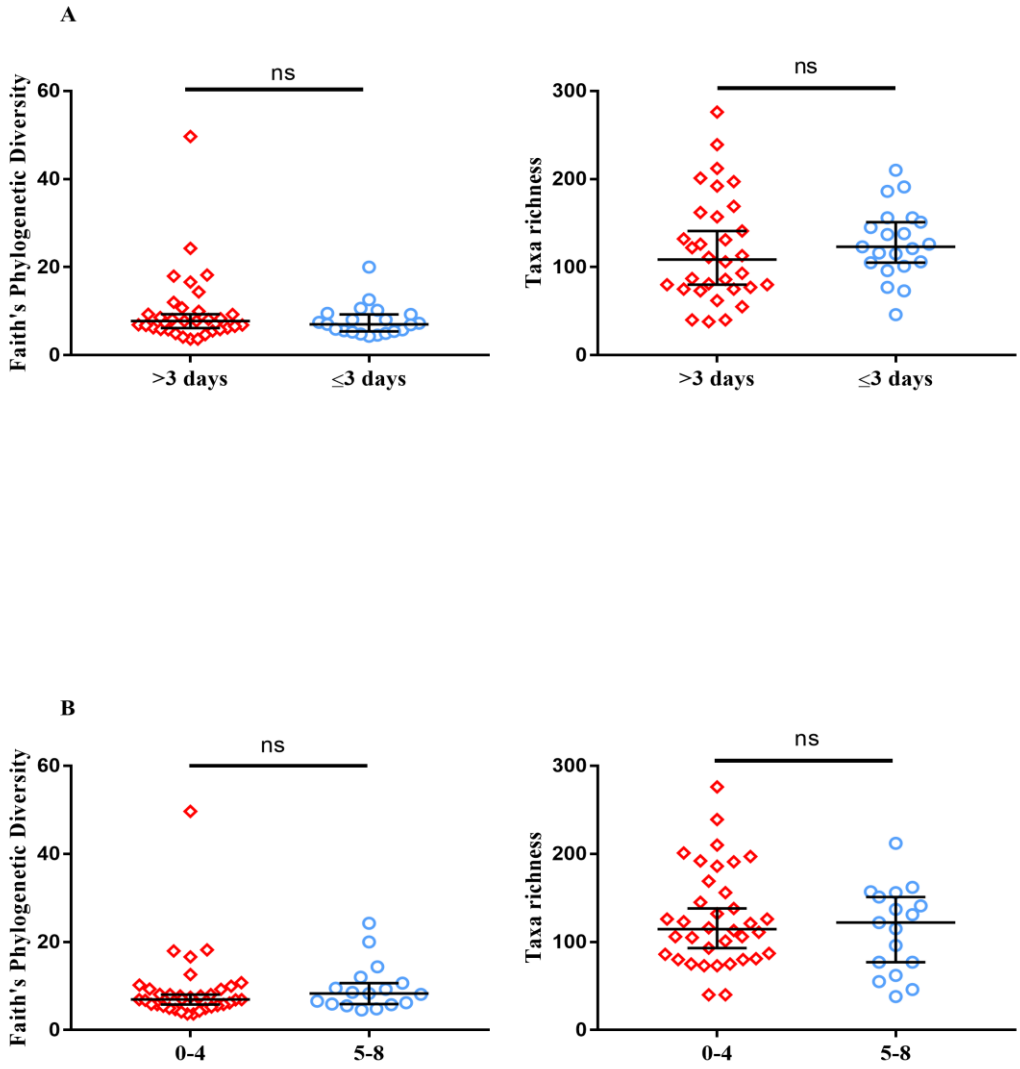


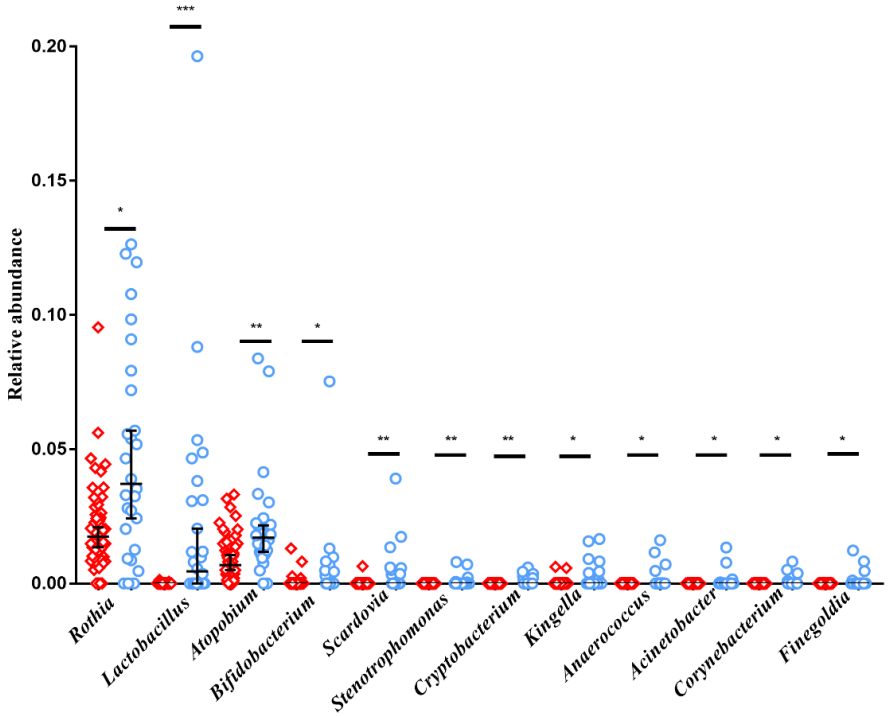
Figure S2. Alpha diversity measures among potential confounders within adult inpatients A. Length of stay B. Charlson Co-morbidity Index. ns, not significant. Bars show medians +/- 95% CIs.

Table S1. PERMANOVA and PERMDISP analysis: significance of variance of Bray-Curtis distance of adult inpatient and healthy population with/without antibiotic exposure.

Source	*df*	SS	MS	Pseudo-F	P(perm)	P(disp)
Inpatient vs Healthy	1	0.184	0.184	8.436	<0.001*	<0.001*
Residual	126	2.753	0.022			
Total	127	2.938				
Antibiotic vs no antibiotic	1	0.067	0.067	2.927	0.014*	0.002*
Residual	126	2.871	0.023			
Total	127	2.938				
Antibiotic vs no antibiotic (healthy)	1	0.019	0.019	1.244	0.263	0.944
Residual	73	1.086	0.014			
Total	74	1.105				
Antibiotic vs no antibiotic (inpatients)	1	0.025	0.025	0.792	0.568	0.061
Residual	52	1.623	0.032			
Total	53	1.648				
Inpatients vs Healthy (no antibiotics)	1	0.095	0.095	5.736	<0.001*	0.018*
Residual	89	1.478	0.017			
Total	90	1.573				
Inpatients vs Healthy (antibiotics)	1	0.066	0.066	1.879	0.077	0.061
Residual	35	1.231	0.035			
Total	36	1.298				

Significant effects ($p < 0.05$) are indicated with an asterisk (*).
df-degrees of freedom, SS-sum of squares, MS-mean squares, P(perm) significance, P(disp) significance based on 9999 permutations.

A.



B.

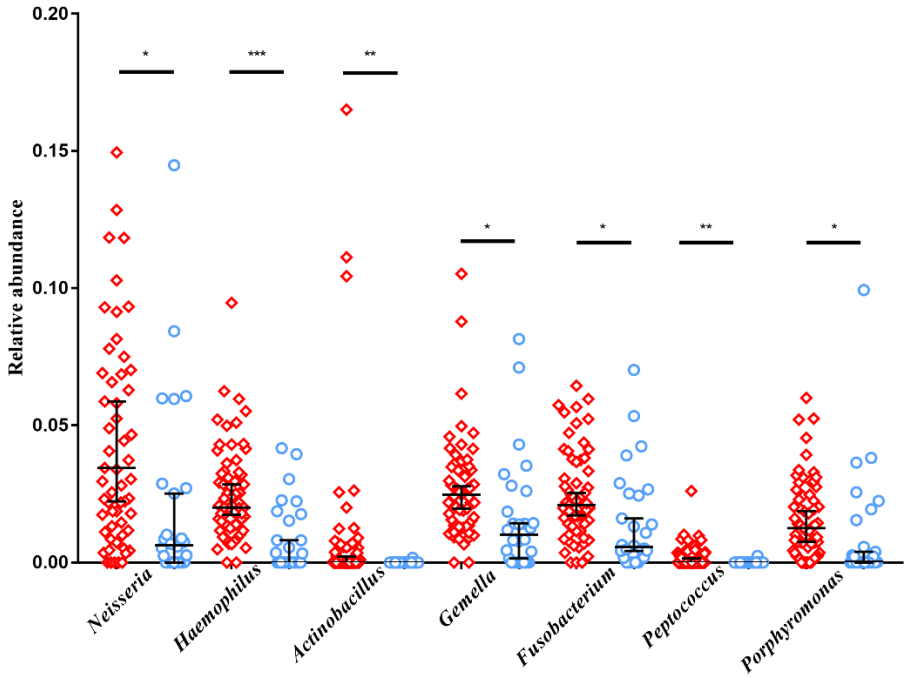


Figure S3A. Distribution of taxa that were significantly over-represented in inpatients in the absence of recent antibiotic exposure. **S3B.** Distribution of taxa that were significantly over-represented in healthy controls in the absence of recent antibiotic exposure

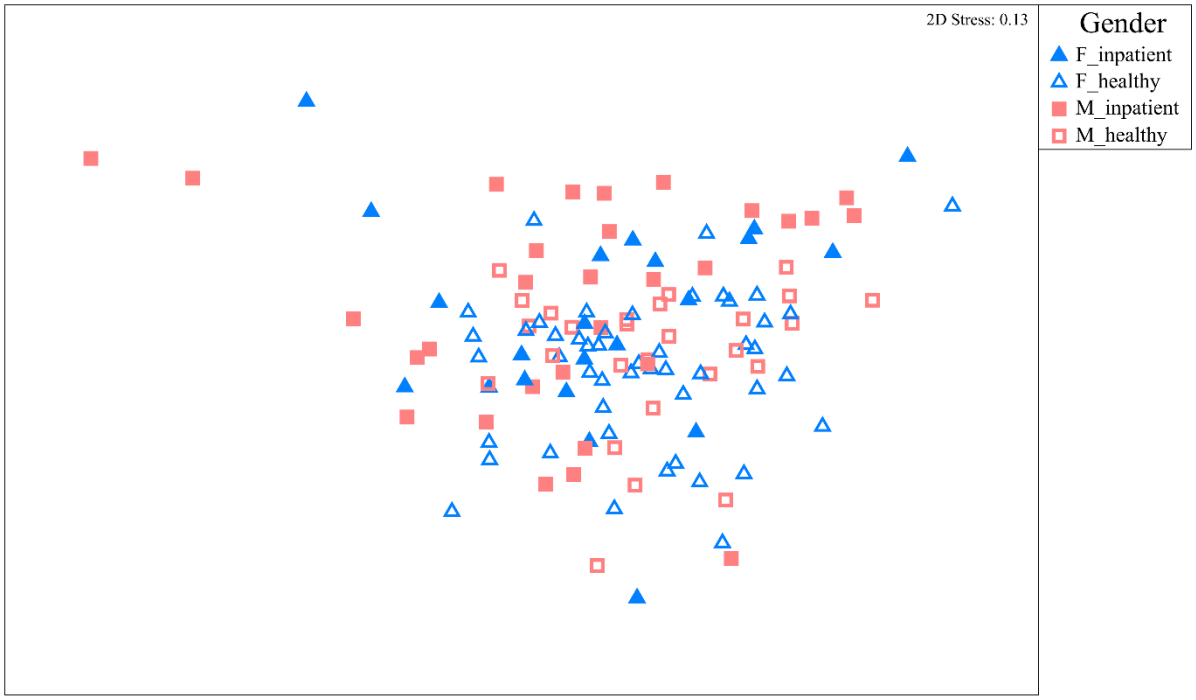


Figure S4. Non-metric multidimensional scaling plot between gender-matched cohort

Table S2. Univariate and multivariate associations between dependent variables (Faith's phylogenetic diversity and Bray-Curtis distance from centroid) and independent variables (age, gender, antibiotic use, and hospital admission status)

	Univariate		Multivariate Model 1[2]		Multivariate Model 2[3]	
	RR[4] (95%CI)	p-value	RR[4] (95%CI)	p-value	RR[4] (95%CI)	p-value
Faith's phylogenetic diversity						
Age (10 years)	0.98 (0.93, 1.03)	0.453	1.07 (0.99, 1.16)	0.098	1.07 (0.99, 1.16)	0.100
Gender						
Female	1.00	NA	1.00	NA	1.00	NA
Male	0.77 (0.61, 0.95)	0.018*	0.81 (0.65, 1.01)	0.060	0.81 (0.65, 1.01)	0.060
Antibiotic use						
No	1.00	NA	1.00	NA	1.00	NA
Yes	0.99 (0.78, 1.27)	0.957			1.10 (0.85, 1.41)	0.471
Hospital admission						
No	1.00	NA	1.00	NA	1.00	NA
Yes	0.77 (0.62, 0.97)	0.025*	0.64 (0.44, 0.92)	0.017*	0.62 (0.42, 0.90)	0.013*
Distance from centroid						
Age (10 years)	1.06 (1.03, 1.09)	<0.001 *	1.02 (0.97, 1.07)	0.455	1.02 (0.97, 1.07)	0.44
Gender						
Female	1.00	NA	1.00	NA	1.00	NA
Male	1.07 (0.93, 1.23)	0.325	1.01 (0.88, 1.15)	0.455	1.01 (0.89, 1.16)	0.847
Antibiotic use						
No	1.00	NA	1.00	NA	1.00	NA
Yes	1.21 (1.04, 1.40)	0.014*			1.10 (0.95, 1.50)	0.213
Hospital admission						
No	1.00	NA	1.00	NA	1.00	NA
Yes	1.32 (1.16, 1.51)	<0.001 *	1.24 (0.99, 1.54)	0.062	1.2 (0.95, 1.50)	0.121

Significant effects ($p < 0.05$) are indicated with an asterisk (*)

1. Assessed using linear regression with log-transformation of alpha diversity and beta diversity
2. Adjusted for age and gender
3. Adjusted for age, gender, and antibiotic use
4. RR= Rate Ratio, NA=Not applicable

Appendix 3: Supplementary Materials from Chapter 3

Supplementary methods

Swab processing

Prior to sampling, each flocked swab in a single sterile swab package was exposed to UV light for a total of 20 mins (10 mins on each side) as a further sterilisation step. Once samples were collected, swabs were immediately stored in 400 µL of sterile sterile (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia) and frozen at -80°C until DNA extraction. To maximise yield, swabs were vortexed in TE storage buffer for 30 sec and solution recovered from swabs by centrifugation (5 min at of 3374 x g). Swabs were then discarded, and DNA extraction performed on TE solution.

DNA extraction

Sample suspended in TE was heated to 95 °C for 1 minute, before being cooled at 4 °C for 2 minutes. Lysozyme (ROCHE, ThermoFisher Scientific, Victoria, Australia) and lysostaphin (Sigma-Aldrich, MO, USA) were then added to a final concentration of 2.9 mg/mL and 0.1 mg/mL, respectively, and samples incubated at 37 °C for 1 hr. Proteinase K (Fermentas, ThermoFisher Scientific, Victoria, Australia) and sodium dodecyl sulphate (Sigma-Aldrich, MO, USA) were then added to a final concentration of 1.2 mg/mL and 1.5 %, w/v, respectively. Following incubation for 1 hr at 50 °C, approximately 0.13 g of washed beads (1:1 of 0.1mm and 1mm silica zirconium beads) was added to the sample prior to a cycle of bead beating at 6.5 m/s for 1 min using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, USA). The supernatant was transferred into a new 2 mL conical screw cap microtube and 100 µL of 5M sodium chloride and 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1; pH8.0; Sigma-Aldrich, MO, USA) were added and samples vortexed for 60 sec. The aqueous-organic layers were separated by centrifugation at 13,000 x g for 20 minutes and 600 µL of the aqueous layer was transferred to a new 2 mL microfuge tube. DNA was precipitated by the addition of 10 M

ammonium acetate and 100% ethanol (Sigma Aldrich, MO, USA) in a 1:10 and 1:1 ratio with sample volume, respectively and recovered using an EZ-10 Spin column in accordance with manufacturer's instructions (Bio Basic, Inc., Ontario, Canada). DNA was eluted in 50 µL UltraPure DNase/RNase-free distilled water (Gibco, ThermoFisher Scientific, Victoria, Australia) three times (total 150 µl) and samples were vacuum concentrated until pellet formation using CHRIST-RVC 2-18 CDplus machine (John Morris Group, Germany). DNA pellets were resuspended in 100 µL UltraPure DNase/RNase-free distilled water. Samples were then stored at -80 °C prior to analysis.

16S rRNA gene amplicon sequencing

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from OP swab DNA using modified universal bacterial primer pairs 515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA-3'). with Illumina adapter overhang sequences as indicated by underline. Amplicons were generated, cleaned, indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) with certain modifications. Briefly, an initial PCR reaction contained at least 12.5 ng of DNA, 0.5 µL of forward primer (1 µM), 0.5 µL of reverse primer (1 µM) and 12.5 µL of 2× KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 µL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 5 min. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according

to the manufacturer's instructions. The final library was paired end sequenced at 2 × 300 bp using a MiSeq Reagent Kit v3 on an Illumina MiSeq platform. Sequencing was performed at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute.

Sequence data processing

The Quantitative Insights Into Microbial Ecology (QIIME2-2018.2) software was used to analyse the 16S rRNA sequence generated from paired-end sequence reads using the QIIME2 bioinformatics pipeline (https://docs.qiime2.org/2018.11/tutorials/moving-pictures). Single-nucleotide variants (SNVs) were assigned to the reads against the SILVA database release 132 (December 2017). After sequence processing and removal of spurious and contaminant reads, a median read depth of 8,867.5 (quartile 1 and quartile 3, 7,203.25 and 12,174.25) was achieved.

Quantitation of bacterial load, specific bacterial taxa and resistance gene carriage.

A quantitative PCR (qPCR) assay targeting the 16S rRNA gene using SYBR Green assays was used to assess total bacterial load as described previously (1). Levels of *vanB* gene were assessed using SYBR Green assays. Levels of the *nuc* gene of *S. aureus* were assessed with the TaqMan assay (2).

For *vanB* SYBR Green qPCR assay, 1 μL of DNA extract, 0.2 μM of each primer, 17.5 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and the appropriate volume of water was added to a 35 μL total reaction volume. Quantitative RT-PCR were performed on three technical replicates, at 10 μL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Cycling conditions for SYBR Green qPCR assays were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 secs and 60 °C for 1 min. Melt curve analysis was then performed at the following conditions: 95 °C for 15 secs, followed by an initial stage temperature of 60 °C for 1 min and a final temperature of 95 °C for 15 secs, with readings recorded at increments of 0.05 °C/s.

For the *nuc* geneTaqMan assay, 1 µL of DNA extract, 0.14 µM of each primer, 0.04 µM of probe, 17.5 µL of 2X KAPA PROBE FAST One-Step Master Mix (Sigma-Aldrich, MO, USA), and the appropriate volume of water was added to a 35 µL total reaction volume. Quantitative RT-PCR were performed on three technical replicates, at 10 µL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Cycling conditions for TaqMan assay were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 secs ,52 °C for 45 secs and 72 °C for 45 secs.

Standard curves were generated for each qPCR reaction based on serial dilutions of *Escherichia coli* genomic DNA for the 16S rRNA gene and bacterial isolates previously identified using either whole genome sequencing or clinical isolates for the rest of the bacterial taxa and resistance genes.

Presence of methicillin resistant *Staphylococcus aureus* (MRSA) was detected using a TaqMan based assay designed to detect the orfXSCCmec junction region which is a modified and expanded version of a previously published method (3) based at South Australia (SA) Pathology molecular diagnostic laboratory.

Supplementary Figures and Tables

Table S1: Primer sequences of antibiotic resistance and positive control (16S rRNA) genes for quantitative PCR.

Gene	Sequence (5' - 3')	Annealing conditions (temperature, extension time)	References
16S	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	60°C, 60s	(1)
nuc gene (*S. aureus*)	AAATTACATAAAGAACCTGCGACA GAATGTCATTGGTTGACCTTTGTA	52°C, 45s	(2) modified for this study
***MRSA* multiplex PCR**		55°C, 15s	SA Pathology
SCC*mec*			
mecii574	GTCAAAAATCATGAACCTCATTACTTATG		(3)
mecii519	ATTTCATATATGTAATTCCTCCACATCTC		(3)
meciv511	CAAATATTATCTCGTAATTTACCTTGTTC		(3)
mecv492	CTCTGCTTTATATTATAAAATTACGGCTG		(3)
mecvii512	CACTTTTTATTCTTCAAAGATTTGAGC		(3)
mecVb	CTAACATTTTCTAATTTATTTAACATA		SA Pathology
mecIX	CGTTCCGATACGTTCAAAAT		SA Pathology
mecX	TTAAATACTAGGAGTACTAGCCATAT		SA Pathology
mecEU	CTCTGATAAGCCATTCATTCATC		SA Pathology
orfX			
Xsau325	GGATCAAACGGCCTGCACA		SA Pathology
orfX LNA	FAM- T[+A]ACA[+C]AAC[+C]CG[+C]ATC[+A]TTTG- BHQ1		SA Pathology
vanB	TGTAGGCTGCGATATTCAAAGCT TTCACAAAGACAGGGTAGGTAAGCG	60°C, 60s	This study*

* Based on SA Pathology Molecular Diagnostic Laboratory Protocol, South Australia

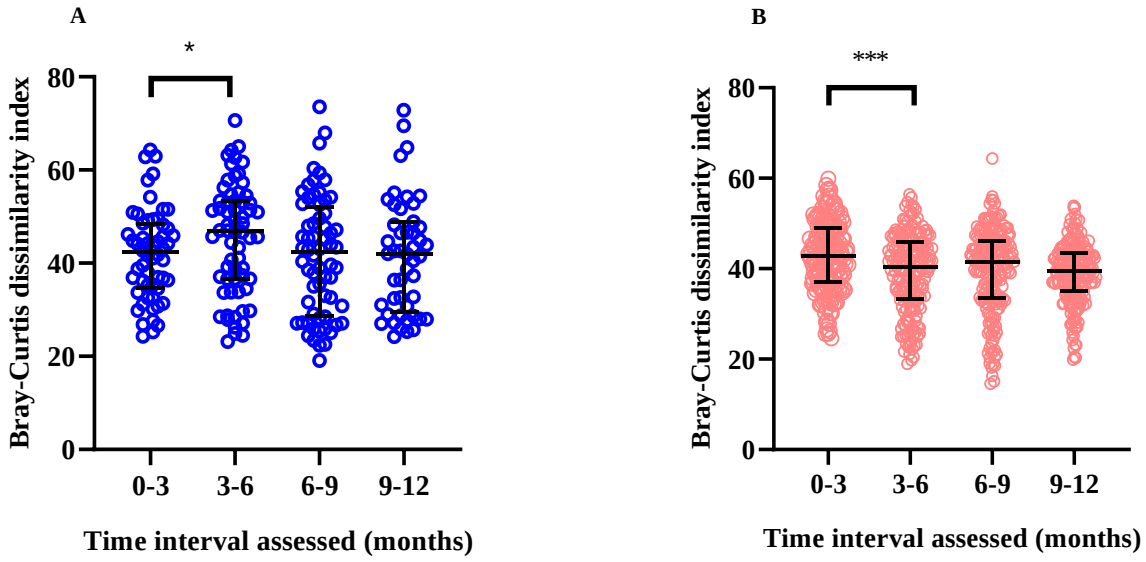


Figure S1: Microbiota composition according to time points (A) common areas and (B) inpatient rooms. Within the common areas, the highest change in microbiota composition was reflected by an increased Bray-Curtis distance (median BC score [IQR]: 46.84 [16.79]). Within the inpatient areas, the greatest change in microbiota composition was between 0-3 months (median BC score [IQR]: 42.75 [12]). Wilcoxon matched-pairs signed rank test *p<0.05; ***p<0.001.

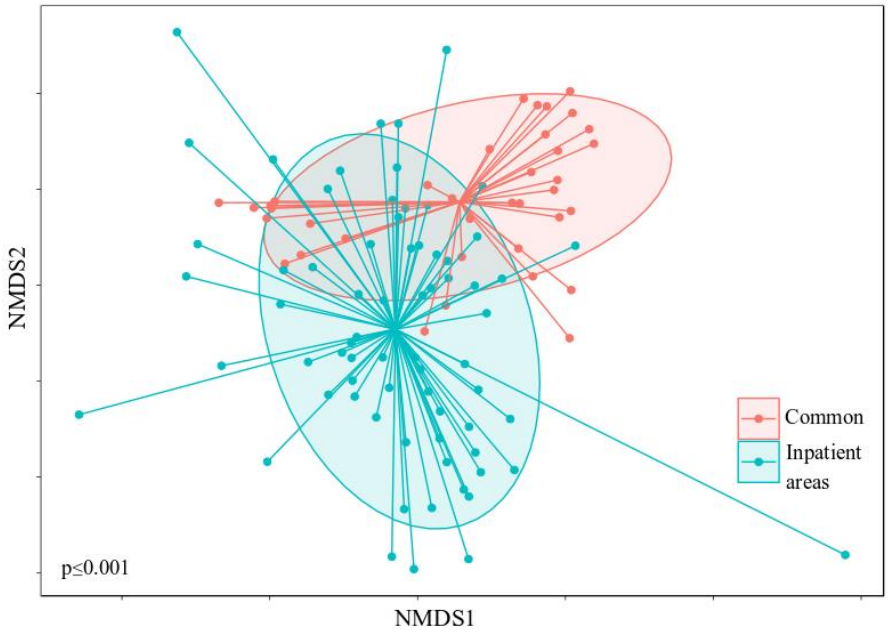


Figure S2: Nonmetric multidimensional scaling (NMDS) ordination plot according to sampling locations at nRAH at all sampling points based on BC distance. Statistical analysis to determine the significance of microbiota composition differences between both groups was performed using the PERMANOVA test.

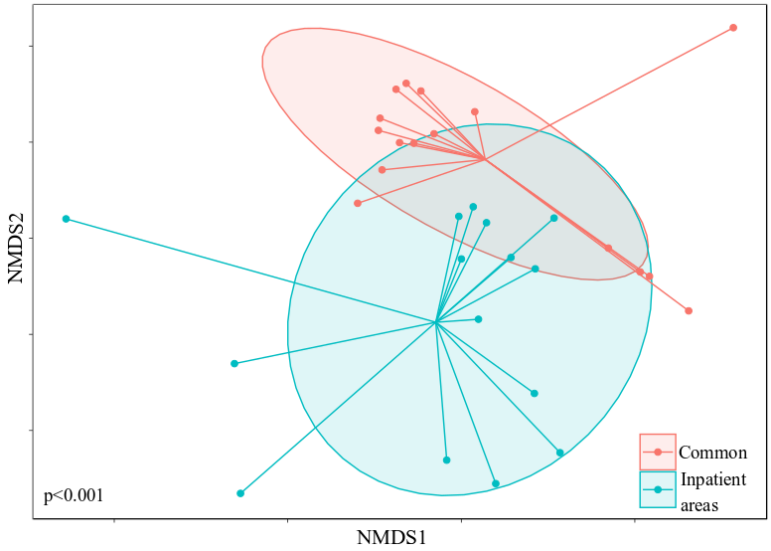


Figure S3: NMDS ordination plot according to sampling locations at oRAH at all sampling points based on BC distance. Statistical analysis to determine the significance of microbiota composition differences between both groups was performed using the PERMANOVA test.

Table S2. PERMANOVA and PERMDISP analysis: significance of variance of Bray-Curtis distance of the new RAH (nRAH) and old RAH (oRAH). O: occupied; UO: unoccupied; nRAH: new hospital; oRAH: old hospital

	PERMANOVA		PERMDISP	
Source	**t-value**	**P(perm)**	**t-value**	**P(disp)**
Common areas				
UO vs 1-year (nRAH)	0.75	0.677	0.05	0.976
UO (nRAH) vs O (oRAH)	1.02	0.329	1.59	0.274
UO (nRAH) vs UO (oRAH)	1.04	0.332	0.27	0.813
1-year (nRAH) vs O (oRAH)	1.13	0.241	1.66	0.241
1-year (nRAH) vs UO (oRAH)	0.64	0.758	0.24	0.879
UO (oRAH) vs O (oRAH)	1.27	0.144	1.25	0.382
Inpatient areas				
UO vs 1-year (nRAH)	1.77	0.001*	0.69	0.511
UO (nRAH) vs O (oRAH)	1.95	≤0.001*	1.28	0.248
1-year (nRAH) vs O (oRAH)	1.20	0.138	0.74	0.498

Significant effects ($p < 0.05$) are indicated with an asterisk (*).

P(perm) significance, P(disp) significance based on 9999 permutations.

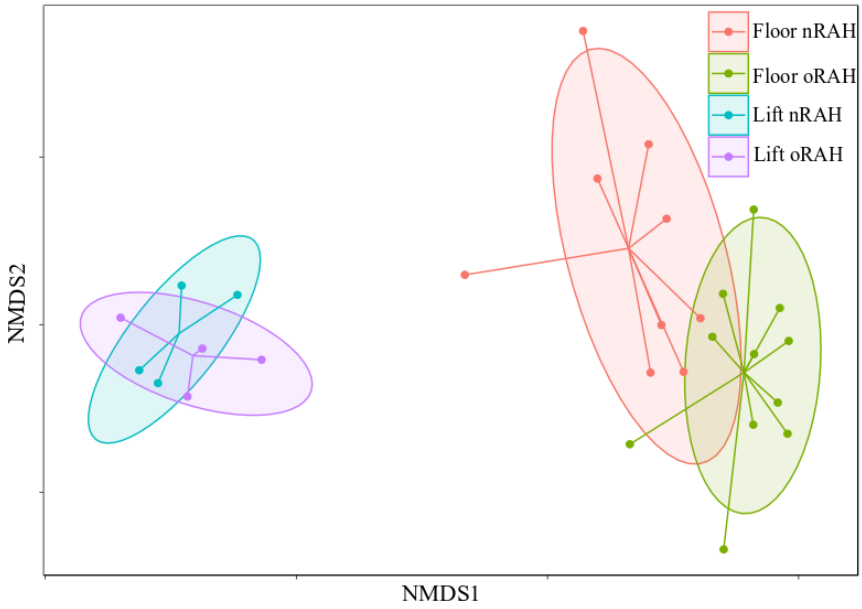


Figure S4: NMDS ordination plot according to sampling sites at oRAH and nRAH based on BC distance demonstrating the separation of samples based on high touch (lift buttons) and high foot traffic (floor samples) areas.

Table S3: Bacterial species and antibiotic resistance genes identified using PCR and 16S sequencing. MRSA: Methicillin-resistant *Staphylococcus aureus*; VRE: Vancomycin-Resistant Enterococci.

Source		***S. aureus***	**MRSA**	****Enterococcus sp.***	***vanB* VRE**
nRAH					
	Common areas	19/40 (47.5%)	14/40 (35.0%)	10/40 (25.0%)	0/40 (0%)
	Inpatient areas	21/70 (30.0%)	12/70 (17.1%)	24/70 (34.3%)	1/70 (1.4%)
Total		**40/110 (36.4%)**	**26/110 (23.6%)**	**34/110 (30.9%)**	**1/110 (0.9%)**
oRAH					
	Common areas	8/16 (50.0%)	3/16 (18.8%)	4/16 (25.0%)	0/16 (0%)
	Inpatient areas	3/16 (18.8%)	0/16 (0%)	8/16 (50.0%)	0/16 (0%)
Total		**11/32 (34.4%)**	**3/32 (9.38%)**	**12/32 (37.5%)**	**0/32 (0%)**

*Identified through 16S sequencing

References

1. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading) 2002;148(Pt 1):257-66.
2. Thomas LC, Gidding HF, Ginn AN, Olma T, Iredell J. Development of a real-time *Staphylococcus aureus* and MRSA (SAM-) PCR for routine blood culture. J Microbiol Methods 2007;68(2):296-302.
3. Huletsky A, Giroux R, Rossbach V, et al. New real-time PCR assay for rapid detection of methicillin-resistant *Staphylococcus aureus* directly from specimens containing a mixture of staphylococci. J Clin Microbiol 2004;42(5):1875-84.

Appendix 4: Supplementary Materials from Chapter 4

Supplementary methods

Swab processing

Rectal swabs were immediately stored frozen at -80°C until DNA extraction. To maximise yield, 400 µL of sterile (10mM Tris, 1mM EDTA) Tris-EDTA (TE) buffer (ROCHE, Victoria, Australia) was added to the swabs and vortexed for 30 sec and solution recovered from swabs by centrifugation (3374 x g for 5 minutes). Swabs were then discarded, and DNA extraction performed on TE solution.

DNA extraction

TE solution containing sample was heated to 95 °C for 1 minute, before being cooled at 4°C for 2 minutes. Lysozyme (ROCHE, ThermoFisher Scientific, Victoria, Australia) and lysostaphin (Sigma-Aldrich, MO, USA) were then added to a final concentration of 2·9 mg/mL and 0.1 mg/mL, respectively, and samples incubated at 37 °C for 1 hr. Proteinase K (Fermentas, ThermoFisher Scientific, Victoria, Australia) and sodium dodecyl sulphate (Sigma-Aldrich, MO, USA) were then added to a final concentration of 1·2 mg/mL and 1·5 %, w/v, respectively. Following incubation for 1 hr at 50 °C, approximately 0·13 g of washed beads (1:1 of 0·1mm and 1mm silica zirconium beads) was added to the sample prior to a cycle of bead beating at 6·5 m/s for 1 min using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, USA). The supernatant was transferred into a new 2 mL conical screw cap microtube and 100 µl of 5M sodium chloride and 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1; saline buffered at pH8·0; Sigma-Aldrich, MO, USA) were added and samples vortexed for 60 sec. The aqueous-organic layers were separated by centrifugation at 13,000 x g for 20 minutes and 600 µl of the aqueous layer was transferred to a new 2 mL microfuge tube. DNA was precipitated by the addition of 10 M ammonium acetate and 100% ethanol (Sigma Aldrich, MO, USA) in a 1:10 and 1:1 ratio with sample volume, respectively and recovered using an EZ-10 Spin column in

accordance with manufacturer's instructions (Bio Basic, Inc., Ontario, Canada). DNA was eluted in 50 µl UltraPure DNase/RNase-free distilled water (Gibco, ThermoFisher Scientific, Victoria, Australia) three times (total 150 µl) and samples were vacuum concentrated until pellet formation using CHRIST-RVC 2-18 CDplus machine (John Morris Group, Germany). DNA pellets were resuspended in 100 µL UltraPure DNase/RNase-free distilled water. Samples were then stored at -80 °C prior to analysis.

16S rRNA gene amplicon sequencing

The V4 hypervariable region of the bacterial 16S rRNA gene was amplified from OP swab DNA using modified universal bacterial primer pairs 515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAA-3'). with Illumina adapter overhang sequences as indicated by underline. Amplicons were generated, cleaned, indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol(https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) with certain modifications. Briefly, an initial PCR reaction contained at least 12·5 ng of DNA, 0·5 µL of forward primer (1 µM), 0·5 µL of reverse primer (1 µM) and 12·5 µL of 2× KAPA HiFi Hotstart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) in a total volume of 25 µL. The PCR reaction was performed on a Veriti 96-well Thermal Cycler (Life Technologies) using the following program: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension step at 72 °C for 5 min. Samples were multiplexed using a dual-index approach with the Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The final library was paired end sequenced at 2 × 300 bp

using a MiSeq Reagent Kit v3 on an Illumina MiSeq platform. Sequencing was performed at the David R Gunn Genomics Facility, South Australian Health and Medical Research Institute.

Sequence data processing

The Quantitative Insights Into Microbial Ecology (QIIME2-2018·2) software was used to analyse the 16S rRNA sequence generated from paired-end sequence reads using the QIIME2 bioinformatics pipeline (https://docs.qiime2.org/2018.11/tutorials/moving-pictures). Single-nucleotide variants (SNVs) were assigned to the reads against the SILVA database release 132 (December 2017).

Metagenomic bioinformatic processing

Illumina paired-end reads were adapter- and quality-filtered using Trimmomatic v0·38 (1). These high-quality interleaved reads were used for *de novo* assembly of contigs of at least 900 bp with IDBA-UD v1.1.1 (2). Gene prediction was performed using MetaGeneMark (3), with genes shorter than 100 bp discarded. A non-redundant gene catalogue of 1,538,641 genes was constructed using CD-HIT (4) with parameters: "-c 0·95 –aS 0·9" (genes with greater than 95% identity and aligned length covering over 90% of the shorter gene were grouped together). Genes with greater than 100 bp were retained and transcribed into amino acids using transeq, part of The European Molecular Biology Open Software Suite (EMBOSS v6.5.7) (5). The transcribed non-redundant gene catalogue was annotated to the comprehensive antibiotic resistance database (CARD) release 2020-03-04 (6), using BLASTP, with parameters: "-task blastp-fast -evalue 1e-10 -qcov_hsp_perc 99 -max_hsps 1 -max_target_seqs 1." High quality reads from each sample were aligned against the gene catalogue, and gene-length normalized read counts were calculated using SOAP (7). For every sample, relative gene abundances were estimated by dividing the number of the gene-length normalized read counts for each gene by the total of reads that uniquely mapped to any gene in the catalogue as previously described (8).

Quantitation of total bacterial load, resistance gene carriage and specific bacterial taxa

Levels of *OXA-1, NDM-7, fusB, rmtB, dfrA14, catB3, mcr-1, CTX-M-14* and *CMY-2* genes were assessed using SYBR Green assays based on previously described primer pairs and previous described conditions (9). Modifications to the qPCR annealing conditions were used for *mcr-1, NDM-7* and *OXA-1* qPCR assays (Table S2). For SYBR Green qPCR assays, 1 μL of DNA extract, 0.2 μM of each primer, 17.5 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, USA) and the appropriate volume of water was added to a 35 μL total reaction volume. Quantitative RT-PCR were performed on three technical replicates, at 10 μL reaction volume per replicate, on a QuantStudio 6 and 7 Flex Real-Time PCR system (Applied Biosystems, Carlsbad, USA). Cycling conditions for SYBR Green qPCR assays were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. Melt curve analysis was then performed at the following conditions: 95°C for 15 secs, followed by an initial stage temperature of 60°C for 1 min and a final temperature of 95°C for 15 secs, with readings recorded at increments of 0·05°C/s. Standard curves were generated for each qPCR reaction based on serial dilutions of DNA from bacterial isolates previously identified using whole genome sequencing.

Supplementary Tables and Figures

Table S1: Patient co-location categories that were assigned to determine spatial relation between patients.

Patient location	Category no.
Patient located within the same bay	1
Patient located in the neighbouring bay	2
Patient located in the opposite bay from the index bay	3
Patient located in the diagonally opposite bay from the index bay	4
Patient located two bays away from the index bay	5
Patient located in a diagonal bay, two bays away from index bay	6
Patient located three bays away from the index bay	7
Patient located in a diagonal bay, three bays away from index bay	8
Patient located furthest away from the index bay	9
Patient located furthest away diagonally from the index bay	10
Patient located in the corridor in comparison to patient located in a bay	11

Table S2: Primer sequences of antibiotic resistance and positive control (16S rRNA) genes for quantitative PCR.

Gene	Sequence (5' - 3')	Annealing conditions (temperature, extension time)	References
OXA-1	CGGATGGTTTGAAGGGTTTATTAT	55°C, 60 s	(10, 11)
	TTTCTTGGCTTTTATGCTTG		
NDM-7	GAATGTCTGGCAGCACACTT	56°C, 60 s	(12)
	GCATTGGCATAAGTCGCAATCC		This study
CTX-M-14	TCAAGCCTGCCGATCTGGT	60°C, 60 s	(13)
	TGATTCTCGCCGCTGAAG		
CMY-2	AAAGCCTCATGGGTGCATAAA	60°C, 60 s	(10)
	ATAGCTTTTGTTTGCCAGCATCA		
mcr-1.0	AGTCCGTTTGTTCTTGTGGC	56°C, 60 s	(14)
	AGATCCTTGGTCTCGGCTTG		
dfrA14	GCTGCGAAAGCGAAAAACGGCGT	60°C, 60 s	This study
	ATCGTCGATAAGTGGAGCGTAGA		(15)
	GGC		
catB3	GCACTCGATGCCTTCCAAAA	60°C, 60 s	(10)
	AGAGCCGATCCAAACGTCAT		
rmtB	GCTTTCTGCGGGCGATGTAA	60°C, 60 s	(16)
	ATGCAATGCCGCGCTCGTAT		
fusB	TGAACTACCTAGTCCGCAA	60°C, 60 s	This study
	TTATATATTTCCGATTTGATGCAAG		(17)

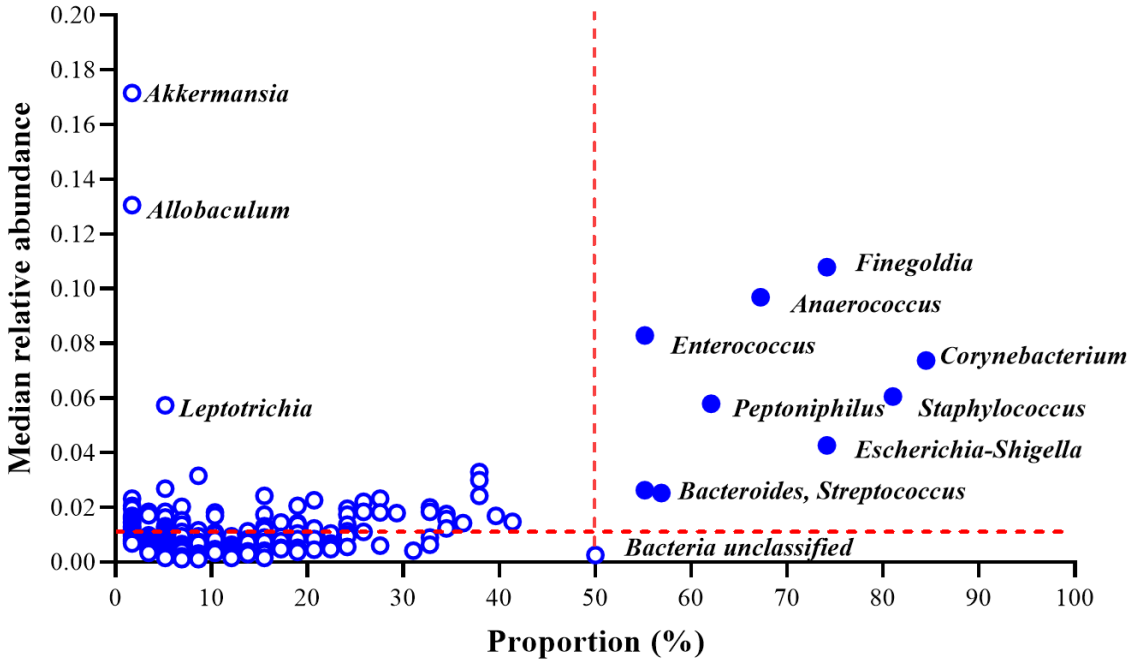


Figure S1: Identification of core microbiota among study population (>50% population, median>0·01).

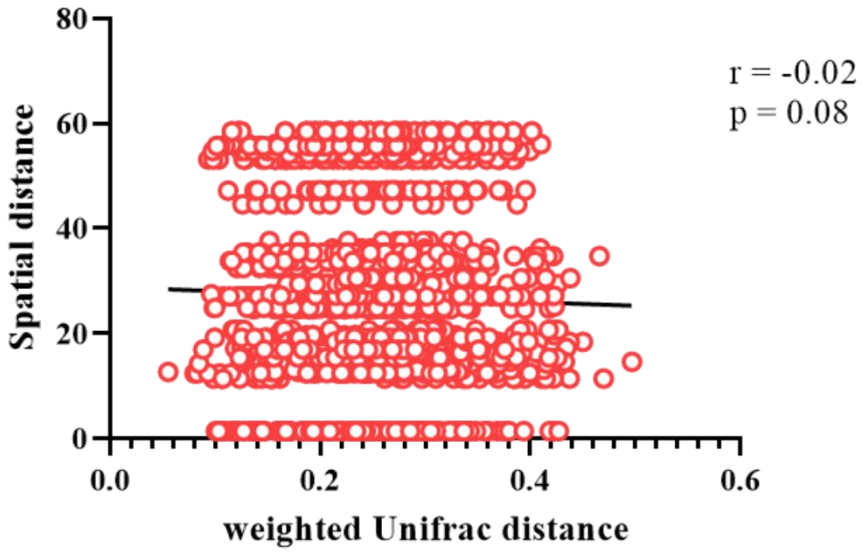


Figure S2: Spearman's correlation of spatial distance between individuals located on the ward (spatial distance) and compositional similarity distance between individual's microbiota (weighted UNIFRAC distance).

Table S3: Antimicrobial resistance genes identified through shotgun metagenomics

Gene name	Gene accession ID	ARO number	ARO class	Evidence of gene mobility	Reference for gene mobility
tetB(P)	AAA20117.1	ARO:3000195	Tetracycline resistance	Yes	(18)
cat type A-13	AAA23018.1	ARO:3004454	Phenicol resistance	Yes	(19)
tetO	AAA23033.2	ARO:3000190	Tetracycline resistance	Yes	(20)
dfrA3	AAA25550.1	ARO:3003105	Diaminopyrimidine resistance	Yes	(21)
cat type A-7	AAA26615.1	ARO:3004457	Phenicol resistance	Yes	(22)
tetX	AAA27471.1	ARO:3000205	Tetracycline resistance	Yes	(23)
vanRA	AAA65953.1	ARO:3002919	Glycopeptide resistance	Yes	(24)
vanSA	AAA65954.1	ARO:3002931	Glycopeptide resistance	Yes	(24)
vanHA	AAA65955.1	ARO:3002942	Glycopeptide resistance	Yes	(24)
vanA	AAA65956.1	ARO:3000010	Glycopeptide resistance	Yes	(24)
dfrD	AAA85213.1	ARO:3002866	Diaminopyrimidine resistance	Yes	(25)
vatB	AAA86871.1	ARO:3002841	Streptogramin resistance	Yes	(26)
vanRB	AAB05622.1	ARO:3002921	Glycopeptide resistance	Yes	(27)
vanSB	AAB05623.1	ARO:3002932	Glycopeptide resistance	Yes	(27)
vanYB	AAB05624.1	ARO:3002956	Glycopeptide resistance	Yes	(27)
vanWB	AAB05625.1	ARO:3002964	Glycopeptide resistance	Yes	(27)
vanHB	AAB05626.1	ARO:3002943	Glycopeptide resistance	Yes	(27)
cat type A-14	AAB23649.1	ARO:3004460	Phenicol resistance	Yes	(28)
carA	AAC32027.1	ARO:3002817	Macrolide resistance	Yes	(29)
vanHF	AAF36802.1	ARO:3002945	Glycopeptide resistance	Yes	(30)
vanF	AAF36803.1	ARO:3002908	Glycopeptide resistance	Yes	(30)
catD	AAF66228.1	ARO:3002682	Phenicol resistance	Yes	(31)
vatE	AAF86220.1	ARO:3002844	Streptogramin resistance	Yes	(26)
mefE	AAK99775.1	ARO:3000614	Macrolide resistance	Yes	(32)
oqxB	AAP43110.2	ARO:3003923	Fluoroquinolone resistance	Yes	(33)

vanSF	AAR84673.1	ARO:3002936	Glycopeptide resistance	Yes	(30)
blaR1	ABU39979.1	ARO:3000217	Beta-lactam resistance	Yes	(34)
mefB	ACJ63262.1	ARO:3003107	Macrolide resistance	Yes	(35)
vanRM	ACL82957.1	ARO:3002928	Glycopeptide resistance	Yes	(36)
vanSM	ACL82958.1	ARO:3002939	Glycopeptide resistance	Yes	(36)
vanHM	ACL82960.1	ARO:3002947	Glycopeptide resistance	Yes	(36)
vanM	ACL82961.1	ARO:3002911	Glycopeptide resistance	Yes	(36)
vgaD	ACX92986.2	ARO:3002832	Streptogramin resistance	Yes	(37)
vanN	AEP40500.1	ARO:3002912	Glycopeptide resistance	Yes	(38)
vanTN	AEP40502.2	ARO:3002975	Glycopeptide resistance	Yes	(38)
vanRN	AEP40503.1	ARO:3002929	Glycopeptide resistance	Yes	(38)
vanSN	AEP40504.1	ARO:3002940	Glycopeptide resistance	Yes	(38)
mecA	AGC51118.1	ARO:3000617	Beta-lactam resistance	Yes	(39)
APH(3')-IIIa	AGV10830.1	ARO:3002647	Aminoglycoside resistance	Yes	(40)
clbA	AGZ55247.1	ARO:3002814	Macrolide-lincosamide-linezolid-phenicol-streptogramin resistance	Yes	(41)
ANT(6)-Ia	AHE40557.1	ARO:3002626	Aminoglycoside resistance	Yes	(42)
clbC	BAD63613.1	ARO:3002816	Linezolid resistance	Yes	(41)
dfrG	BAE15963.1	ARO:3002868	Diaminopyrimidine resistance	Yes	(43)
mecB	BAI83385.1	ARO:3003440	Beta-lactam resistance	Yes	(44)
mefC	BAL43360.1	ARO:3003745	Macrolide resistance	Yes	(45)
tetW	CAA10975.1	ARO:3000194	Tetracycline resistance	Yes	(46)
cat type A-7	CAA63498.2	ARO:3004458	Phenicol resistance	Yes	(47)
tetQ	CAA79727.1	ARO:3000191	Tetracycline resistance	Yes	(48)
lsaB	CAE18141.1	ARO:3003111	Clindamycin resistance	Yes	(49)
dfrA20	CAE53424.1	ARO:3003016	Diaminopyrimidine resistance	Yes	(50)
tetM	CAQ49384.1	ARO:3000186	Tetracycline resistance	Yes	(51)
tet44	CBH51823.1	ARO:3000556	Tetracycline resistance	Yes	(52)
ANT(6)-Ib	CBH51824.1	ARO:3002629	Aminoglycoside resistance	Yes	(52)
dfrK	CBL80435.1	ARO:3002869	Diaminopyrimidine resistance	Yes	(53)
vgaE	CBY88983.1	ARO:3002833	Streptogramin resistance	Yes	(54)

tetA	NP_862226.1	ARO:3004639	Tetracycline resistance	Yes	(18)
AAC(6')-Ie-APH(2'')-Ia	AAA88548.1	ARO:3002597	Aminoglycoside resistance	Yes	(55)
mecC	CCC86795.1	ARO:3001209	Beta-lactam resistance	Yes	(56)
cfr(B)	CDF47262.1	ARO:3004649	Macrolide-lincosamide-streptogramin resistance	Yes	(57)
tet(X4)	QBQ69719.1	ARO:3004720	Tetracycline resistance	Yes	(58)
APH(3')-VIa	CAA30578.1	ARO:3002652	Aminoglycoside resistance	Yes	(59)
CMY-2	CAA62957.1	ARO:3002013	Beta-lactam resistance	Yes	(60)
ACI-1	CAB51471.1	ARO:3004359	Beta-lactam resistance	Yes	(61)
TEM-1	CAD09800.1	ARO:3000873	Beta-lactam resistance	Yes	(62)
fexA	CAD70268.1	ARO:3002704	Efflux pump conferring antibiotic resistance	Yes	(63)
APH(3')-Ia	CAE51638.1	ARO:3002641	Aminoglycoside resistance	Yes	(64)
catB10	CAI47810.1	ARO:3003110	Phenicol resistance	Yes	(65)
rmtB	CAP07796.1	ARO:3000860	Aminoglycoside resistance	Yes	(66)
dfrA21	CAP69659.1	ARO:3003017	Diaminopyrimidine resistance	Yes	(67)
dfrA22	CAX16467.1	ARO:3003018	Diaminopyrimidine resistance	Yes	(68)
vgaC	CBL58195.1	ARO:3002831	Streptogramin resistance	Yes	(69)
clbB	BAH45481.1	ARO:3002815	Macrolide-streptogramin-lincosamide resistance	Yes	(41)
catB6	CAA11473.1	ARO:3002678	Phenicol resistance	Yes	(70)
APH(6)-Ic	CAA25854.1	ARO:3002659	Aminoglycoside resistance	Yes	(71)
ANT(3'')-IIa	CAA26199.1	ARO:3004089	Aminoglycoside resistance	Yes	(72)
ermA	CAA26964.1	ARO:3000347	Macrolide-lincosamide-streptogramin resistance	Yes	(73)
AAC(6')-Iad	BAD12078.1	ARO:3002572	Aminoglycoside resistance	Yes	(74)
sat1	BAD95494.1	ARO:3002895	Nucleoside antibiotic resistance	Yes	(75)
fosA6	AMQ12811.1	ARO:3004111	Fosfomycin resistance	Yes	(76)
sul4	AUI09862.1	ARO:3004361	Sulfonamide antibiotic resistance	Yes	(77)
mcr-1.9	AVA31022.1	ARO:3004507	Peptide antibiotic resistance	Yes	(78)
mcr-8	AVX52225.1	ARO:3004516	Peptide antibiotic resistance	Yes	(79)
dfrA15	AHB39758.1	ARO:3003013	Diaminopyrimidine resistance	Yes	(80)
catB11	AID93387.1	ARO:3004660	Phenicol resistance	Yes	(81)
optrA	AKA86814	ARO:3003746	Mactrolide-streptogramin-lincosamide	Yes	(82)

mcr-1	AKF16168	ARO:3003689	Peptide antibiotic resistance	Yes	(83)
AAC(6')-Ib7	AKN19287.1	ARO:3002578	Aminoglycoside resistance	Yes	(84)
fosB4	ALM24139.1	ARO:3004671	Fosfomycin resistance	Yes	(85)
tet(W/N/W)	AMP42147.1	ARO:3004442	Tetracycline resistance	Yes	(86)
armA	ADC55560.1	ARO:3000858	Aminoglycoside resistance	Yes	(87)
dfrA12	ADG84870.1	ARO:3002858	Diaminopyrimidine resistance	Yes	(88)
OXA-181	AEP16366.1	ARO:3001784	Beta-lactam resistance	Yes	(89)
OXA-347	AET35493.1	ARO:3001777	Beta-lactam resistance	Yes	(90)
OXA-1	AFB82783.1	ARO:3001396	Beta-lactam resistance	Yes	(91)
catA8	AFN69318.1	ARO:3004658	Phenicol resistance	Yes	(92)
NDM-7	AFQ31613.1	ARO:3002357	Beta-lactam resistance	Yes	(93)
catB3	AFQ93498.1	ARO:3002676	Phenicol resistance	Yes	(94)
lnuD	ABR14060.1	ARO:3002838	Lincosamide resistance	Yes	(95)
AAC(3)-IId	ABS70977.1	ARO:3004623	Aminoglycoside resistance	Yes	(96)
msrE	ACB05808.1	ARO:3003109	Macrolide,streptogramin,lincosamide resistance	Yes	(97)
dfrA14	ACI32877.1	ARO:3002859	Diaminopyrimidine resistance	Yes	(98)
sul3	ACJ63260.1	ARO:3000413	Sulfonamide resistance	Yes	(99)
qnrS1	ABF47469.1	ARO:3002790	Fluoroquinolone resistance	Yes	(100)
linG	ABG65740.1	ARO:3002879	Lincosamide resistance	Yes	(101)
dfrA17	ABG91835.1	ARO:3002860	Diaminopyrimidine resistance	Yes	(102)
vgaALC	ABH10964.1	ARO:3002830	Streptogramin resistance	Yes	(103)
mphE	ABI20451.1	ARO:3003741	Macrolide resistance	Yes	(104)
CTX-M-55	ABI34705.1	ARO:3001917	Beta-lactam resistance	Yes	(105)
aad(6)	AAU10334.1	ARO:3002628	Aminoglycoside resistance	Yes	(106)
tet(39)	AAW66497.1	ARO:3000566	Efflux pump conferring antibiotic resistance	Yes	(107)
OXA-68	AAW81339.1	ARO:3001616	Beta-lactam resistance	Yes	(108)
qacH	AAZ42322.1	ARO:3003836	Efflux pump conferring antibiotic resistance	Yes	(109)
dfrA5	ABB89122.1	ARO:3002861	Diaminopyrimidine resistance	Yes	(88)
qnrB1	ABC86904.2	ARO:3002714	Fluroquinolone resistance	Yes	(110)
mel	AAL73129.1	ARO:3000616	Efflux pump conferring antibiotic resistance	Yes	(32)
tet(D)	AAL75563.1	ARO:3000168	Efflux pump conferring antibiotic resistance	Yes	(18)

tet(A)	AAN06707.1	ARO:3000165	Efflux pump conferring antibiotic resistance	Yes	(18)
catB8	AAO52851.1	ARO:3004456	Phenicol resistance	Yes	(111)
dfrA1	AAP74961.2	ARO:3002854	Diaminopyrimidine resistance	Yes	(112)
SHV-28	AAG15384.1	ARO:3001086	Beta-lactam resistance	Yes	(113)
floR	AAG16656.1	ARO:3002705	Phenicol resistance	Yes	(114)
sul2	AAL59753.1	ARO:3000412	Sulfonamide resistance	Yes	(115)
tet(J)	AAD12753.1	ARO:3000177	Efflux pump conferring antibiotic resistance	Yes	(18)
aadA5	AAF17880.1	ARO:3002605	Aminoglycoside resistance	Yes	(116)
CTX-M-14	AAF72530.1	ARO:3001877	Beta-lactam resistance	Yes	(117)
cmx	AAG03380.1	ARO:3002703	Efflux pump conferring antibiotic resistance	Yes	(118)
tet(K)	AAB28795.1	ARO:3000178	Efflux pump conferring antibiotic resistance	Yes	(18)
sat4	AAB53445.1	ARO:3002897	Nucleoside antibiotic	Yes	(119)
vgaB	AAB95639.1	ARO:3000118	Streptogramin resistance	Yes	(120)
aadA3	AAC14728.1	ARO:3002603	Aminoglycoside resistance	Yes	(116)
APH(6)-Id	AAC23556.1	ARO:3002660	Aminoglycoside resistance	Yes	(121)
ANT(2'')-Ia	AAC64365.1	ARO:3000230	Aminoglycoside resistance	Yes	(122)
arr-2	AAC64366.1	ARO:3002847	Rifamycin resistance	Yes	(123)
tetA(P)	AAA20116.1	ARO:3000180	Efflux pump conferring antibiotic resistance	Yes	(18)
tet(L)	AAA22851.1	ARO:3000179	Efflux pump conferring antibiotic resistance	Yes	(18)
tetS	AAA25293.1	ARO:3000192	Tetracycline resistance	Yes	(124)
lnuA	AAA26652.1	ARO:3002835	Lincosamide resistance	Yes	(125)
ermT	AAA98096.1	ARO:3000595	Macrolide-lincosamide-streptogramin resistance	Yes	(126)
AAC(6')-Ia	AAA98298.1	ARO:3002545	Aminoglycoside resistance	Yes	(127)
cepA	AAA21532.1	ARO:3003559	Beta-lactam resistance	No	N/A
catQ	AAA23215.1	ARO:3002687	Phenicol resistance	No	N/A
vanC	AAA24786.1	ARO:3000368	Glycopeptide resistance	No	N/A
oleC	AAA26793	ARO:3003748	Macrolide resistance	No	N/A
tlrC	AAA26832.1	ARO:3002827	Efflux pump conferring antibiotic resistance	No	N/A
oleB	AAA50325.1	ARO:3003036	Efflux pump conferring antibiotic resistance	No	N/A
mexB	AAA74437.1	ARO:3000378	Efflux pump conferring antibiotic resistance	No	N/A
bcrA	AAA99504.1	ARO:3002987	Bacitracin resistance	No	N/A

tsnR	AAA99931.1	ARO:3003060	Peptide antibiotic resistance	No	N/A
mexC	AAB41956.1	ARO:3000800	Efflux pump conferring antibiotic resistance	No	N/A
mexD	AAB41957.1	ARO:3000801	Efflux pump conferring antibiotic resistance	No	N/A
ceoB	AAB58161.1	ARO:3003010	Efflux pump conferring antibiotic resistance	No	N/A
rosB	AAC60780.1	ARO:3003049	Efflux pump conferring antibiotic resistance	No	N/A
rosA	AAC60781.1	ARO:3003048	Peptide antibiotic resistance	No	N/A
acrB	AAC73564.1	ARO:3000216	Efflux pump conferring antibiotic resistance	No	N/A
kdpE	AAC73788.1	ARO:3003841	Aminoglycoside resistance	No	N/A
msbA	AAC74000.1	ARO:3003950	Efflux pump conferring antibiotic resistance	No	N/A
mdtH	AAC74149.2	ARO:3001216	Efflux pump conferring antibiotic resistance	No	N/A
mdtA	AAC75135.2	ARO:3000792	Efflux pump conferring antibiotic resistance	No	N/A
mdtB	AAC75136.1	ARO:3000793	Efflux pump conferring antibiotic resistance	No	N/A
mdtC	AAC75137.1	ARO:3000794	Efflux pump conferring antibiotic resistance	No	N/A
yojI	AAC75271.1	ARO:3003952	Peptide antibiotic resistance	No	N/A
pmrF	AAC75314.1	ARO:3003578	Peptide antibiotic resistance	No	N/A
evgS	AAC75429.1	ARO:3000833	Efflux pump conferring antibiotic resistance	No	N/A
emrR	AAC75731.1	ARO:3000516	Efflux pump conferring antibiotic resistance	No	N/A
emrB	AAC75733.1	ARO:3000074	Efflux pump conferring antibiotic resistance	No	N/A
bacA	AAC76093.1	ARO:3002986	Peptide antibiotic resistance	No	N/A
acrS	AAC76296.1	ARO:3000656	Efflux pump conferring antibiotic resistance	No	N/A
acrE	AAC76297.1	ARO:3000499	Efflux pump conferring antibiotic resistance	No	N/A
acrF	AAC76298.1	ARO:3000502	Efflux pump conferring antibiotic resistance	No	N/A
mdtF	AAC76539.1	ARO:3000796	Efflux pump conferring antibiotic resistance	No	N/A
vanTC	AAD22403.1	ARO:3002970	Glycopeptide resistance	No	N/A
smeB	AAD51345.1	ARO:3003052	Efflux pump conferring antibiotic resistance	No	N/A
smeC	AAD51346.1	ARO:3003053	Efflux pump conferring antibiotic resistance	No	N/A
smeS	AAD51347.1	ARO:3003067	Efflux pump conferring antibiotic resistance	No	N/A
smeR	AAD51348.1	ARO:3003066	Efflux pump conferring antibiotic resistance	No	N/A
tetT	AAF01499.1	ARO:3000193	Tetracycline resistance	No	N/A
mtrD	AAF42062.1	ARO:3000811	Efflux pump conferring antibiotic resistance	No	N/A
mtrC	AAF42063.1	ARO:3000810	Efflux pump conferring antibiotic resistance	No	N/A

novA	AAF67494.2	ARO:3002522	Aminocoumarin resistance	No	N/A
vanRC	AAF86641.1	ARO:3002922	Glycopeptide resistance	No	N/A
vanSC	AAF86642.1	ARO:3002933	Glycopeptide resistance	No	N/A
triB	AAG03547.1	ARO:3003680	Triclosan resistance	No	N/A
oprM	AAG03816.1	ARO:3000379	Efflux pump conferring antibiotic resistance	No	N/A
mexE	AAG05881.1	ARO:3000803	Efflux pump conferring antibiotic resistance	No	N/A
mexF	AAG05882.1	ARO:3000804	Efflux pump conferring antibiotic resistance	No	N/A
arnA	AAG06942.1	ARO:3002985	Polymyxin resistance	No	N/A
mexK	AAG07064.1	ARO:3003693	Efflux pump conferring antibiotic resistance	No	N/A
mexJ	AAG07065.1	ARO:3003692	Efflux pump conferring antibiotic resistance	No	N/A
mexL	AAG07066.1	ARO:3003710	Efflux pump conferring antibiotic resistance	No	N/A
mexG	AAG07592.1	ARO:3000806	Efflux pump conferring antibiotic resistance	No	N/A
mexH	AAG07593.1	ARO:3000807	Efflux pump conferring antibiotic resistance	No	N/A
mexI	AAG07594.1	ARO:3000808	Efflux pump conferring antibiotic resistance	No	N/A
patB	AAK76136.1	ARO:3000025	Fluoroquinolone resistance	No	N/A
patA	AAK76137.1	ARO:3000024	Fluoroquinolone resistance	No	N/A
pmrA	AAK99679.1	ARO:3000822	Efflux pump conferring antibiotic resistance	No	N/A
adeA	AAL14439.1	ARO:3000774	Efflux pump conferring antibiotic resistance	No	N/A
adeB	AAL14440.1	ARO:3000775	Efflux pump conferring antibiotic resistance	No	N/A
mdsC	AAL19304.1	ARO:3000791	Efflux pump conferring antibiotic resistance	No	N/A
mdsB	AAL19305.1	ARO:3000790	Efflux pump conferring antibiotic resistance	No	N/A
mdsA	AAL19306.1	ARO:3000789	Efflux pump conferring antibiotic resistance	No	N/A
sdiA	AAL20862.1	ARO:3000826	Efflux pump conferring antibiotic resistance	No	N/A
vanE	AAL27442.1	ARO:3002907	Glycopeptide resistance	No	N/A
vanTE	AAL27444.1	ARO:3002971	Glycopeptide resistance	No	N/A
vanRE	AAL27445.1	ARO:3002924	Glycopeptide resistance	No	N/A
vanSE	AAL27446.1	ARO:3002935	Glycopeptide resistance	No	N/A
vanD	AAM09849.1	ARO:3000005	Glycopeptide resistance	No	N/A
vanHD	AAM09850.1	ARO:3002944	Glycopeptide resistance	No	N/A
vanRD	AAM09851.1	ARO:3002923	Glycopeptide resistance	No	N/A
vanXD	AAM09852.1	ARO:3003070	Glycopeptide resistance	No	N/A

mprF	AAN00989.1	ARO:3003774	Peptide antibiotic resistance	No	N/A
murA	AAN28945	ARO:3003785	Fosfomycin resistance	No	N/A
lsaA	AAO43110.1	ARO:3000300	Efflux pump conferring antibiotic resistance	No	N/A
parY	AAO47226.2	ARO:3003318	Aminocoumarin resistance	No	N/A
vanRF	AAR84672.1	ARO:3002925	Glycopeptide resistance	No	N/A
mepA	AAU95768.1	ARO:3000026	Efflux pump conferring antibiotic resistance	No	N/A
macA	AAV85981.1	ARO:3000533	Macrolide resistance	No	N/A
macB	AAV85982.1	ARO:3000535	Macrolide resistance	No	N/A
adeJ	AAX14802.1	ARO:3000781	Efflux pump conferring antibiotic resistance	No	N/A
adeK	AAX14803.1	ARO:3000782	Efflux pump conferring antibiotic resistance	No	N/A
vanUG	ABA71726.1	ARO:3004253	Glycopeptide resistance	No	N/A
vanRG	ABA71727.1	ARO:3002926	Glycopeptide resistance	No	N/A
vanSG	ABA71728.1	ARO:3002937	Glycopeptide resistance	No	N/A
vanWG	ABA71730.1	ARO:3002965	Glycopeptide resistance	No	N/A
vanG	ABA71731.1	ARO:3002909	Glycopeptide resistance	No	N/A
vanXYG	ABA71732.1	ARO:3003069	Glycopeptide resistance	No	N/A
vanTG	ABA71733.1	ARO:3002972	Glycopeptide resistance	No	N/A
arlS	ABD30512.1	ARO:3000839	Efflux pump conferring antibiotic resistance	No	N/A
mprF	ABG86067.1	ARO:3003773	Peptide antibiotic resistance	No	N/A
cmeB	ABS43151.1	ARO:3000784	Efflux pump conferring antibiotic resistance	No	N/A
cmeA	ABS43901.1	ARO:3000783	Efflux pump conferring antibiotic resistance	No	N/A
mdtG	ABV18113.1	ARO:3001329	Efflux pump conferring antibiotic resistance	No	N/A
lmrC	ABX00624.1	ARO:3002881	Lincosamide resistance	No	N/A
vanL	ABX54687.1	ARO:3002910	Glycopeptide resistance	No	N/A
vanTrL	ABX54690.1	ARO:3002974	Glycopeptide resistance	No	N/A
vanRL	ABX54691.1	ARO:3002927	Glycopeptide resistance	No	N/A
tet32	ACH87088.1	ARO:3000196	Tetracycline resistance	No	N/A
adeI	ACJ41739.1	ARO:3000780	Efflux pump conferring antibiotic resistance	No	N/A
abeS	ACJ59254.1	ARO:3000768	Efflux pump conferring antibiotic resistance	No	N/A
tolC	ACN32294.1	ARO:3000237	Efflux pump conferring antibiotic resistance	No	N/A
cfxA6	ACT97371.1	ARO:3003097	Beta-lactam resistance	No	N/A

cipA	ACX65640.1	ARO:3003907	Macrolide- lincosamide-linezolid-phenicol-streptogramin resistance	No	N/A
adeR	ADM92605.1	ARO:3000553	Aminoglycoside resistance	No	N/A
adeS	ADM92606.1	ARO:3000549	Aminoglycoside resistance	No	N/A
hmrM	ADO96486.1	ARO:3003953	Efflux pump conferring antibiotic resistance	No	N/A
ileS	ADP36409.1	ARO:3003730	Mupirocin resistance	No	N/A
lsaC	AEA37904.1	ARO:3003112	Efflux pump conferring antibiotic resistance	No	N/A
mdfA	AFH35853.1	ARO:3001328	Efflux pump conferring antibiotic resistance	No	N/A
salA	AGN74946	ARO:3003749	Lincosamide-streptogramin resistance	No	N/A
adeN	AGV28567.1	ARO:3000559	Efflux pump conferring antibiotic resistance	No	N/A
vanHO	AHA41499.1	ARO:3002948	Glycopeptide resistance	No	N/A
vanO	AHA41500.1	ARO:3002913	Glycopeptide resistance	No	N/A
vanSO	AHA41504.1	ARO:3002941	Glycopeptide resistance	No	N/A
vanRO	AHA41505.1	ARO:3002930	Glycopeptide resistance	No	N/A
rlmA(II)	AJD73064.1	ARO:3001301	Macrolide resistance	No	N/A
adeL	ALH22601.1	ARO:3000620	Efflux pump conferring antibiotic resistance	No	N/A
adeC	ALX99516.1	ARO:3003811	Efflux pump conferring antibiotic resistance	No	N/A
mdtK	AML99881.1	ARO:3001327	Efflux pump conferring antibiotic resistance	No	N/A
gadW	ANK04027.1	ARO:3003838	Efflux pump conferring antibiotic resistance	No	N/A
tetA(58)	APB03214.1	ARO:3003980	Tetracycline resistance	No	N/A
catU	APB03217.1	ARO:3003983	Phenicol resistance	No	N/A
TaeA	APB03219.1	ARO:3003986	Efflux pump conferring antibiotic resistance	No	N/A
arlR	ATC67679.1	ARO:3000838	Efflux pump conferring antibiotic resistance	No	N/A
lmrD	AYV52072.1	ARO:3002882	Lincosamide resistance	No	N/A
emrY	BAA11237.1	ARO:3000254	Efflux pump conferring antibiotic resistance	No	N/A
baeS	BAA15934.1	ARO:3000829	Efflux pump conferring antibiotic resistance	No	N/A
baeR	BAA15935.1	ARO:3000828	Efflux pump conferring antibiotic resistance	No	N/A
acrD	BAA16344.1	ARO:3000491	Efflux pump conferring antibiotic resistance	No	N/A
emrA	BAA16547.1	ARO:3000027	Efflux pump conferring antibiotic resistance	No	N/A
mexY	BAA34300.1	ARO:3003033	Efflux pump conferring antibiotic resistance	No	N/A
AAC(3)-Xa	BAA78619.1	ARO:3002544	Aminoglycoside resistance	No	N/A

H-NS	BAB35162.1	ARO:3000676	Efflux pump conferring antibiotic resistance	No	N/A
evgA	BAB36671.1	ARO:3000832	Efflux pump conferring antibiotic resistance	No	N/A
abeM	BAD89844.2	ARO:3000753	Efflux pump conferring antibiotic resistance	No	N/A
mexN	BAE06006.1	ARO:3003705	Efflux pump conferring antibiotic resistance	No	N/A
gadX	BAE77778.1	ARO:3000508	Efflux pump conferring antibiotic resistance	No	N/A
mdtE	BAE77781.1	ARO:3000795	Efflux pump conferring antibiotic resistance	No	N/A
CRP	BAE77933.1	ARO:3000518	Efflux pump conferring antibiotic resistance	No	N/A
mdtP	BAE78082.1	ARO:3003550	Efflux pump conferring antibiotic resistance	No	N/A
mdtO	BAE78083.1	ARO:3003549	Efflux pump conferring antibiotic resistance	No	N/A
mdtN	BAE78084.1	ARO:3003548	Efflux pump conferring antibiotic resistance	No	N/A
vanWI	BAE83690.1	ARO:3003724	Glycopeptide resistance	No	N/A
vanRI	BAE85478.1	ARO:3003728	Glycopeptide resistance	No	N/A
vanKI	BAE85481.1	ARO:3003727	Glycopeptide resistance	No	N/A
mtrE	CAA64891.1	ARO:3000812	Efflux pump conferring antibiotic resistance	No	N/A
ykkD	CAB13167.1	ARO:3003064	Efflux pump conferring antibiotic resistance	No	N/A
smeE	CAC14595.1	ARO:3003056	Efflux pump conferring antibiotic resistance	No	N/A
smeF	CAC14596.1	ARO:3003057	Efflux pump conferring antibiotic resistance	No	N/A
acrA	CAC41008.1	ARO:3004041	Efflux pump conferring antibiotic resistance	No	N/A
mprF	CAC99773.1	ARO:3003770	Peptide antibiotic resistance	No	N/A
tet36	CAD55718.1	ARO:3000197	tetracycline resistance	No	N/A
cdeA	CAE00499.1	ARO:3003835	Efflux pump conferring antibiotic resistance	No	N/A
amrB	CAH35802.1	ARO:3002983	Efflux pump conferring antibiotic resistance	No	N/A
adeH	CAJ77855.1	ARO:3000779	Efflux pump conferring antibiotic resistance	No	N/A
adeF	CAJ77856.1	ARO:3000777	Efflux pump conferring antibiotic resistance	No	N/A
adeG	CAJ77857.1	ARO:3000778	Efflux pump conferring antibiotic resistance	No	N/A
mprF	CAX52582.1	ARO:3003324	Peptide antibiotic resistance	No	N/A
mtrA	CCP46065.1	ARO:3000816	Efflux pump conferring antibiotic resistance	No	N/A
efrA	CDO61513.1	ARO:3003948	Efflux pump conferring antibiotic resistance	No	N/A
efrB	CDO61516.1	ARO:3003949	Efflux pump conferring antibiotic resistance	No	N/A
lmrB	KIX81495.1	ARO:3002813	Efflux pump conferring antibiotic resistance	No	N/A
CpxR	SIP52035.1	ARO:3004054	Efflux pump conferring antibiotic resistance	No	N/A

abcA	XP_753111.1	ARO:3003942	Efflux pump conferring antibiotic resistance	No	N/A
catB	AAA73865.1	ARO:3002674	Phenicol resistance	No	N/A
acrA	AAC73565.1	ARO:3004043	Efflux pump conferring antibiotic resistance	No	N/A
ugd	AAC75089.1	ARO:3003577	Peptide antibiotic resistance	No	N/A
mdtM	AAC77293.1	ARO:3001214	Efflux pump conferring antibiotic resistance	No	N/A
farA	AAF40763.1	ARO:3003961	Efflux pump conferring antibiotic resistance	No	N/A
farB	AAF40764.1	ARO:3003962	Efflux pump conferring antibiotic resistance	No	N/A
vanXYC	AAF61331.1	ARO:3002966	Glycopeptide resistance	No	N/A
vatF	AAF63432	ARO:3003744	Streptogramin resistance	No	N/A
PmpM	AAG04750.1	ARO:3004077	Efflux pump conferring antibiotic resistance	No	N/A
soxR	AAG05661.1	ARO:3004107	Efflux pump conferring antibiotic resistance	No	N/A
oprN	AAG05883.1	ARO:3000805	Efflux pump conferring antibiotic resistance	No	N/A
opmB	AAG05913.1	ARO:3004072	Efflux pump conferring antibiotic resistance	No	N/A
muxC	AAG05914	ARO:3004075	Efflux pump conferring antibiotic resistance	No	N/A
muxB	AAG05915.1	ARO:3004074	Efflux pump conferring antibiotic resistance	No	N/A
efpA	CCP45647.1	ARO:3003955	Efflux pump conferring antibiotic resistance	No	N/A
norB	CCQ22388.1	ARO:3000421	Efflux pump conferring antibiotic resistance	No	N/A
oprZ	EGP45230	ARO:3004142	Efflux pump conferring antibiotic resistance	No	N/A
axyY	EGP45231.1	ARO:3004144	Efflux pump conferring antibiotic resistance	No	N/A
kpnG	EHL92831.1	ARO:3004588	Efflux pump conferring antibiotic resistance	No	N/A
dfrE	EOD99669.1	ARO:3002875	Diaminopyrimidine resistance	No	N/A
kpnH	EOU56998.1	ARO:3004597	Efflux pump conferring antibiotic resistance	No	N/A
vanI	KTE89608.1	ARO:3003723	Glycopeptide resistance	No	N/A
vmlR	NP_388442.1	ARO:3004476	Macrolide-lincosamide-streptogramin resistance	No	N/A
mprF	Q8FW76	ARO:3003772	Peptide antibiotic resistance	No	N/A
erm(K)	WP_010896559.1	ARO:3004643	Macrolide-lincosamide-streptogramin resistance	No	N/A
otr(A)	CAA37477.1	ARO:3002891	Tetracycline resistance	No	N/A
mupA	CAA53189	ARO:3000521	Mupirocin resistance	No	N/A
APH(3')-Iib	CAA62365.1	ARO:3002645	Aminoglycoside resistance	No	N/A
emrE	CAA77936.1	ARO:3004039	Efflux pump conferring antibiotic resistance	No	N/A
nmcr	CAA79966.1	ARO:3003665	Beta-lactam resistance	No	N/A

tmrB	CAB12108.2	ARO:3003059	Nucleoside antibitoic resistance	No	N/A
aadK	CAB14620.1	ARO:3002627	Aminoglycoside resistance	No	N/A
smeD	CAC14594.1	ARO:3003055	Efflux pump conferring antibiotic resistance	No	N/A
lpeB	CAH14033.1	ARO:3004100	Efflux pump conferring antibiotic resistance	No	N/A
cmlB1	CAL30186.1	ARO:3002699	Efflux pump conferring antibiotic resistance	No	N/A
dfrA26	CAL48457.1	ARO:3002857	Diaminopyrimidine resistance	No	N/A
cfrC	CAL84423.1	ARO:3004146	Macrolide-lincosamide-streptogramin resistance	No	N/A
tet(40)	CAM12479.1	ARO:3000567	Efflux pump conferring antibiotic resistance	No	N/A
ampC1	CBJ02047.1	ARO:3004611	Beta-lactam resistance	No	N/A
opmE	BAE06009.1	ARO:3003700	Efflux pump conferring antibiotic resistance	No	N/A
eptA	BAE78116.1	ARO:3003576	Peptide antibiotic resistance	No	N/A
pgpB	BAG33043.1	ARO:3003920	Peptide antibiotic resistance	No	N/A
efmA	BAG75524.1	ARO:3003954	Efflux pump conferring antibiotic resistance	No	N/A
kpnE	BAH63251.1	ARO:3004580	Efflux pump conferring antibiotic resistance	No	N/A
kpnF	BAH63252.1	ARO:3004583	Efflux pump conferring antibiotic resistance	No	N/A
qacA	BAJ09383.1	ARO:3003046	Efflux pump conferring antibiotic resistance	No	N/A
ampH	BAJ42218.1	ARO:3004612	Beta-lactam resistance	No	N/A
oprA	BAM10414.1	ARO:3003039	Efflux pump conferring antibiotic resistance	No	N/A
ompK37	CAA09666.1	ARO:3004122	Beta-lactam resistance	No	N/A
cpxA	BAB38260.1	ARO:3000830	Efflux pump conferring antibiotic resistance	No	N/A
mgrA	BAB41874.1	ARO:3000815	Efflux pump conferring antibiotic resistance	No	N/A
DHA-1	BAB43543.1	ARO:3002132	Beta-lactam resistance	No	N/A
mepR	BAB56495.1	ARO:3000746	Efflux pump conferring antibiotic resistance	No	N/A
emeA	BAC11911.1	ARO:3003551	Efflux pump conferring antibiotic resistance	No	N/A
rpoB2	BAD59497.1	ARO:3000501	Rifamycin resistance	No	N/A
mexM	BAE06005.1	ARO:3003704	Efflux pump conferring antibiotic resistance	No	N/A
mexP	BAE06007.1	ARO:3003698	Efflux pump conferring antibiotic resistance	No	N/A
mexQ	BAE06008.1	ARO:3003699	Efflux pump conferring antibiotic resistance	No	N/A
mphC	BAA34540.1	ARO:3000319	Macrolide resistance	No	N/A
tetA(60)	ANZ79240.1	ARO:3004035	Efflux pump conferring antibiotic resistance	No	N/A
tetB(60)	ANZ79241.1	ARO:3004036	Efflux pump conferring antibiotic resistance	No	N/A

tetB(48)	APB03215.1	ARO:3003981	Efflux pump conferring antibiotic resistance	No	N/A
LlmA	APB03216.1	ARO:3003982	Lincosamide resistance	No	N/A
rphB	APB03222.1	ARO:3003992	Rifamycin resistance	No	N/A
qepA4	AQX36338.1	ARO:3004379	Efflux pump conferring antibiotic resistance	No	N/A
mecD	AQX82857.1	ARO:3004185	Beta-lactam resistance	No	N/A
catV	ATL63235.1	ARO:3004357	Phenicol resistance	No	N/A
poxtA	AVI44920.1	ARO:3004470	Macrolide-lincosamide-streptogramin resistance	No	N/A
fusF	AVL76727.1	ARO:3004663	Fusidic acid resistance	No	N/A
tcr3	BAA07390.1	ARO:3002893	Tetracycline resistance	No	N/A
rphA	AIA08936.1	ARO:3000444	Rifamycin resistance	No	N/A
ADC-76	ALA14811.1	ARO:3003882	Beta-lactam resistance	No	N/A
bcr-1	ALV80601.1	ARO:3003801	Efflux pump conferring antibiotic resistance	No	N/A
tet(43)	ACS83748.1	ARO:3000573	Efflux pump conferring antibiotic resistance	No	N/A
cblA-1	ACT97415.1	ARO:3002999	Beta-lactam resistance	No	N/A
rbpA	ADV91011.1	ARO:3000245	Rifamycin resistance	No	N/A
PER-7	AEI54993.1	ARO:3002369	Beta-lactam resistance	No	N/A
tet(45)	AEM62948.1	ARO:3003196	Efflux pump conferring antibiotic resistance	No	N/A
lin	AEO25219.1	ARO:3004651	Lincosamide resistance	No	N/A
tetA(46)	AET10444.1	ARO:3004032	Efflux pump conferring antibiotic resistance	No	N/A
tetB(46)	AET10445.1	ARO:3004033	Efflux pump conferring antibiotic resistance	No	N/A
basS	AEX49906.1	ARO:3003583	Peptide antibiotic resistance	No	N/A
mupB	AEY83581	ARO:3000510	mupirocin resistance	No	N/A
ramA	AFK13828.1	ARO:3000823	Efflux pump conferring antibiotic resistance	No	N/A
facT	AFK80333.1	ARO:3001313	Efflux pump conferring antibiotic resistance	No	N/A
rgt1438	AFO53532.1	ARO:3002883	Rifamycin resistance	No	N/A
BRP(MBL)	AGH88989.1	ARO:3001205	Beta-lactam resistance	No	N/A
CMY-104	AGR82311.1	ARO:3002116	Beta-lactam resistance	No	N/A
lnuE	AGT57825	ARO:3003762	Lincosamide resistance	No	N/A
vga(E)	AHB37625.1	ARO:3004715	Streptogramin resistance	No	N/A
mecR1	ABQ47844.1	ARO:3000215	Beta-lactam resistance	No	N/A
blaI	ABU39978.1	ARO:3000160	Beta-lactam resistance	No	N/A

PC1	ABX30738.1	ARO:3000621	Beta-lactam resistance	No	N/A
tet(42)	ACD35503.1	ARO:3000572	Efflux pump conferring antibiotic resistance	No	N/A
mtrR	ACF30254.1	ARO:3000817	Efflux pump conferring antibiotic resistance	No	N/A
abaQ	ACJ41547.2	ARO:3004574	Efflux pump conferring antibiotic resistance	No	N/A
VEB-7	ACO56763.1	ARO:3002376	Beta-lactam resistance	No	N/A
PDC-7	ACQ82812.1	ARO:3002506	Beta-lactam resistance	No	N/A
amvA	ACQ82816.1	ARO:3004577	Efflux pump conferring antibiotic resistance	No	N/A
lmrP	ABF33001.1	ARO:3003969	Efflux pump conferring antibiotic resistance	No	N/A
abaF	ABO11759.2	ARO:3004573	Efflux pump conferring antibiotic resistance	No	N/A
ermR	AAU93796.1	ARO:3000594	Macrolide-lincosamide-streptogramin resistance	No	N/A
CfxA4	AAV37205.1	ARO:3003005	Beta-lactam resistance	No	N/A
tet(38)	AAV80464.1	ARO:3000565	Tetracycline resistance	No	N/A
lmrS	AAW38464.1	ARO:3004572	Efflux pump conferring antibiotic resistance	No	N/A
lnuC	AAY32951.1	ARO:3002837	Lincosamide resistance	No	N/A
mgtA	ABA28305.2	ARO:3000462	Macrolide resistance	No	N/A
oleI	ABA42118.2	ARO:3000866	Macrolide resistance	No	N/A
oleD	ABA42119.2	ARO:3000865	Macrolide resistance	No	N/A
catB9	AAL68645.1	ARO:3002681	Phenicol resistance	No	N/A
dfrC	AAO04716.1	ARO:3002865	Diaminopyrimidine resistance	No	N/A
oqxA	AAP43109.1	ARO:3003922	Fluoroquinolone resistance	No	N/A
omp38	AAP82271	ARO:3004123	Beta-lactam resistance	No	N/A
tet(41)	AAP93922.1	ARO:3000569	Efflux pump conferring antibiotic resistance	No	N/A
qacB	AAQ10694.1	ARO:3003047	Efflux pump conferring antibiotic resistance	No	N/A
OXA-50	AAQ76277.1	ARO:3001796	Beta-lactam resistance	No	N/A
otrC	AAR96051.1	ARO:3002894	Efflux pump conferring antibiotic resistance	No	N/A
norA	AAS68233.1	ARO:3000391	Efflux pump conferring antibiotic resistance	No	N/A
chrB	AAS79458.1	ARO:3001302	Macrolide-lincosamide resistance	No	N/A
mexV	AAG07762.1	ARO:3003030	Efflux pump conferring antibiotic resistance	No	N/A
mexW	AAG07763.1	ARO:3003031	Efflux pump conferring antibiotic resistance	No	N/A
opmH	AAG08359.1	ARO:3003682	Efflux pump conferring antibiotic resistance	No	N/A
emrE	AAG08375.1	ARO:3004038	Efflux pump conferring antibiotic resistance	No	N/A

mphB	AAG57600.1	ARO:3000318	Macrolide resistance	No	N/A
msrC	AAK01167.1	ARO:3002819	Macrolide-lincosamide-streptogramin resistance	No	N/A
tet(35)	AAK37619.1	ARO:3000481	Efflux pump conferring antibiotic resistance	No	N/A
APH(2'')-IIa	AAK63040.1	ARO:3002635	Aminoglycoside resistance	No	N/A
emtA	AAL02176.1	ARO:3004669	Macrolide- lincosamide resistance	No	N/A
fusB	AAL12234.1	ARO:3003552	Fusidic acid resistance	No	N/A
golS	AAL19308.1	ARO:3000504	Efflux pump conferring antibiotic resistance	No	N/A
BlaEC family class C beta-lactamanse	AAC77110.1	ARO:3004290	Beta-lactam resistance	No	N/A
otr(B)	AAD04032.1	ARO:3002892	Tetracycline resistance	No	N/A
mef(En2)	AAF74725.1	ARO:3004659	Macrolide resistance	No	N/A
ermB	AAF86219.1	ARO:3000375	Macrolide-lincosamide-streptogramin resistance	No	N/A
triC	AAG03548.1	ARO:3003681	Triclosan resistance	No	N/A
mexA	AAG03814.1	ARO:3000377	Efflux pump conferring antibiotic resistance	No	N/A
catB7	AAG04095.1	ARO:3002679	Phenicol resistance	No	N/A
fosA	AAG04518.1	ARO:3000149	Fosfomycin resistance	No	N/A
muxA	AAG05916.1	ARO:3004073	Efflux pump conferring antibiotic resistance	No	N/A
opmD	AAG07595.1	ARO:3000809	Efflux pump conferring antibiotic resistance	No	N/A
ermX	AAA98484.1	ARO:3000596	Macrolide-lincosamide-streptogramin resistance	No	N/A
cmlv	AAB36568.1	ARO:3002700	Phenicol resistance	No	N/A
oprJ	AAB41958.1	ARO:3000802	Efflux pump conferring antibiotic resistance	No	N/A
AAC(6')-Ii	AAB63533.1	ARO:3002556	Aminoglycoside resistance	No	N/A
tet(V)	AAB84282.1	ARO:3000181	Tetracycline resistance	No	N/A
blt	AAC36944.1	ARO:3003006	Efflux pump conferring antibiotic resistance	No	N/A
cmrA	AAC45805.1	ARO:3002702	Phenicol resistance	No	N/A
rpoB mutants	A1A317	ARO:3004480	Rifamycin resistance	No	N/A
catA4	AAA25655.1	ARO:3004657	Phenicol resistance	No	N/A
aadS	AAA27459.1	ARO:3004683	Aminoglycoside resistance	No	N/A
ermF	AAA88675.1	ARO:3000498	Macrolide-lincosamide-streptogramin resistance	No	N/A

Table S4: Antibiotic use according to class of antibiotic during study period and annual hospital use 2017-2018.

Class of antibiotic	% of usage
During study period	
Cephalosporins	23·7
Anti-TB agents (first and second line)	20·3
Fluoroquinolones	16·9
Penicillins and combination penicillin	13·6
Combination cephalosporins	8·5
Nitroimidazoles	5·1
Aminoglycosides	3·4
Carbapenems	3·4
Rifaximin	3·4
Macrolides	1·7
Annual hospital use	
Penicillins and combination penicillins	35
Cephalosporins	25
Co-trimoxazole	12
Anti-TB agents (first and second line)	10
Fluoroquinolones	6
Macrolides	3
Rifamycins	3
Lincomycins	2·4
Aminoglycosides	2
Carbapenems	1
Glycopeptides	0·3
Others	0·3

Table S5: Significant correlations between detected AMR genes and bacterial taxa (n=24 patients).

Gene	Antibiotic resistance	Gene Regulator	Phylum: Taxa	Spearman's r	P value
ACI-1 †	Beta-lactam resistance	No	F: *Ruminococcaceae UCG-014* *	0.83	<0.01
basS	Peptide antibiotic resistance	No	F: *Erysipelotrichaceae UCG-003* *	0.74	<0.01
blaR1 †	Beta-lactam resistance	Yes	F: *Ruminiclostridium 6* * F: *Megamonas* * F: *Ruminococcus 1* * A: *Enterorhabdus* *	0.65 0.80 0.84 0.84	0.03 <0.01 <0.01 <0.01
catB10	Phenicol resistance	No	F: *Ruminococcaceae NK4A214 group* ** F: *Ruminiclostridium 6* **	0.76 0.79	<0.01 <0.01
catV	Phenicol resistance	No	F: *Subdoligranulum* * F: *Megamonas* * A: *Enterorhabdus* ** F: *Ruminococcus 1* *	0.65 0.67 0.74 0.74	0.04 0.03 <0.01 <0.01
cblA-1	Beta-lactam resistance	No	A: *Olsenella* **	0.81	<0.01
clbA	Macrolide-lincosamide-linezolid-phenicol-streptogramin resistance	No	B: *Prevotella 7* *	0.68	0.02
dfrA1	Diaminopyrimidine resistance	No	A: *Olsenella* ** F: *Clostridioides* ** F: *Moryella* **	0.66 0.74 0.70	0.03 <0.01 0.13
dfrA15	Diaminopyrimidine resistance	No	A: *Senegalimassilia* **	0.65	0.04
farA	Efflux pump conferring antibiotic resistance	No	P: *Desulfovibrio* * F: *Peptococcus* ** F: *Lachnospiraceae NK4A136 group* ** P: Burkholderiaceae_unclassified * F: *Ruminococcaceae UCG-004* ** B: *Barnesiella* * F: *Oscillibacter* **	0.64 0.64 0.76 0.78 0.83 0.79 0.83	0.04 0.04 <0.01 <0.01 <0.01 <0.01 <0.01
fexA †	Efflux pump conferring antibiotic resistance	No	F: *Tyzzerella 4* * F: *(Ruminococcus) torques group* *	0.67 0.66	0.02 0.02
lnuA †	Lincosamide resistance	No	F: *Weissella* * F: *Ruminococcaceae.NK4A214 group* *	0.71 0.68	<0.01 0.02
mcr-1	Peptide antibiotic resistance	No	F: *Clostridioides* **	0.83	<0.01

mef(B) †	Macrolide resistance	No	F: *Ruminococcaceae NK4A214 group* *	0.65	0.03
mexL	Efflux pump conferring antibiotic resistance		F: *Ruminococcaceae UCG-002* **	0.66	0.03
nmcr	Beta-lactam resistance	Yes	F: *Peptostreptococcus* *	0.73	<0.01
opmE	Efflux pump conferring antibiotic resistance	No	B: *Alloprevotella* * F: *Lachnospiraceae UCG-004* *	0.75 0.78	<0.01 <0.01
oprA	Efflux pump conferring antibiotic resistance	No	F: *Dielma* *	0.86	<0.01
optrA †	Mactrolide-streptogramin-lincosamide resistance	No	F: *Catenibacterium* *	0.65	0.04
OXA-181	Beta-lactam resistance	No	F: *Dielma* *	0.68	0.02
pgpB	Peptide antibiotic resistance	No	B: *Prevotella 9* *	0.64	0.04
poxtA	Macrolide-lincosamide-streptogramin resistance	No	A: *Corynebacterium* *	-0.64	0.04
qacB	Efflux pump conferring antibiotic resistance	No	F: *Dielma* *	0.74	<0.01
salA	Lincosamide-streptogramin resistance	No	F: *Hungatella* * F: Family XIII_unclassified *	0.68 0.75	0.02 <0.01
sat1 †	Nucleoside antibiotic resistance	No	F: *Clostridioides* * F: *Moryella* *	0.81 0.64	<0.01 0.04
smeD	Efflux pump conferring antibiotic resistance	No	F: *Megasphaera* **	0.69	0.02
sul3	Sulfonamide resistance	No	F: *Erysipelatoclostridium* **	0.64	0.04
tetA(46)	Efflux pump conferring antibiotic resistance	No	A: *Collinsella* *	0.68	0.02
tetA(60)	Efflux pump conferring antibiotic resistance	No	F: *Mogibacterium* *	0.64	0.04
tetA(P) †	Efflux pump conferring antibiotic resistance	No	F: *Murdochiella* *	0.77	<0.01
tetB(48)	Efflux pump conferring antibiotic resistance	No	F: *Ruminococcaceae NK4A214 group* *	0.65	0.03
tet(D)	Efflux pump conferring antibiotic resistance	No	F: *(Ruminococcus) torques group* ** F: *Moryella* **	0.70 0.64	0.01 0.04
tet32	Tetracycline resistance	No	F: *Subdoligranulum* *	0.64	0.04
tlrC	Efflux pump conferring antibiotic resistance	No	B: *Prevotella 7* ** F: Family XIII_unclassified *	0.71 0.68	0.01 0.02

tmrB	Nucleoside antibiotic resistance	No	F: Peptostreptococcaceae_unclassified *	0.67	0.02
vanE	Glycopeptide resistance	No	B: *Prevotella 9* **	0.65	0.03
vanHA	Glycopeptide resistance	No	F: *Catenibacterium* *	0.81	<0.01
vanO	Glycopeptide resistance	No	F: *Megamonas* *	0.79	<0.01
vanRD	Glycopeptide resistance	No	F: *Subdoligranulum* *	0.76	<0.01
vanSA	Glycopeptide resistance	No	A: Corynebacteriaceae_unclassified *	0.64	0.04
vanSM	Glycopeptide resistance	No	F: *Megasphaera* *	0.65	0.04
vanSN	Glycopeptide resistance	No	F: *Catenibacterium* * F: *Ruminococcaceae NK4A214 group* * F: *Ruminiclostridium 6* *	0.67 0.68 0.68	0.03 0.02 0.02
vanWB	Glycopeptide resistance	No	F: *Megamonas* *	0.67	0.02
vanWG	Glycopeptide resistance	No	F: *Lachnospiraceae NK4A136 group* * F: *Megamonas* *	0.64 0.74	0.04 <0.01
vgaC †	Mactrolide-streptogramin-lincosamide resistance	No	F: *Ruminococcus 1* * A: *Enterorhabdus* *	0.65 0.65	0.04 0.04
vgaE †	Mactrolide-streptogramin-lincosamide resistance	No	P: *Desulfovibrio* ** F: *Ruminococcus* * F: *Ruminiclostridium 6* * B: *Odoribacter* ** F: *Solobacterium* *	0.64 0.83 0.64 0.79 0.84	0.04 <0.01 0.04 <0.01 <0.01

Phylum: F- Firmicutes; A-Actinobacteria; B- Bacteroidetes; P- Proteobacteria

* Possible association/No data with resistance gene

** No association with resistance gene

† Evidence of gene mobility

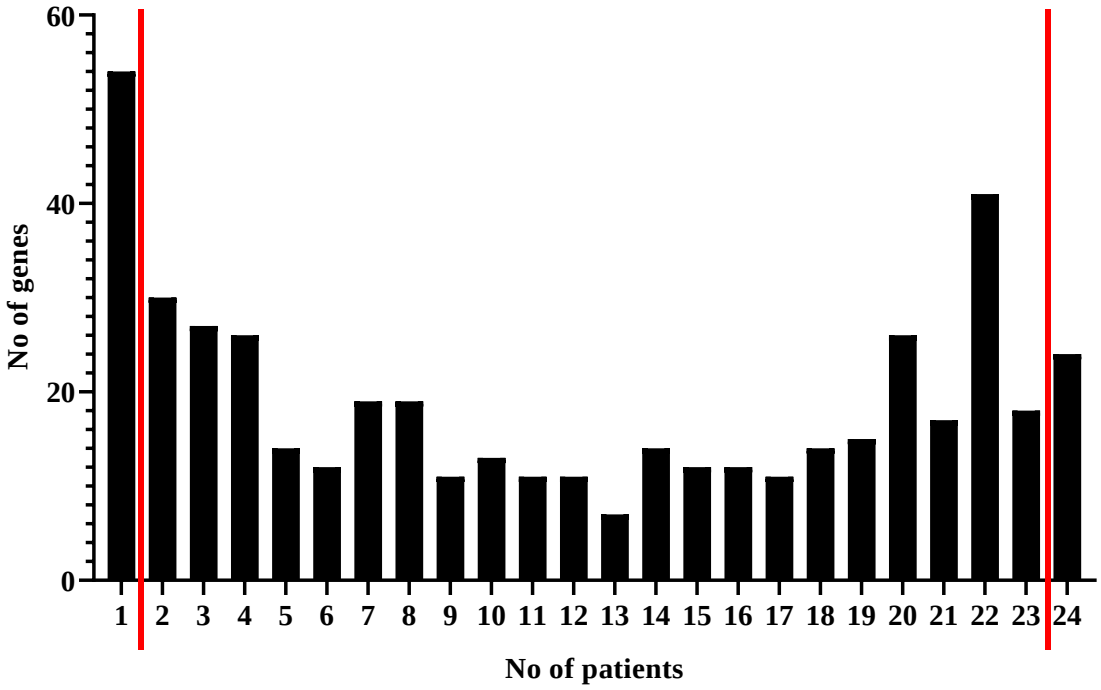


Figure S3: Distribution of detected resistance genes within patient cohort. The number of patients (of a total of 24) in whom a specific number of resistance genes were detected is shown. Genes that detected in all patients, or in only a single individual only, were excluded from analysis of potential inter-patient transmission.

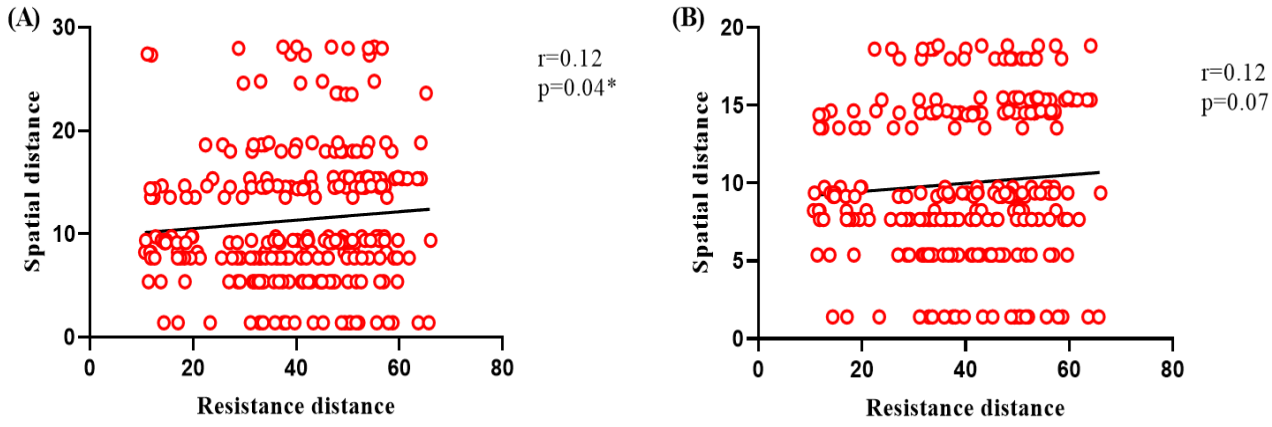


Figure S4: Spearman's correlation of spatial distance between individuals located on the ward (spatial distance) and resistance gene presence/absence similarity (resistance distance). (A) Resistance genes present in patients located within the bay and corridor (B) resistance genes present in patients located within the bay.

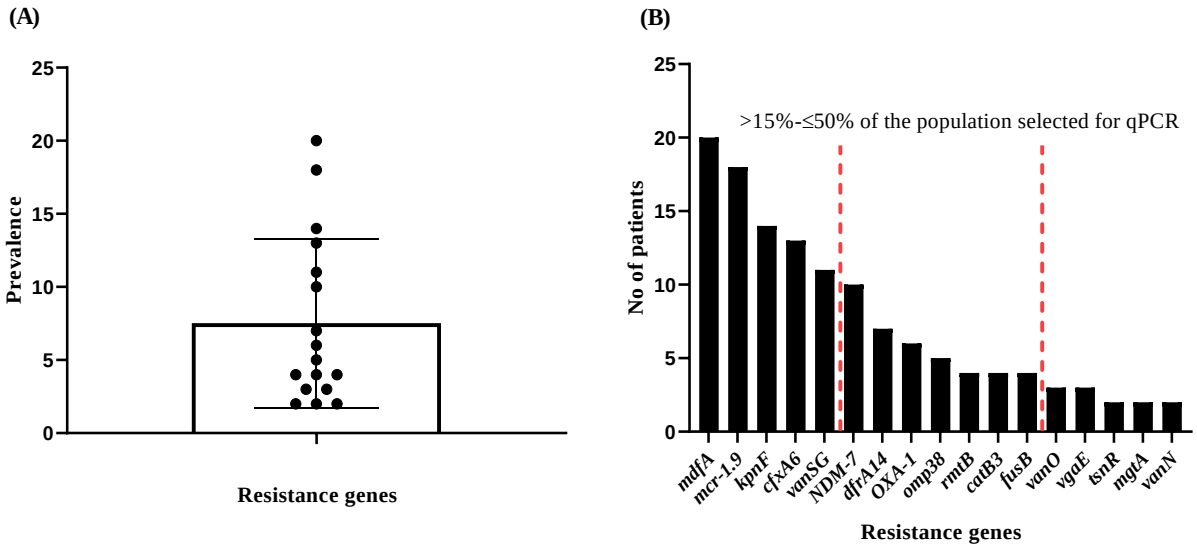


Figure S5: (A) Overall distribution of resistance genes identified through DISTLM according to their prevalence (B) Prevalence of individual genes as detected by metagenomic analysis. Genes present in >15% and <50% were selected for validation by quantitative PCR.

Table S6: Proportion of genes detected in shotgun metagenomic sequencing and qPCR analysis.

Gene	Proportion detected in shotgun metagenomics (n=24)	Proportion detected in qPCR (n=59)
OXA-1	25·0%	24·0%
CTX-M-14	16·7%	25·4%
CMY-2	45·8%	31·0%
mcr-1.0	12·5%	5·1%
NDM-7	41·7%	30·5%
drfA14	29·2%	28·8%
catB3	16·7%	22·0%
fusB	16·7%	40·7%
rmtB	16·7%	15·3%

Table S7: Distribution of resistance genes according to patient location on the ward.

Location	***OXA-1***	***NDM-7***	***mcr-1·0***	***CTX-M-14***	***CMY-2***	***dfrA14***	***catB3***	***fusB***	***rmtB***
Private rooms	0	0	0	0	0	50·0%	0	0	0
Bay 1	11·0%	0	0	22·2%	11·0%	22·2%	11·0%	66·7%	0
Bay 2	0	37·5%	0	25·0%	37·5%	0	0	25·0%	12·5%
HDU	0	25·0%	25·0%	0	25·0%	0	0	0	25·0%
Bay 4	12·5%	0	0	37·5%	12·5%	0	0	25·0%	0
Bay 5	50·0%	75·0%	25·0%	50·0%	100·0%	50·0%	50·0%	75·0%	25·0%
Bay 6	28·6%	28·6%	14·3%	14·3%	14·3%	42·9%	28·6%	57·1%	14·3%
Bay 7	66·7%	50·0%	0	33·3%	33·3%	50·0%	66·7%	83·3%	50·0%
Bay 8	50·0%	75·0%	0	0	75·0%	50·0%	50·0%	50·0%	25·0%
Patients within corridor	28·6%	42·9%	0	42·9%	28·6%	57·1%	28·6%	0	14·3%

Table S8: Univariate analysis of clinical variables and resistance genes.

Genes	Length of hospital stay[+]	Use of antibiotics[+]	Use of gram-negative antibiotics[+]	Patients with TB[+]	Patients with bacterial sepsis[+]	Presence of bacterial sepsis and MDR TB[+]
OXA-1	0·25	0·60	0·43	0·02	0·29	0·28
NDM-7	0·02	0·08	0·40	0·06	0·50	0·27
catB3	0·21	0·11	0·85	<0·01	0·84	0·88
dfrA14	0·28	0·31	0·20	<0·01	0·18	0·39
rmtB	0·81	0·27	0·62	0·06	0·65	0·47
fusB	0·62	0·78	0·90	0·87	0·50	0·46
CTX-M-14	0·94	0·15	0·19	0·84	0·34	0·34
CMY-2	0·04	0·03	0·19	0·51	0·08	0·10
mcr-1·0	0·35	0·96	0·95	0·96	0·93	0·94

[+]Adjusted for age

TB: tuberculosis; MDR TB: multi-drug resistant tuberculosis

Table S9: Multivariate regression analysis of clinical variables and resistance genes.

Genes	Length of hospital stay[+]	Patients with TB[+]	Patients with bacterial sepsis[+]
OXA-1	0·60	0·03	0·79
NDM-7	0·04	0·13	0·13
catB3	0·45	<0·01	0·37
dfrA14	0·72	0·02	0·58
rmtB	0·89	0·03	0·27
fusB	0·60	0·61	0·47
CTX-M-14	0·91	0·64	0·29
CMY-2	0·03	0·59	0·03
mcr-1·0	0·41	0·61	0·36

[+]Adjusted for age, length of stay, patients with TB and presence of bacterial sepsis

TB: tuberculosis

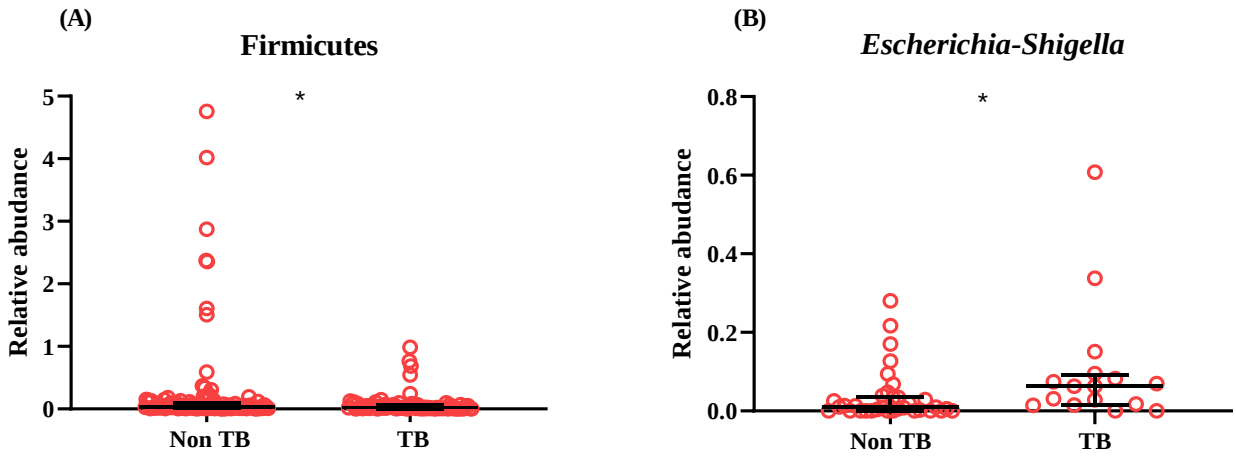


Figure S6: (A) Levels of Firmicutes between patients with and without TB (B) Levels of *Escherichia-Shigella* between patients with and without TB.

Table S10: Correlation between relative abundance of specific taxa and absolute abundance of associated AMR genes.

Taxa	Gene	Spearman r	P value
Escherichia-Shigella	*OXA-1*	0.43	<0·01
	NDM-7	0.47	<0·01
	dfrA14	0.36	<0·01
	rmtB	0.13	0.33
	catB3	0.47	<0·01
Pseudomonas sp.	*OXA-1*	-0.18	0.20
	catB3	-0.17	0.20
Acinetobacter sp.	*rmtB*	-0.02	0.86
	catB3	-0.09	0.49
Staphylococcus sp.	*fusB*	0.23	0.07
Morganella sp.	*OXA-1*	0.19	0.15
	dfrA14	-0.01	0.98
	rmtB	0.13	0.31
	catB3	0.04	0.76
Proteus sp.	*OXA-1*	-0.04	0.78
	dfrA14	0.18	0.16
	rmtB	0.05	0.72
	catB3	0.18	0.17

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Appendix 5: Statement of authorship Chapter 3

STATEMENT OF AUTHORSHIP

Title of paper	How occupancy and space-use shape the hospital microbiome: changes in environmental microbiology during transition from a major tertiary hospital to a newly built 800-bed facility
Publication Status	Under review
Publication Details	Journal of Infection

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Contribution to the paper	Concept and design of project Sample processing and analysis Bioinformatics processing and data analysis Drafting and revision of manuscript
Overall percentage (%)	80%
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	
Date	20 Feb 2021

Co-author contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

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Appendix 6: Statement of authorship Chapter 4

STATEMENT OF AUTHORSHIP

Title of paper	Transmission of antimicrobial resistance in an open-plan hospital ward: A cross-sectional metagenomic study of resistome dispersion in a lower middle-income setting.
Publication Status	Published March 2021
Publication Details	Antimicrobial Resistance and Infection Control

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