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Characterisation of common environmental healthcare associated infection pathogens and their genetic relatedness to isolates causing severe infection in a neonatal care unit at a tertiary level hospital in Soweto

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CHARACTERISATION OF COMMON ENVIRONMENTAL HEALTHCARE ASSOCIATED INFECTION PATHOGENS AND THEIR GENETIC RELATEDNESS TO ISOLATES CAUSING SEVERE INFECTION IN A NEONATAL CARE UNIT AT A TERTIARY LEVEL HOSPITAL IN SOWETO

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment for the degree of Masters of Medicine (Microbiology)

Johannesburg, 2022

CANDIDATES DECLARATION

I, Michel Le Grange declare that this research report is my own work. It is being submitted for the degree of Masters in Medicine (Microbiology) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



…….. (signature)

The 24 day of May, 2022

ABSTRACT

Introduction: Multidrug-resistant (MDR) healthcare associated infections (HAI) cause debilitating and life-threatening infections in hospitalised patients. Neonates are at high-risk for HAI. The environment may act as a reservoir for MDR HAI organisms, and evaluation for relatedness between MDR environmental organisms and clinical specimens is vital. Molecular techniques provide the best method for assessing relatedness.

Setting: Neonatal unit at a tertiary academic institution in Gauteng, South Africa

Objective: To sample high-touch environmental surfaces to determine microbial bioburden, isolate multidrug-resistant organisms (MDROs) and determine genetic relatedness of MDR environmental organism and clinical MDROs by macro-restriction analysis

Methods: In a point-prevalence environmental sampling survey of the neonatal unit, 112 environmental samples were taken and processed for microbial bioburden and the presence of MDROs. Neonatal blood culture samples of MDROs were collected over four weeks. Macro-restriction analysis was performed to determine genetic relatedness of environmental and clinical isolates

Results: Microbial bioburden demonstrated variation in the unit. Greater microbial bioburden was recorded in overcrowded cubicles. Environmental sample isolates included 52 MDROs: 5 MRSA; 4 CRE; 19 ESBL Enterobacterales, and 24 XDR *A. baumannii.* Isolation of a MDR ESKAPE organism was not related to the microbial bioburden of the surface sampled (p-value= 0.22). Genetic relatedness of environmental and clinic XDR *A. baumannii* isolates was found, however a temporal relationship was unable to be demonstrated.

Conclusion: Microbial bioburden and distribution of MDR ESKAPE organisms was described. The microbial burden and isolation of MDR ESKAPE organisms do not correlate. Organisms are transferred from high touch surfaces to infants in the unit, most likely via health care worker and caregiver hands. Genetic relatedness of environmental and clinical isolates emphasises the importance of infection prevention and control measures.

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TABLE OF CONTENTS

CONTENTS PAGE

CANDIDATES DECLARATION……...i

ABSTRACT……ii

ACKNOWLEDGEMENTS……iii

LIST OF FIGURES……vi

LIST OF IMAGES…….....vii

LIST OF TABLES……viii

ABBREVIATIONS……ix

1. INTRODUCTION……...1

1.1. General Introduction……..1

1.2. Literature Review……1

1.3 Study Aims and Objectives…….……6

2. METHODS……..…7

2.1. Setting……...7

2.2.Study Procedures……8

2.2.1 Environmental sampling methods……..9

2.2.2 Sample processing and culture methods……..12

2.2.3 Bacterial identification and antimicrobial susceptibility testing…….....15

2.2.4 Macro-restriction analysis……17

2.2.5 Data processing……18

3. RESULTS……18

3.1 Threshold of detection from environmental sampling methods……18

3.2 Environmental Sampling……19

3.2.1 Microbial bioburden……..19

3.2.2 Environmental organisms……21

3.2.3 ESKAPE + C organisms……..22

3.2.4 Distribution of MDRO isolates……25

3.2.4.1 XDR *Acinetobacter baumannii* distribution……28

3.2.4.2 MRSA distribution……..28

3.2.4.3 ESBL *Enterobacter cloacae*……28

3.2.4.4 MDRO *Klebsiella pneumoniae*……28

CONTENTS **PAGE**

3.3 Microbial bioburden and MDRO ESKAPE organisms……29

3.4 Clinical isolates……29

3.5 Macro restriction analysis……...31

3.5.1 Methicillin-resistant *Staphylococcus aureus*……..31

3.5.2 Extended-spectrum beta-lactamase producing *Klebsiella pneumoniae*…….33

3.5.3 Extensively drug-resistant *Acinetobacter baumannii*……..35

4. DISCUSSION……..38

4.1 Limit of detection from environmental sampling methods……..38

4.2 Environmental Sampling……..39

4.2.1 Distribution of MDRO isolates…….42

4.2.2 Microbial bioburden and MDRO ESKAPE organisms…….44

4.3 Macro-restriction analysis……45

4.3.1 Methicillin-resistant *Staphylococcus aureus*……45

4.3.2 Extended-spectrum beta-lactamase producing *Klebsiella pneumoniae*……..46

4.3.3 Extensively drug-resistant *Acinetobacter baumannii*……..46

4.4. Limitations…..……48

5. CONCLUSION……50

6. APPENDICES……51

Appendix 1 - Ethics clearance letter……51

Appendix 2 - Environmental Sampling Taking Log……...52

Appendix 3 - Quality control template and expected outcomes…….....53

Appendix 4 - Standard operating procedure – Pulsed-field gel electrophoresis for MRSA……..54

Appendix 5 - Standard operating procedure – Pulsed-field gel electrophoresis for Enterobacterales……57

Appendix 6 - Standard operating procedure – Pulsed-field gel electrophoresis for *Acinetobacter* baumannii……60

Appendix 7 - Consolidated data sheet……63

Appendix 8 - Working card for processing environmental sample…….67

7. REFERENCES……...69

LIST OF FIGURES

FIGURE **PAGE**

Figure 3.1 Spatial distribution of MDR ESKAPE isolates in the neonatal unit……..27

Figure 3.2 Line diagram showing date of collection of clinical isolates..…….30

LIST OF IMAGES

IMAGE **PAGE**

Image 3.1 MRSA PFGE Gel…….32

Image 3.2 ESBL *Klebsiella pneumoniae* PFGE gel……..34

Image 3.3 XDR *Acinetobacter baumannii* PFGE Gel……...37

LIST OF TABLES

TABLE NUMBER PAGE

Table 1.1 Survival of hospital pathogens on dry hospital surfaces……4

Table 2.1 Morphology and rapid test identification of the organism…….15

Table 2.2 Criteria for interpreting PFGE patterns……18

Table 3.1 Summary of overall microbial bioburden in the unit……..20

Table 3.2 Microbial Bioburden in the NICU and TICU……20

Table 3.3 Microbial bioburden in different TICU cubicles……..20

Table 3.4 Microbial bioburden of basin surfaces……21

Table 3.5 Number of ESKAPE+C organisms isolated……22

Table 3.6 MDR ESKAPE organism resistance pattern……..23

Table 3.7 CRE *Klebsiella pneumoniae* antibiotic susceptibility results……23

Table 3.8 ESBL *Klebsiella pneumoniae* antibiotic susceptibility results…..24

Table 3.9 ESBL *Enterobacter cloacae* antibiotic susceptibility results…….24

Table 3.10 XDR *Acinetobacter baumannii* antibiotic susceptibility results..24

Table 3.11 Microbial bioburden of samples that isolated MDR ESKAPE organisms vs. samples that did not isolate MDR ESKAPE organisms……..29

Table 3.12 Microbial bioburden stratification and MDRO isolation……...29

Table 3.13 List of MDRO clinical isolates……30

Table 3.14 List of PFGE isolates for MRSA isolates……..31

Table 3.15 List of PFGE isolates for ESBL *Klebsiella pneumoniae* ……33

Table 3.16 List of PFGE isolates for XDR *Acinetobacter baumannii* ……..36

ABBREVIATIONS

°C: degrees Celsius

µl: microlitre

ASM: American Society of Microbiology

CA: California

CEO: Chief Executive Officer

cfu: colony forming unit

CLSI: Clinical Laboratory and Standards Institute

cm^2: centimetre squared

CMID: Clinical Microbiology and Infectious Diseases

CPAP: Continuous positive airway pressure

CRE: Carbapenem-resistant Enterobacterales

DMP: Diagnostic Medical Products

DNA: Deoxyribonucleic acid

ESBL: Extended spectrum beta-lactamase

ESKAPE: *Enterococcus* spp, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter* spp

ESKAPE+C: *Enterococcus* spp, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter* spp and *Candida auris*

GERMS-SA: Group for Enteric, Respiratory and Meningeal Diseases Surveillance in South Africa

HAI: Hospital Acquired Infection

ICSL: Infection Control Services Laboratory

ICU: Intensive care unit

ivi: intravenous injection

IDSA: Infectious Disease Society of America

MDR: Multidrug-resistant

MDRO: Multidrug-resistant organism

MIC: Minimum inhibitory concentration

ml: millilitre

MRSA: Methicillin-resistant *Staphylococcus aureus*

N/A: Not available

NICD: National Institute of Communicable Diseases

NICU: Neonatal Intensive Care Unit

NLF: Non-lactose fermenter

PFGE: Pulsed-field gel electrophoresis

ppm: parts per million

R: Resistant

S: Susceptible

SDD: Susceptible Dose Dependant

TICU: Transitional Intensive Care Unit

TNTC: Too numerous to count

USA: United States of America

VRE: Vancomycin Resistant Enterococci

WGS: Whole genome sequencing

Wits: University of the Witwatersrand

XDR: Extensively drug-resistant

1. INTRODUCTION

1.1 General Introduction

Healthcare associated infections (HAI) are considered the most frequent adverse events threatening patient's safety worldwide, causing significant morbidity and mortality.(1) Endemic HAI represents a major burden and safety issue for patients in resource-constrained countries compared to developed countries.(1) Not only do patients that acquire HAI have a higher mortality risk, but those that survive have longer hospital admission, further increasing health care costs.(2) Several factors promote the acquisition of infections in hospitalised patients. These include decreased immunity, prolonged hospital stay, especially for preterm infants, increasing variety of invasive medical procedures, overcrowding, and poor adherence to infection prevention and control (IPC) practices.(1,2) In one review, 75% of paediatric HAI bloodstream infections were caused by *Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Enterococcus* spp*, Acinetobacter* spp and *Pseudomonas aeruginosa.*(2) Hospital environmental surfaces are often contaminated with microorganisms, and these surfaces can act as a reservoir and vehicles for the transfer of HAI pathogens.(3)

1.2 Literature review

The burden of multidrug-resistant organisms (MDROs) has increased worldwide.(4) MDROs are defined as organisms exhibiting microbial antibiotic resistance to at least one antibiotic in three or more antimicrobial categories.(5) There is a high prevalence of multidrug resistance amongst the "ESKAPE" bacteria, defined by the Infectious Diseases Society of America (IDSA) as *Enterococcus* spp*, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.(4) There is a significant prevalence of MDR ESKAPE organisms in patients who acquire an HAI in South Africa's healthcare facilities.(6) In the most recently available GERMS-SA (Group for Enteric, Respiratory and Meningeal Diseases Surveillance in South Africa) for 2019, 20% of all carbapenem-resistant Enterobacterales bacteraemia occurred in infants less than one year of age, and 40% of all *Acinetobacter baumannii* bacteraemia occurred in infants less than one year of age.(7)

Candida auris is an emerging pathogenic fungus, with some isolates found to be resistant to all three major classes of antifungal agents.(8) *Candida auris* invasive infections are associated with high mortality.(8) *Candida auris* was first identified in South Africa in 2009. Since then, laboratory-based fungal surveillance has noted a sustained and real increase in cases at the national reference mycology laboratory at the National Institute of Communicable Diseases (NICD).(9,10) The last published results indicated that *Candida auris* is the third most common cause of candidaemia in South Africa.(10) Prior to the emergence of *Candida auris,* there were multiple undetected outbreaks of azole-resistant *Candida parapsilosis* in neonatal intensive care units (NICU) in the Gauteng province.(11) The ability of *Candida auris* strains to survive on dry and moist surfaces and further whole genome sequencing investigations between environmental and clinical isolates suggests that transmission from the environment is possible.(8,12) *Candida* spp often contaminate floor surfaces and sink drains.(12)

The transmission of pathogens causing HAI occurs via three methods: contact, droplet and airborne.(13) Contaminated environmental surfaces are involved in contact spread. Direct contact spread involves direct physical transfer of microorganisms from person to person, which may be from patient to patient or healthcare worker to patient.(13) Indirect, direct spread involves contact with an inanimate object or hospital surface contaminated with microorganisms.(13) The healthcare worker may act as an intermediary between the contaminated surface and the patient. For many years it was thought that the environment did not contribute to the hospital acquisition of HAI.(14) This was due to historic studies showing that HAI rates were not associated with the levels of the general microbial contamination. Currently, there is no consensus on acceptable levels of microbial contamination of environmental surfaces in health care settings.(15) There is increasing evidence from various sources suggesting that contaminated surfaces play a role in HAI transmission.(14) These suggest an increased risk of acquiring a HAI with vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA)*, Acinetobacter* spp, *Pseudomonas* spp or *Clostridioides difficile* infection if admitted to a room that was previously occupied by a patient that was known to be infected or colonised with one of these pathogens.(16) Factors that influence the transfer of microorganisms onto surfaces and cross-contamination are type of

organism, source and destination surfaces, moisture level and size of the inoculum.(13)

Microorganisms are shed into the hospital environment from infected or colonised patients and staff.(17) Patients are the primary source of contamination; therefore, surfaces close to the patient and that are touched frequently by healthcare workers have a higher frequency of contamination. These are the so-called high-touch surfaces from which HAI may spread via healthcare workers. Infected patients shed more than colonised patients.(17)

The concentration of pathogens causing HAI are often much less present in the environment than that present on the patient (1-100 cfu/cm^2 vs 10^3 cfu/cm^2 on skin), and special enrichment techniques are required to culture them.(17) The mere presence of a HAI pathogen on a surface does not automatically result in infection transmission, but the infectious dose of most HAI pathogens seems to be relatively low; therefore, the presence of a pathogen at any concentration may pose a risk.(17) In 2004, Dancer *et al* proposed a standard in health care settings of a total aerobic colony count of <5 cfu/cm^2 for hand-touch surface cleanliness.(18) This was extrapolated from the United States Department of Agriculture and international food standards of food preparation surfaces. In addition, they proposed that the isolation of select indicator organisms at any number should generate an evaluation and intervention of hospital surface cleaning.(18) The indicator organisms suggested were MRSA, *Clostridioides difficile*, VRE, and select Gram negative organisms.(18) The basis of an aerobic colony count of >5 cfu/cm^2 for hand-touched surfaces was based on three suppositions. The first is that a high aerobic colony count suggests inadequate environmental cleaning, the second that a heavy microbial bioburden may mask the identification of an indicator organism and thirdly, a heavy concentration of an environmental organism that is epidemiologically linked to a pathogen, may suggest an increased likelihood of the presence of the pathogen (e.g. coagulase-negative staphylococci and *Staphylococcus aureus*).(18) This standard does not take into account the patient susceptibility to infection or cleanliness requirements of different hospital environments(19). The lack of true knowledge about the hospital environmental microbial bioburden on surfaces leads to difficulty assessing the achievable microbial bioburden that will impact patient safety and assessing cleaning and disinfecting procedures to reduce microbial bioburden(20).

Microbiological studies have shown that important hospital pathogens can survive for long periods on surfaces in the right conditions, and some pathogens may persist even after appropriate cleaning of surfaces.(14)

Table 1.1 Survival of hospital pathogens on dry hospital surfaces

ORGANISM	SURVIVAL TIME
Acinetobacter spp	3 days to 11 months
Enterococcus spp including VRE	5 days to >46 months
Pseudomonas aeruginosa	6 hours to 16 months
Klebsiella spp	2 hours to >30 months
Staphylococcus aureus including MRSA	7 days to >12 months

(Adapted from Otter *et al*, 2013)

Hospital surfaces may serve as a source for HAI with MDROs. A study in Malaysia looking at the distribution and antibiotic resistance in clinically important bacteria in the environment found genetically related MDR *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter* spp. and *Enterobacter* spp*,* from various sampling sites.(21) In the neonatal ICU, predominantly *Acinetobacter* spp and *K. pneumoniae* were isolated.(21) In units where carbapenem-resistant *Acinetobacter baumannii* was endemic, environmental contamination was frequent if patients were also colonised/infected.(22) In a study in a respiratory ICU, almost 40% of environmental and clinical samples of *Acinetobacter baumannii* isolates were found to be in the same cluster on pulsed-field gel electrophoresis.(23) Higher proportions of potentially pathogenic organisms are isolated from sanitary facilities.(19) There is a poorly understood and under-appreciated association between pathogens that survive and persist better in water sources and HAI infections. The minimisation of exposure to hospital tap water is recommended.(24) Exposure to water aerosols from sinks in patients' wards, may act as a source of contamination and patient colonisation. Hopman *et al* found a reduction in patient colonisation with Gram negative bacilli after removing sinks from patient areas and introducing "water-free" patient care.(25) An investigation of a neonatal *Pseudomonas aeruginosa* outbreak in Northern Ireland demonstrated organisms in biofilms in the plumbing could serve as a reservoir for an outbreak strain of *Pseudomonas aeruginosa.*(26) The more complex the plumbing system, the more likely the isolation of biofilm and organisms,(26) Rethinking of the layout of plumbing and water drainage systems in the hospital built environment may be required to prevent hospital waterborne HAI infections.(27)

Evidence on how to sample hospital environmental surfaces are inconsistent, and there is no guidance or legislation on how to proceed.(28) A review of sampling techniques found that MDRO and clinically significant microorganisms are frequently isolated from hospital environmental surfaces and may therefore represent a risk to vulnerable patients.(28) It is difficult to draw conclusions on environmental sampling due to the many variables present. Different swab materials showed inconsistent results for the isolation of bacteria.(29) Other variables that affect environmental sampling include: the technique used, use of wetting agents, transport media, neutralising broths, processing techniques, organisms targeted and the environment.(28) Swabs and sponges are the most commonly used environmental sampling materials in both healthcare and non-healthcare settings.(28) Swabs and sponges are cost-effective, can sample various surface types and irregular surfaces, variable surface sizes, pick up a variety of organisms and work over a range of microbial burden levels.(28) Disadvantages of swabs and sponges include variability in sampling, area sampled, pressure during sampling and swab material. Swabs and sponges also require an extraction step in processing and suffer from variability in the recovery of viable organisms from the sampled surfaces.(28) Sample extraction is affected by the solution used for extraction and the method used.(28) Vortexing has been demonstrated to have good extraction recovery for swabs, and agitation is a viable method for sponges.(28) Sample enrichment in a nutrient broth is helpful for slower-growing organisms, stressed organisms, and to recover specific organisms from swab or sponge samples.(28) Targeting for specific organisms requires inoculation of the enriched broth onto selective media.(28) The use of an enrichment broth only allows the determination of whether an organism is present or absent.(28) Many environmental sampling techniques are only evaluated in the laboratory setting.(28) Colony forming units (cfu) per cm^2, often do not reflect the true risk to the patient, as studies show that surfaces with the highest bioburden are not always the surfaces with the most MDRO.(28)

Linking microorganisms from environmental samples with clinical isolates by molecular epidemiology is important whenever it is possible to do so.(15) Macro-restriction analysis by pulsed-field gel electrophoresis (PFGE) is a powerful genotyping technique used to determine the relatedness of a group of bacterial isolates.(30) PFGE is used to separate large DNA fragments after digestion with a unique restriction enzyme and applying it to a gel matrix under an electric field that

periodically changes direction. It provides a good representation of the entire bacterial chromosome in a single gel, with a reproducible restriction profile.(31,32) The PFGE profile must be interpreted taking into account environmental factors and the epidemiological window at the time of sampling.(33) Point mutations, insertions, deletions, and loss or acquisition of plasmids account for fragment differences in different profiles.(34)

Advances in whole genome sequencing (WGS) have lowered the cost and time for sequencing a bacterial genome, but costs are still high.(34) The additional need for highly trained bioinformatic staff and data processing and interpretation skills adds to cost and limits availability.(34) WGS has surpassed other molecular techniques to become the reference method for assessing the genetic relatedness of microorganisms.(33)

The neonatal unit at the tertiary level hospital where environmental sampling was performed experiences a high burden of hospital-acquired infections with MDRO isolates, particularly XDR (extensively drug-resistant) *Acinetobacter baumannii* and CRE (carbapenem-resistant Enterobacterales) *Klebsiella pneumoniae.* (Wadula J, 2021, personal communication, March)

1.3. Study Aims and Objectives

This study aimed to determine the microbiological burden of high-touch surfaces and terminally disinfected incubators of the neonatal intensive care unit (NICU) and the transitional intensive care unit (TICU) with a particular interest in MDRO "ESKAPE" organisms and *Candida auris*. These environmental samples were compared to available clinical MDRO isolates of severe infection (blood culture) for genetic relatedness by macro-restriction analysis.

The study objectives were:

- To sample high-touch surfaces, equipment and terminally cleaned incubators and determine the microbiological bioburden via quantitative culture methods.
- To identify and characterise the antimicrobial resistance of MDR ESKAPE organisms and *Candida auris* (MDR ESKAPE+C) present on the environmental surfaces sampled, even if present in low numbers.

- Identify clinical isolates of MDR ESKAPE+C organism from blood cultures and CSF cultures taken from patients admitted to NICU and TICU and temporally related to the environmental sampling period.
- To determine the possible genetic relatedness of MDR ESKAPE+C organisms from the environment and clinical samples by macro-restriction analysis using pulsed-field gel electrophoresis (PFGE).

2. METHODS

Ethics approval was granted by the Human Research Ethics Committee - Medical, of the University of the Witwatersrand. Permission to perform the study was also obtained from the postgraduate committee of the University of the Witwatersrand. Ethics approval was obtained before any study procedures commenced. The ethics approval number is M200228 (see appendix 1). Consent and approval from the unit head and the hospital CEO were also obtained.

2.1 Setting

A point-prevalence sampling survey was conducted in the Neonatal Intensive Care Unit (NICU) and the high care unit, called the Transitional Intensive Care Unit (TICU) at a tertiary level hospital in Soweto. This unit serves the population of Soweto and surrounding areas. The unit caters for the majority of 30 000 neonates born per year and requiring admission from this region, and it is one of the two referral centres for surgical services for southern Gauteng and North West provinces. The NICU and TICU share the same physical building space, with no physical barrier between the units, but only a centrally placed nursing station. The NICU offers critical ill premature and term neonates services, often requiring ventilation. The NICU has two isolation cubicles with single beds and two open cubicles. One cubicle has six beds, and the other cubicle has four beds. The TICU cares for less ill neonates and functions as a step-down facility for the NICU. Maximal ventilatory support in the TICU is continuous positive airway pressure (CPAP). The TICU has five cubicles. Four cubicles house between 8 -10 beds, and 1 cubicle is used as an isolation cubicle as needed with a maximum of 3 beds available. Each bed has its own monitor unit, with a touch screen display, individual saturation probe, heart rate monitors and blood pressure cuff. A clean new module is attached to the monitor if

the neonate is undergoing invasive monitoring. Infusion pumps are cleaned and attached to the bed or near the bed as needed. In the NICU, each neonatal bed has its own mobile multifunctional bedstand, which is colloquially known as a “trolley”. This is used as a writing surface when making notes, for the temporary placement of medication and feeds before administration and as the preparation surface for other procedures. In the TICU cubicles, only 3 of these mobile trolleys are shared amongst the patients in the cubicle. Each cubicle in both the NICU and TICU have a “medication station”, which acts as a store for unopened syringes and needles that can be used to administer medications and urgent medications (ivi fluids). The medical station also has a 200ml ivi bag (normal saline or neonatal solution) that is accessed with a new needle and syringe when fluids are needed to dilute medications administered to an infant. There are no individual vials of dilution fluid. The NICU and TICU have 51 beds, with an almost always 100% bed occupancy, and the TICU is often overcrowded, above the bed capacity of the unit. Prescribed intravenous medications for neonates in the NICU and TICU (antibiotics, infusions, etc.) are prepared by the pharmacy department in a dedicated cleanroom located outside the NICU and TICU unit entrance.

2.2 Study Procedures

The study comprised of two components: the first was a point prevalence sampling of the environment of the NICU and TICU done over 1 day on 29 July 2021. To minimise the Hawthorne effect, only the head of unit knew the date of sampling.(35) Other staff were informed on arrival on the day of sampling to avoid changes in staff behaviour before sampling. The second component was collecting clinical microbiological samples of MDR ESKAPE+C organisms cultured from blood and cerebral-spinal fluid (CSF) of patients in the NICU and TICU. Duplicate samples from the same patient were not included. The clinical sample sites were chosen because they represented severe invasive infection and were less likely to be confused with colonisation. Clinical samples were collected over 4 weeks from 5 July 2021 until 30 July 2021. This period was chosen for clinical samples to detect possible clonal transmission and a temporal relationship between clinical and environmental isolates. The date of environmental sampling and the period of collection of clinical samples was restricted due to operational requirements and temporary COVID restrictions implemented, that limited non-essential research in the hospital. It was agreed upon with the head of the unit to minimise any potential disturbance to the usual running of

the unit. Genetic relatedness was determined between environmental MDR ESKAPE+C isolates and clinical MDR ESKAPE+C isolates by macro-restriction analysis using pulsed-field gel electrophoresis (PFGE). Only MDR ESKAPE+C organisms that had greater than two environmental and one clinical isolate were processed for macro-restriction analysis using pulsed-field gel electrophoresis.

2.2.1 Environmental sampling methods

Environmental samples were collected using environmental swabs (PurBlu Swab Sampler with Hi-Cap neutralising broth 5ml; World Bioproducts, Libertyville, IL, USA) for small surfaces (≤100cm^2) and sponges (EZ Reach sponge sampler with Hi-Cap neutralising broth 10ml; World Bioproducts, Libertyville, IL, USA) for surfaces larger than 100cm^2, and multiple surfaces where applicable. The swab and sponge consisted of a biocide-free polyurethane head that resisted tearing and flaking while sampling. The Hi-Cap neutralising broth neutralises residual sanitisers, including quaternary compounds, free chlorine molecules, acidic compounds and peroxyacetic acid. There are no nutrients in the broth, and it does not promote bacterial growth, therefore representing the sampled bacterial load.

To evaluate the bacterial limit of detection for MDR ESKAPE+C of the swab and sponge sampling technique, a preliminary evaluation was performed at the Infection Control Services Laboratory (ICSL) at the University of Witwatersrand. An isolate representative of each MDR ESKAPE+C was obtained from the collection of MDR organisms stored at the ICSL. The *Candida auris* isolate was obtained from a clinical specimen isolate. Each organism was suspended in brain heart infusion (BHI) broth and incubated aerobically at 35^{o}C±2^{o}C in ambient air for 18 hours. An inoculum of each turbid broth was streaked on a 5% blood agar plate and incubated at 35^{o}C±2^{o}C for 18-24 hours for individual colony growth. For each organism, a 0,5 McFarland standard was made in normal saline. A 0,5 McFarland standard represents approximately 1-1,5x10^{8}cfu.(35) Each bacterial and fungal McFarland suspension was serially diluted by a factor of 10, to a dilution of 1x10^{-8}, representing 1cfu/ml. The serial dilutions were made by diluting 1ml of each suspension into 9ml of normal saline. One millilitre of each dilution at concentrations of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} was inoculated onto separate plastic test surfaces and allowed to dry. This was done for each organism. The surface was sampled and processed as described below. The lowest concentration at which the swab and sponge sampling detected organisms

were recorded as the expected threshold of detection. Once the threshold of detection was determined, mixed cultures were prepared and evaluated to ensure that different organisms were picked up adequately by the sampling process from mixed communities of organisms.

On the day of sampling the NICU and TICU, the head nurse and doctors of the unit were informed that environmental sampling would be occurring, to be aware of who we were and the reason for our presence. Sampling was done to ensure that there was no to minimal disruption to the normal work of the unit and no patient interaction. Before sampling, hand hygiene was practised, and sterile gloves and a plastic apron were worn. Repeat hand hygiene; new gloves and apron were applied when moving to a new cubicle.

Environmental sampling was targeted at high-touch surfaces and areas where potential environmental contamination may occur. High-touch surfaces near the neonate were sampled as a composite with one sponge. The high-touch surfaces per composite sponge sample included: patient monitors screens, infusion pumps and ventilators or CPAP machines in use. In the NICU, multifunctional trolleys were included with the composite of each neonate, as each neonate had its own trolley. All these high-touch surfaces were within proximity to the neonate and could be reached and touched by a healthcare worker while attending the neonate. An arbitrary distance of 1 metre was used to delineate the extent of proximity. The inside of incubators and cots were not sampled, nor were monitoring devices attached to the patient, as these would most likely represent the organisms colonising the neonate.

Clean incubators that had been terminally disinfected were sampled to evaluate residual microbial bioburden post terminal disinfection. Terminal disinfection refers to the cleaning and disinfection processes undertaken on an incubator after a neonate has been discharged. As per the unit's protocol, terminal disinfection of incubators included removing the incubator from the unit and washing the incubator with warm water and detergent to remove any gross contamination with body fluids. After rinsing and drying the incubator, it was then wiped down with a hypochlorite solution with 5000ppm concentration and again allowed to dry. After this step, the incubators are cleaned with a commercial sporicidal wipe (Clinell sporicidal wipes; Gama Healthcare, Watford, UK). After terminal cleaning, the incubators are left in a passage to be taken for use when the next patient is admitted.

Common use areas, with potential for environmental contamination, in each sampled cubicle included: medication station, mobile trolleys, the hand basin and drain, tap handles, the water outlet faucet and hand wash bottles (soap bottles and alcohol-based hand sanitisers). Once-off common area environmental sampling for possible contamination included: the nursing station, the probe of the ultrasound machine, blood glucose meter and the door handle to the unit's entrance. The medication preparation room in the pharmacy anteroom was also sampled. This was where all intravenous injection (ivi) medications were prepared before delivery in the unit.

In total, 115 specimens were taken. Three samples were negative environmental controls, leaving 112 environmental samples (mixture of sponges and swab samples) representative of environmental sampling. Environmental negative controls were taken by opening the swab or sponge, removing it, exposing it to air (not sampling a surface) and returning it into the neutralising broth. Negative environmental controls were controlled at the beginning, middle and end of the sampling time. They were performed to exclude sample contamination.

Small areas (<100cm^2) were sampled with the PurBlu Swab Sampler with Hi-Cap neutralising broth 5ml (World Bioproducts, Libertyville, IL, USA). This swab sampler had a screw-top lid, with the tip of the swab immersed in the 5ml of neutralising broth. For both swab and sponge sampling techniques, the sampling technique protocol was adapted from the American Society of Microbiology (ASM) Clinical Microbiology Procedures Handbook, Fourth Edition, Section 13 (36) and the manufacturer's instructions. Briefly, for swab sampling, after opening the swab sampler, the excess neutralising broth was removed by wringing out the excess fluid by pressing and rotating the swab on the inside surface of the tube, above the level of the neutralising broth. The surface was then sampled, slowly rotating the moistened swab while pulling the swab in close parallel streaks, with enough pressure to cause the stick of the swab to bend and exert a force on the surface. This motion was then repeated perpendicular to the original streaks. The swab was then replaced into neutralising broth, and the test tube screwed closed. The tube was then gently shaken end to end. The samples were taken using a sterile template on flat surfaces, so a known surface area was sampled. The surface area was estimated using a grid on irregular surfaces (tap handles, sink drainage holes).

Large surface areas were sampled with EZ Reach sponge sampler with Hi-Cap neutralising broth 10ml (World Bioproducts, Libertyville, IL, USA). The sponges were prepared in sterile bags with 10ml of Hi-Cap neutralising broth. After opening the sample bag, the excess neutralising broth was squeezed out of the sponge by applying pressure from the outside of the bag. The sponge wipe was then removed by the handle, ensuring not to touch the inside of the bag to prevent contamination. The surface to be sampled was streaked with one side of the sponge, with consistent pressure. The sponge was then rotated onto its opposite side and the same area was streaked perpendicularly to the original streaks. The sponge was then placed back into the bag after removal of the handle attached to the sponge. The bag was then folded and sealed with wire ties on the top of the bag. The sponge was mixed into the neutralising broth present in the bag.

All samples were labelled and recorded on the Environmental Sampling Tracking Log (Appendix 2). The information recorded on the Environmental Sampling Tracking Log included the date, the sampler, sample method (swab or sponge), area in cm^2, cubicle, site sampled (bed and equipment), the sample episode number (ID) and the date of processing, once processed in the laboratory. All samples were taken to the laboratory on the day of sampling, and laboratory processing started.

2.2.2 Sample processing and culture methods

All samples were taken to the ICSL at the University of Witwatersrand Department of Clinical Microbiology and Infectious Diseases (CMID) on the day of sampling and processed immediately on arrival. The laboratory analysis of the samples aimed to quantify the microbial bioburden in colony-forming units per millimetre and cm^2 and identify ESKAPE+C organisms and determine resistance expressed by the organisms.

Physical dissociation methods were applied to the sponge and swab environmental samples to separate bacterial aggregates and allow for a more representative microbial count, of the original bioburden. Swab samples were vortexed for 30 seconds, and sponges were processed in the stomacher for 60 seconds. The diluent Hi-cap neutralising broth was used for further processing.

For the quantitative culture to determine the bioburden in cfu/ml and cfu/$cm^{2,}$ the agar spread plate method was used, also adapted from the ASM Clinical Microbiology

Procedures Handbook. Serial 10-fold dilutions of the diluent were made up to a 1:1000 concentration. These were made by adding 1ml of diluent to 9ml of normal saline.

- Original diluent
- 1:10 dilution (add 1ml diluent to 9ml normal saline)
- 1:100 dilution (add 1ml of 1:10 dilution to 9ml saline)
- 1:1000 dilution (add 1ml of 1:100 dilution to 9ml saline)

After preparation of the dilutions, 0.1ml of the original diluent and each dilution was inoculated onto a 5% blood agar plate surface (DMP, Sandringham, South Africa); therefore, there were four 5% blood agar plates. The inoculum was distributed on the plate surface with a sterile spreader, placed in a biological safety cabinet to dry, covered with an agar plate lid, and inverted for incubation. The plates were incubated in ambient air at 35^{o}C±2^{o}C and reviewed at 24 hours for growth. If there was no or minimal growth, the plates were incubated for a further 24 hours and reviewed for colony count. Colony counts were performed when growth was noted on the plates. Growth was considered sufficient when distinct isolated colonies could be seen. The plate and dilution that had colony growth, but less than 300 colonies were chosen, and the number of separate distinct colonies were counted. If there was greater than 300 colonies, the count was recorded as “too numerous to count”, as counts above 300 start showing areas of confluent growth and errors in counting can occur. The number of colonies counted at a specific dilution was the colony-forming units at that set dilution. Colony-forming units/millilitre (cfu/ml) was calculated using the formula:

- Cfu/ml = number of colonies counted ÷ volume inoculated (0.1ml) x dilution (at which the colonies were counted).(37)

The colony-forming units per cm^2 were calculated using the following formula:

- Cfu/cm^2 = (number of colonies counted x volume of original suspension) ÷ (total surface area x dilution factor).(37,38)

The cfu/cm^2 was rounded to the nearest whole number. Samples with less than 1 cfu/cm^2 were recorded at “<1”.

Sample enrichment in brain-heart infusion (BHI) nutrient broth was performed to enhance the growth of MDR ESKAPE+C organisms that may have been present in low numbers and to be processed on selective and differential media to separate

them from heterogeneous microbial populations present in the environmental samples. One millilitre of diluent was placed in 10ml BHI nutrient broth and incubated aerobically at 35^{o}C±2^{o}C. The broth was reviewed at 24 hours for turbidity. If the broth was still clear at 24 hours, it was incubated for a further 24 hours. If it was still clear at 48 hours, a Gram stain was performed, and if no bacteria or yeast was seen, the broth was considered negative for growth. If the broth became turbid at any time, a Gram stain was performed, and organism staining reaction and morphology were recorded. After Gram staining, a 0.1ml loop was streaked on the selective and differential media that was listed below and chosen to isolate the ESKAPE+C organisms:

- 5% blood with nalidixic acid and colistin (DMP, Sandringham, South Africa) for *Enterococcus* spp
- Commercial colorex *Staphylococcus aureus* agar (MediaMage, Roodepoort, South Africa) for *Staphylococcus. aureus*
- MacConkey agar (DMP, Sandringham, South Africa) for *Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter* spp and *Pseudomonas aeruginosa*
- Sabouraud Dextrose agar with chloromycetin (DMP, Sandringham, South Africa) for *Candida auris*

The inoculated plates were incubated aerobically at 35^{o}C±2^{o}C and reviewed after 18 to 24 hours of incubation and then inspected for the morphological features and rapid tests for the target ESKAPE+C organisms. The Sabouraud Dextrose agar plate was incubated for a further 48 hours if there was no fungal growth, and the plate was not overgrown with other bacteria at 24 hours.

There had to be at least two morphologically identical colonies present on the culture plates to ensure there were sufficient colonies to perform rapid biochemical tests (see table 2.1 below) and to plate out for growth on 5% blood agar plates. Colonies identified as possible ESKAPE+C organisms were plated onto 5% blood agar and incubated at 35^{o}C±2^{o}C before proceeding with formal identification and antimicrobial susceptibility testing.

All reagents, equipment and media were quality controlled as per procedural laboratory's standard operating procedures. Reagents, equipment, and media were within their expiration date and in good working order. See appendix 3 for the quality

control testing template. Each new batch of agar plates or reagents were quality controlled.

2.2.3 Bacterial identification and antimicrobial susceptibility testing

Organism growth on selective and differential media was compared to Gram stain findings, colony morphology and rapid tests were performed as per table 2.1

Table 2.1 Morphology and rapid test identification of the organism

Organism	Media	Colonial Morphology	Rapid Test
Staphylococcus aureus	Colorex Staph	Lilac colonies	Catalase positive Coagulase (Prolex kit*) positive
Enterococcus spp	5% blood agar with nalidixic acid and colistin	Small, grey, convex colonies with entire border. Variable haemolysis patterns	PYR - positive
Klebsiella pneumoniae	MacConkey agar	Lactose fermenter (pink) mucoid colonies	Rapid Indole negative Oxidase negative
Acinetobacter baumannii	MacConkey agar	Non-lactose fermenter. Salmon pink, opaque, small convex colonies with entire borders	Oxidase negative
Enterobacter spp	MacConkey agar	Lactose fermenter. flat colonies with entire borders, occasional NLF[+]	Rapid Indole negative Oxidase negative
Pseudomonas aeruginosa	MacConkey agar	Grape odour, non-lactose fermenter, translucent colonies with spreading edges	Oxidase Positive
Candida auris	Sabouraud Dextrose agar with chloromycetin	"Bread" smell. Creamy white, convex colonies	Germ tube negative

* Prolex™ Staph Xtra Latex Kit (Pro-Lab Diagnostics, Merseyside, United Kingdom)
[+] NLF – Non-lactose fermenter

Colonies identified as possible ESKAPE+C organisms were further identified using the Vitek 2 system (BioMérieux, Marcy-l'Étoile, France). Antimicrobial susceptibility testing was also performed on the automated Vitek 2 platform (BioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions and interpreted according to Clinical Laboratory Standards Institute (CLSI) M100 32nd ed. 2021 criteria.(39)

Gram positive organisms (*Staphylococcus aureus* and *Enterococcus faecalis/faecium)* were processed on the AST-P603 card. For *Enterococcus faecalis/faecium,* the antibiotics reported were: benzylpenicillin, ampicillin, vancomycin, teicoplanin, linezolid, ciprofloxacin, clindamycin, erythromycin, tetracycline, gentamicin high level and streptomycin high level. Enterococci were

considered vancomycin-resistant if the antibiotic susceptibility report had a vancomycin MIC greater than or equal to 8µg/ml.

Staphylococcus aureus antibiotics reported were: benzylpenicillin, oxacillin, cefoxitin screen, vancomycin, teicoplanin, linezolid, co-trimoxazole, erythromycin, clindamycin, MLS inducible test, ciprofloxacin, rifampicin, and gentamicin. Isolates were considered methicillin-resistant if the cefoxitin screen was positive and the oxacillin MIC was greater than or equal to 4µg/ml.

Gram negative bacilli organisms were processed on the Vitek AST-N255 card. For *Klebsiella pneumoniae* and *Enterobacter* spp, the antibiotics reported were ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime, ertapenem, meropenem, imipenem, gentamicin, amikacin, ciprofloxacin, tigecycline, nitrofurantoin and co-trimoxazole. An isolate was considered an ESBL-producing organism if the MIC to ceftazidime was greater than or equal to 8µg/ml and/or cefotaxime was greater than or equal to 2µg/ml. This was also reviewed in the context of other antimicrobial susceptibilities. It would be expected that an ESBL-producing isolate would also be resistant to ampicillin and first and second-generation cephalosporins. An isolate was considered carbapenem-resistant if the MIC was not susceptible to any of the carbapenems tested; ertapenem greater than 0,5µl/ml and meropenem and imipenem greater than 1µl/ml. Carbapenem-resistant isolates were tested for carbapenemase enzymes using a lateral flow chromogenic assay, RESIST-4 O.N.K.V (Coris bioConcept, Gembloux, Belgium), which tests for KPC, OXA-48, NDM and VIM carbapenemases.

For *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* the antibiotics reported were cefotaxime, ceftazidime, cefepime, piperacillin/tazobactam, amikacin, gentamicin, meropenem, imipenem, ciprofloxacin, tigecycline and co-trimoxazole. Multidrug resistance was determined by exhibiting microbial antibiotic resistance to at least one antibiotic in three or more antimicrobial categories, and extensively drug resistance was determined by showing resistance to all available and tested antimicrobial categories except one. Colistin testing is not verifiable on the VITEK 2 platform. Colistin broth microdilution was not performed due to the limited availability of the test and prioritisation for clinical samples by the ICSL. Lack of funds to outsource the testing and the rarity of colistin resistance reported from clinical

samples (unpublished laboratory surveillance data) were further reasons for not performing colistin broth microdilution.

Antibiotic susceptibilities were interpreted using the MIC values generated by the Vitek 2 system. All bacterial identifications and antibiotic susceptibility tests were processed with an accompanying purity plate on 5% blood agar that was reviewed to ensure mono-microbial growth and that no contaminating bacteria were present to influence the antimicrobial susceptibility results or identification of the organism. MDR isolates were stored on semisolid agar for macro-restriction analysis.

2.2.4 Macro-restriction analysis

Macro-restriction analysis using pulsed-field gel electrophoresis (PFGE) was performed. Whole genome sequencing was not affordable nor readily available for the funding and scope of this study. The PFGE procedure was performed according to the NHLS standard operating procedure NJHF0249 used in the ICSL laboratory of the NHLS and the University of the Witwatersrand. The detailed procedures are in the appendices (appendix 4 MRSA; appendix 5 Gram negative Enterobacterales; appendix 6 *Acinetobacter baumannii*). Each organism processed for macro-restriction analysis followed that organism's specific protocol. An overview of the process is described. Samples for macro-restriction analysis were plated out from semi-solid agar onto 5% blood agar, and a single colony was sub-cultured into BHI broth for 24 hours. The number of bacteria to be processed per isolate was normalised using a spectrometer to a set optical density. The organism was suspended in a PFGE agarose plug; these are known as plug slices. The plug slices were processed for cell lysis using the appropriate buffer, digestion enzyme (if applicable), and proteinase K. The plug slices were washed after cell lysis. The remaining DNA in the plug slices was digested with the organism appropriate restriction enzyme. Isolates were then separated on a Pulsed Field Electrophoresis Gel (1 - 1,5% concentration, dependant on organism) with appropriate switch time and time-period for optimal separation of DNA fragments, as determined previously by the laboratory. The PFGE gel was stained with ethidium bromide and visualised using a UV transilluminator. The image was captured using the laboratory GelDoc system (Bio-Rad, Hercules CA, USA) for further evaluation. Criteria for assessing the relatedness of organisms are in the table 2.2 below, as described by Tenover.(30)

Table 2.2 Criteria for interpreting PFGE patterns

Category	Number genetic differences compared to the outbreak strain	Typical number of fragment differences	Epidemiological interpretation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate probably part of the outbreak
Possibly related	2	4-6	Isolate possibly part of the outbreak
Different	≥3	≥7	Isolate not part of the outbreak

2.2.5 Data processing

Data were collected and collated in Excel 365 (Microsoft, Redmond WA, USA). Data from the environmental sampling log was transferred onto the collated Excel spreadsheet (Appendix 7) with data from the laboratory working card (Appendix 8) used during specimen processing. Before analysis, the data were prepared to ensure there were no missing data and presented in a manner that calculations were easy to perform. Outlying values from water sources and biologically contaminated surfaces with extremely high cfu/cm^2 were identified and excluded from analysis. These cfu/cm^2 were raised to unique microenvironmental niches and their inclusion would severely skew the data. The laboratory working cards and Vitek 2 antimicrobial susceptibility reports were filed with easy reference if further data were required. A descriptive data analysis was performed, and the information was displayed in tables. When comparing two sets of data, the Student t-test using an independent samples t-test methodology was used. The p-value was calculated using the t-scores. A statistical p-value of >0.05 would be considered a significant result. All calculations and data analysis were done using MedCalc® Statistical Software version 20.023 (MedCalc Software Ltd, Ostend, Belgium).

3. RESULTS

3.1 Threshold of detection from environmental sampling methods

Sampling by both the swab and the sponge methods did not yield growth of the targeted MDR ESKAPE+C organisms at a dilution of 1x10^{-8}, suggesting that concentrations of 1cfu/ml are below the threshold of detection for this method of

sampling with these commercial methods. The bacterial isolates were positive at dilutions of $1x10^{-7}$ and $1x10^{-6}$ for *Enterococcus faecium, Pseudomonas aeruginosa* and *Acinetobacter baumannii* by the sponge sampling method and were negative at $1x10^{-7}$ dilution when sampled with the swab method, placing the threshold of detection for bacterial isolates between 10 and 100 cfu/ml. The *Candida auris* isolate was only detectable at a dilution of $1x10^{-5}$ dilution, suggesting a threshold of detection of 1000cfu/ml. The sampling was positive after growth in BHI enrichment broth. Mixing the isolates did not decrease the threshold of detection of the individual organisms. Spread plate processing for colony counts did not grow any colonies for all isolates at the detection threshold.

3.2 Environmental Sampling

A total of 115 samples were processed. Three of these samples were control samples, which were all negative for growth. There was a total of 112 environmental samples. Of the environmental samples, 81 were sponges and 31 were swabs. Eight of the environmental samples failed to grow any organisms on aerobic spread plates nor from BHI broth. Therefore, this left 104 samples to evaluate for MDR ESKAPE+C organisms. A further 5 samples had contaminated aerobic spread plates, preventing evaluation for environmental bioburden. This left 99 specimens for microbial bioburden evaluation.

3.2.1 Microbial bioburden

Of the isolates that were available to assess aerobic spread plate colony counts, 11/99 (11,1%) had <1 cfu/cm2 and 8/99 (8,1%) were too numerous to count and thus unable to calculate colony counts. Therefore 80/99 (80,8%) were available to assess for colony counts. Of the 8 samples that were too numerous to count, 6 were from water sources (basin drain, tap faucet). One sample was from a composite high-touch surfaces in TICU cubicle D bed 3. At the time of sampling, dried blood was present on one of the surfaces (infusion pump) that made up the composite. The other area sampled that had a colony count too numerous to count was the baby weigh station in TICU cubicle D, which had dried clear secretions.

Table 3.1 Summary of overall microbial bioburden in the unit

Included swabs	Mean (cfu/cm^2)	Median (cfu/cm^2)	Range (cfu/cm^2)	Standard Deviation
All Swabs	4,32x10^4	2,40x10	1 – 2,74x10^6	± 3,06x10^5
Outliers removed	1,44x10^2	1,80x10	1 – 2x10^3	± 3,92x10^2
Outside cubicles	7,5	2,5	1 – 2,50x10	± 1,10x10

Of the 80 isolates with a colony-forming unit count the mean cfu/cm^2 was 4,32x10^4 cfu/cm^2. The mean was positively skewed by high colony counts from water sources, from which a colony count was possible, and an infusion pump contaminated with blood at the time of sampling in TICU, cubicle B, bed 9. The mean without these outlying values was 1,44x10^2 cfu/cm^2. Areas outside the cubicles (nursing stations, pharmacy, main door handle, ultrasound machine) had a lower mean microbial bioburden of 7,5 cfu/cm^2, with no growth from the pharmacy preparation area nor the transfer trolley used to transport medication to the ward.

Table 3.2 Microbial Bioburden in the NICU and TICU (excluding water basins)

Area Sampled	Mean (cfu/cm^2)	Median (cfu/cm^2)	Range (cfu/cm^2)	Standard Deviation
NICU	8,6	3,5	1 – 4,2x10	± 1,04x10
TICU	1,61x10^3	2,2x10	1 – 3,42x10^4	± 5,61x10^3

The TICU was more crowded than the NICU, with incubators and open cribs closer together and more throughput traffic with medical staff and mothers. The NICU had a set number of beds available, as detailed earlier. The TICU exceeded bed capacity in each cubicle. Cubicle A had 11 patients when designed for 10, cubicle B had 13 patients when designed for 10, cubicle C had 13 patients when designed for 10 and Cubicle D had 10 patients when designed for 8. The mean microbial bioburden in the TICU (minus water basins) was 1,61x10^3 cfu/cm^2 compared to the NICU mean of 8,6 cfu/cm^2. This difference fell short of statistical significance with a p-value of 0.06.

Table 3.3 Microbial bioburden in different TICU cubicles (excluding water basins)

TICU cubical	Mean (cfu/cm^2)	Median (cfu/cm^2)	Range (cfu/cm^2)	Standard Deviation
TICU A	1,17x10	5,5	1 – 8,5x10	± 2,33x10
TICU B	4,11x10^3	2,6x10	2 – 3,42x10^4	± 9,92x10^3
TICU C	3,78x10^2	6,4X10	12 – 2,33x10^3	± 2,23x10^2
TICU D	8,96x10^2	2,4x10	2 – 8,10x10^3	± 2,11x10^3

The microbial bioburden was not evenly distributed amongst the cubicles in the TICU, with higher bioburdens predictably occurring in the most overcrowded cubicles.

TICU cubicles B, C and D all had the mean positively skewed due to samples that isolated high microbial bioburden counts. Both cubicle B and cubicle C had equipment contaminated with blood, and cubicle D had a weigh station with a high microbial bioburden count. The TICU isolation cubicle was not assessed for descriptive statistics because too few samples were collected from the cubicle (9 samples, of which 3 were from the water basin) to perform meaningful statistical calculations. There was no statistically significant difference between the microbial bioburden between different TICU cubicles. Multiple t-tests were conducted, and all returned with a p-value > 0,1.

The water basins had higher microbial counts than other areas of the NICU and TICU. There was no association between the basin drain, tap faucet, and the tap handles microbial bioburden, as shown in the table 3.4 below.

Table 3.4 Microbial bioburden of basin surfaces

Basin Location	Drain microbial bioburden cfu/cm^2	Tap faucet microbial bioburden cfu/cm^2	Tap handles microbial bioburden cfu/cm^2
Entrance	TNTC*	No Growth	No Growth
NICU cubicle A	7,4x10	2,8x10^5	1,1x10^2
NICU Isolation B	2,9x10^3	TNTC	<1
NICU Isolation C	TNTC	Unable to calculate (contaminated)	2,5x10
NICU cubicle D	5,0x10^2	8,1x10^3	<1
TICU Cubicle A	1,9x10^2	No Growth	3,5x10
TICU Cubicle B	2,2x10	TNTC	2,0x10^2
TICU Cubicle C	1,2x10^4	4,8x10^5	2,8x10
TICU Cubicle D	TNTC	2,7x10^6	1,9x10^2
TICU isolation	TNTC	No Growth	6,4x10

* TNTC – Too numerous to count

There were 5 terminally disinfected incubators available for sampling. Two were open neonatal incubators, and three were closed incubators. All terminally disinfected incubators had a low microbial bioburden of less than 3 cfu/cm^2. All the terminally disinfected incubators isolated environmental organisms from broth consisting of *Micrococcus* spp, *Bacillus* spp and coagulase-negative staphylococci.

3.2.2 Environmental organisms

Whether an MDR ESKAPE+C organism was isolated or not, all the specimens grew environmental organisms. Common environmental organisms isolated included coagulase-negative staphylococci, *Bacillus* spp, and *Micrococcus* spp. Identification

of environmental organisms was based on Gram stain morphology, growth characteristics and rapid tests where applicable.

Although the study primarily focused on identifying ESKAPE+C isolates, other environmental organisms were occasionally identified, including potential neonatal disease-causing organisms such as *Elizabethkingae meningoseptica, Proteus* spp and CRE *Serratia marcescens.* Other environmental organisms isolated included *Pantoa* spp, *Sphingomonas paucimobilis,* and *Delftia acidovorans.* None of these organisms were isolated in clinical samples collected during the study period.

3.2.3 ESKAPE + C organisms

Of the 112 environmental samples processed (115 minus 3 controls), 55/112 (49,1%) isolated one or more ESKAPE+C organisms. Of the 55 samples 49/112 (43,8%) isolated a MDR ESKAPE+C. There were no environmental samples that isolated *Candida auris* nor *Pseudomonas aeruginosa.* In total, there were 74 ESKAPE isolates. Of these ESKAPE organisms, 22/74 (29.7%) did not show acquired antimicrobial resistance, and the remaining 52/74 (70,3%) demonstrated acquired drug resistance.

Of these 74 ESKAPE organisms, 56/74 (75,7%) were isolated from the composite sponges of surfaces in close proximity to the neonate. This pattern of isolation from proximity surfaces was also present for MDR ESKAPE organisms. Of the 52 MDR ESKAPE organisms isolated, 36/52 (69,2%) were isolated from samples taken in close proximity to the neonate. The number of isolates per organism is shown in table 3.5

Table 3.5 Number of ESKAPE+C organisms isolated

ESKAPE isolate	No acquired resistance	Acquired resistance detected
Enterococcus spp	21	0
Staphylococcus aureus	0	5
Klebsiella pneumoniae	0	7
Enterobacter spp	1	16
Acinetobacter baumannii	0	24

The 21 enterococci isolated consisted of 5 *Enterococcus faecalis* isolates, 15 *Enterococcus faecium* isolates and 1 *Enterococcus gallinarum* isolate. The *E. faecalis* and *E. faecium* isolates were susceptible to vancomycin with vancomycin MICs ranging between 0.5µg/ml to 2µg/ml. The *E gallinarum* vancomycin MIC was

tested at the 4µg/ml breakpoint. The *E. faecium* isolates were resistant to ampicillin, whereas the *E. faecalis* and *E. gallinarum* were susceptible to ampicillin.

Table 3.6 MDR ESKAPE organism resistance pattern

ESKAPE isolate	Resistance pattern	Number of isolates
Staphylococcus aureus	MRSA	5
Klebsiella pneumoniae	ESBL	3
	CRE	4
Enterobacter cloacae	ESBL	16
	CRE	0
Acinetobacter baumannii	MDR	0
	XDR	24

The vancomycin minimum inhibitory concentration (MIC) as determined by Vitek 2 susceptibility testing was 1µg/ml for three of the five MRSA isolates. The other 2 MRSA isolates had results at the lower end of Vitek 2 vancomycin susceptibility testing of less than or equal to 0.5µg/ml.

Table 3.7 CRE *Klebsiella pneumoniae* antibiotic susceptibility results

Antibiotic	MIC (µg/ml)	Interpretation	Antibiotic	MIC (µg/ml)	Interpretation
Ampicillin	N/A		Ertapenem	≥8	R
Amoxicillin-Clavulanic acid	≥32	R	Imipenem	8*	R
Piperacillin-tazobactam	≥128	R	Meropenem	≥16	R
Cefuroxime	≥64	R	Amikacin	≤2	S
Cefoxitin	8	S	Gentamicin	≥16	R
Cefotaxime	≥64	R	Ciprofloxacin	0,5	I
Ceftazidime	≥64	R	Co-trimoxazole	≥320	R
Cefepime	32	R	Tigecycline	2	N/A

* See text; R -Resistant; S- Susceptible; I – Intermediate; N/A – not available

The 4 CRE *Klebsiella pneumoniae* isolates were tested for carbapenemase production using RESIST-4 O.N.K.V (Coris bioConcept, Gembloux, Belgium) lateral flow assay. All 4 were negative for KPC, OXA-48, NDM and VIM carbapenemases, for which the assay tests. The four CRE *Klebsiella pneumoniae* isolates antibiotic susceptibilities are as per table 3.7 except for one isolate with a MIC against imipenem that was ≥16µg/ml. All 4 CRE isolates were resistant to gentamicin, but all four tested susceptible to amikacin.

Table 3.8 ESBL *Klebsiella pneumoniae* antibiotic susceptibility results

Antibiotic	MIC (µg/ml)	Interpretation	Antibiotic	MIC (µg/ml)	Interpretation
Ampicillin	N/A		Ertapenem	≤0,5	S
Amoxicillin-Clavulanic acid	≥32	R	Imipenem	≤0,25	S
Piperacillin-tazobactam	64	I	Meropenem	≤0,25	S
Cefuroxime	≥64	R	Amikacin	16	S
Cefoxitin	≤4	S	Gentamicin	≤1	S
Cefotaxime	≥64	R	Ciprofloxacin	≤0,25	S
Ceftazidime	16	R	Co-trimoxazole	≤20	S
Cefepime	8	SDD	Tigecycline	≤0,5	N/A

* See text; R -Resistant; S- Susceptible; I – Intermediate; SDD – Susceptible Dose Dependant; N/A – not available

The three ESBL *Klebsiella pneumoniae* had identical antibiotic susceptibility profiles.

Table 3.9 ESBL *Enterobacter cloacae* antibiotic susceptibility results.

Antibiotic	MIC (µg/ml)	Interpretation	Antibiotic	MIC (µg/ml)	Interpretation
Ampicillin	≥32	N/A	Ertapenem	≤0,5	S
Amoxicillin-Clavulanic acid	≥32	R	Imipenem	≤0,25	S
Piperacillin-tazobactam	8*	S	Meropenem	≤0,25	S
Cefuroxime	≥64	R	Amikacin	≤2	S
Cefoxitin	≥64	R	Gentamicin	≥64	R
Cefotaxime	≥64	R	Ciprofloxacin	1	R
Ceftazidime	≥64	R	Co-trimoxazole	≥320	R
Cefepime	2*	S	Tigecycline	1	N/A

* See text; R -Resistant; S- Susceptible; I – Intermediate; SDD – Susceptible Dose Dependant; N/A – not available

All the *Enterobacter* spp isolated were identified as *Enterobacter cloacae* complex. Most of the 16 ESBL *Enterobacter cloacae* isolates showed the antibiotic susceptibility pattern shown in table 3.9 above. Four of the 16 ESBL *Enterobacter cloacae* isolates had a cefepime MIC of 4µg/ml, interpreted as SDD. Against piperacillin-tazobactam six were intermediate with a MIC of 32-64µg/ml.

Table 3.10 XDR *Acinetobacter baumannii* antibiotic susceptibility results

Antibiotic	MIC (µg/ml)	Interpretation	Antibiotic	MIC (µg/ml)	Interpretation
Piperacillin-tazobactam	≥128	R	Imipenem	≥16	R
Ceftazidime	≥64	R	Meropenem	≥16	R
Cefepime	≥64	R	Gentamicin	≥16*	R
Ciprofloxacin	≥4	R	Tigecycline	4	N/A

* See text; R -Resistant; S- Susceptible; I – Intermediate; N/A – not available

Most of the XDR *Acinetobacter baumannii* tested with the same results as above except for 3 isolates, 2 of which were intermediate (MIC 8µg/ml) for gentamicin, and

one was susceptible (MIC <1µg/ml). Vitek 2 does provide a colistin MIC value, but this is not resulted as it is not reliable and can give incorrect results compared to colistin broth microdilution. Manual amikacin antimicrobial susceptibility testing was not done.

3.2.4 Distribution of MDRO isolates

The distribution of the MDRO isolates in the unit was not equal. There were no isolates of *Candida auris* therefore I will henceforth refer to ESKAPE organisms and not ESKAPE+C. The NICU had 9/52 (17,3%) MDR ESKAPE organisms, with the remainder of the isolates being isolated from the TICU. Within the TICU cubicles, the distribution of the different isolates was not uniform, with species clustering in different cubicles.

Of the MDR ESKAPE organisms that were isolated, 36/52 (69,2%) were from high-touch surfaces in proximity to the neonate. High-touch surfaces in proximity to the neonate included infusion pumps, ventilators, CPAP machines, monitor screens and warmers. The MDR ESKAPE organisms isolated from the environment were XDR *Acinetobacter baumannii* 24/52 (46,2%), ESBL *Enterobacter cloacae* 16/52 (30,8%), CRE and ESBL *Klebsiella pneumoniae* 7/52 (13,4%) and MRSA 5/52(9,6%).

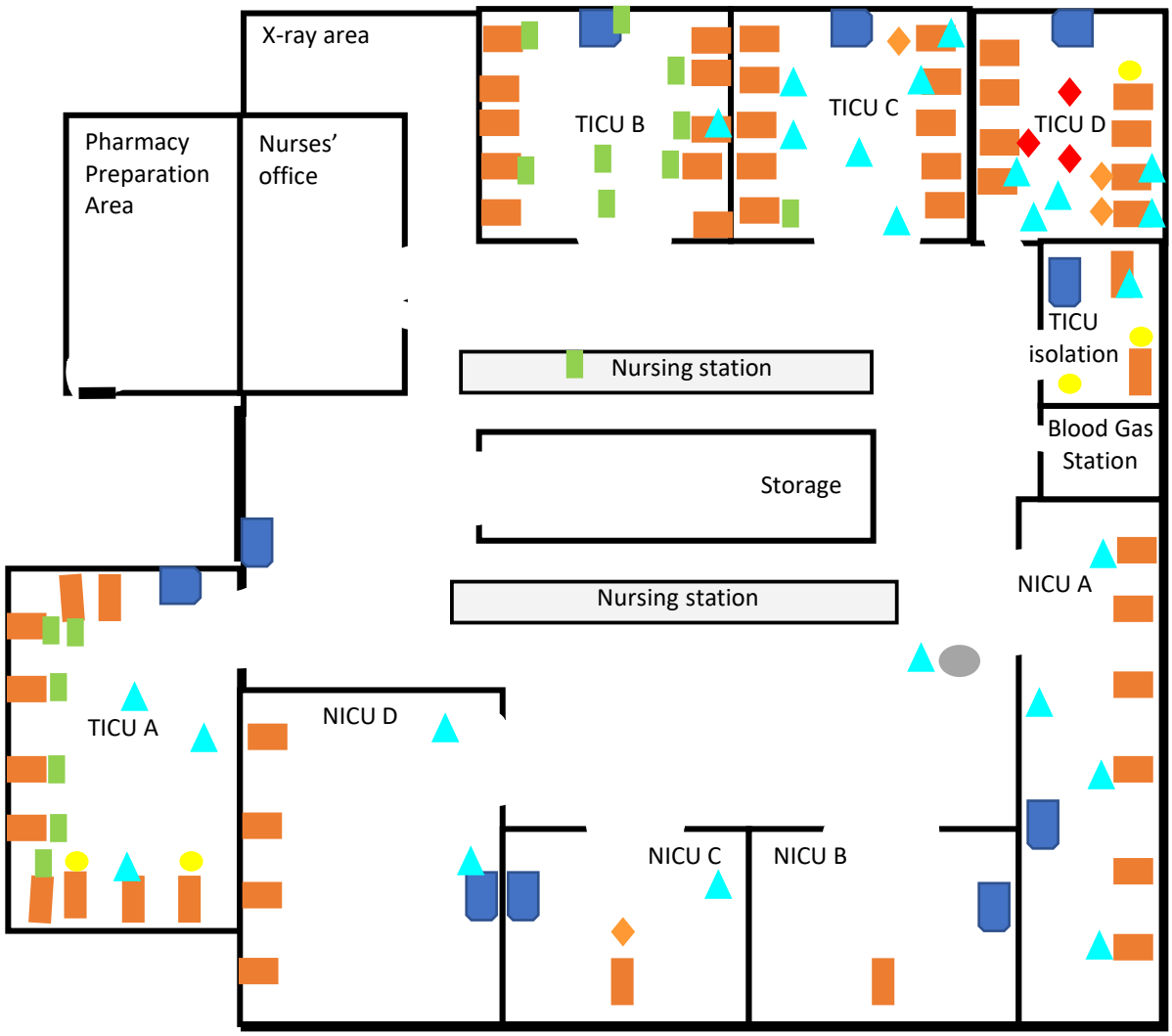
Frequently used surfaces or equipment that may act as environmental reservoirs or vehicles for the transmission of the MDRO ESKAPE organisms were also sampled. The following sampled surfaces yielded one or more MDRO ESKAPE organisms. Please see accompanying figure 3.1 (page 27).

- Glucose meter. There is supposed to be one glucose meter per cubicle in the NICU/TICU. That means there are meant to be ten glucose meters. Only one glucose meter was in use for the entire unit. from which XDR *Acinetobacter baumannii* was isolated.
- There were medication stations in each cubicle as described earlier, where dilutions and ivi medications could be drawn up. An MDR ESKAPE organism was isolated from six out of ten medication stations.
    - NICU A – XDR *Acinetobacter baumannii*
    - NICU C (isolation) – XDR *Acinetobacter baumannii*
    - NICU D – XDR *Acinetobacter baumannii*
    - TICU A – XDR *Acinetobacter baumannii*

    - TICU B – ESBL *Enterobacter cloacae*
    - TICU C – XDR *Acinetobacter baumannii*
- Multifunctional trolleys in the TICU cubicles that multiple staff members use grew MDR ESKAPE organisms in the following cubicles.
    - TICU B – ESBL *Enterobacter cloacae*
    - TICU D – XDR *Acinetobacter baumannii*
    - TICU isolation cubicle (isolation for XDR *Acinetobacter baumannii* infection/colonisation*)* – Methicillin-resistant *Staphylococcus aureus*
- From the neonate weigh station in TICU D, both an ESBL *Klebsiella pneumoniae* and an XDR *Acinetobacter baumannii* were isolated.
- The handwash bottle (alcohol plus chlorhexidine) in TICU D grew an ESBL *Klebsiella pneumoniae* and an XDR *Acinetobacter baumannii.*
- In addition to the drains of the basins isolating CRE *Serratia marcescens,* the taps of the following basins grew MDR ESKAPE organisms
    - NICU D – XDR *Acinetobacter baumannii*
    - TICU B – ESBL *Enterobacter cloacae*

From the nursing station of the TICU, an ESBL *Enterobacter cloacae* was isolated*.*

Figure 3.1 Spatial distribution of MDR ESKAPE isolates in the neonatal unit



Key to diagram

- Wash Basin
- Incubator
- MRSA
- ESBL *Klebsiella pneumoniae*
- CRE *Klebsiella pneumoniae*
- ESBL *Enterobacter cloacae*
- XDR *Acinetobacter baumannii*
- Glucose meter

***Please review together with text that follows. Summary of MDRO distribution**

- XDR *A. baumannii* is found throughout the unit
- MRSA is in two groups in TICU A and on the opposite side of the unit
- ESBL *E. cloacae* is found in TICU A and B only
- ESBL *K. pneumoniae is* found in TICU D only
- CRE *K. pneumoniae* is found in the TICU and NICU

3.2.4.1 *XDR Acinetobacter baumannii distribution*

XDR *Acinetobacter baumannii* was distributed throughout the unit in both the NICU and the TICU. It was found on high-touch surfaces near the neonates and on environmental surfaces that may act as reservoirs for future transmission (medication stations, trolleys, basin tap handle). It was isolated from the only glucose meter in the unit.

3.2.4.2 *MRSA distribution*

The 5 MRSA isolates were found in two groups on opposite unit ends. One group consisted of 2 isolates were from high-touch areas in TICU A. These two isolates were found from beds one patient apart. The second group was isolated from TICU isolation (which was being used for *Acinetobacter baumannii* infected neonates) and one isolate from high-touch surfaces in TICU D, which is next to the TICU isolation cubicle.

3.2.4.3 *ESBL Enterobacter cloacae*

The ESBL *Enterobacter cloacae* isolates were clustered in TICU cubicle A and TICU cubicle B. The isolates from TICU cubicle A were all isolated from high-touch surfaces surrounding beds 4 to 9. In TICU B, *Enterobacter cloacae* were also isolated from high-touch surfaces, the medication table, multifunctional trolley surface and the tap handles of the hand wash basin in the cubicle. A single sample from high-touch surfaces in the TICU cubicle C bed also isolated ESBL *Enterobacter cloacae*. A sample from the nursing station for TICU, an everyday use area for the entire TICU, grew an ESBL *Enterobacter cloacae*.

3.2.4.4 *MDRO Klebsiella pneumoniae*

Three ESBL-producing *Klebsiella pneumoniae* isolates were grown from environmental samples. All three were found in TICU cubicle D. One isolate was found on high-touch surfaces surrounding bed 2. The other samples were found on the unit's communal hand wash bottle (alcohol and chlorhexidine-based hand rub) and on the weigh station present in the unit.

Four CRE *Klebsiella pneumoniae* were isolated. They were more spread out within the unit. Two were found from high-touch surfaces in TICU cubicle D, from beds 7 and 8. One was found in TICU cubicle C from high-touch surfaces of bed 6. TICU

cubicles C and D are adjacent to each other. One isolate was found on the high-touch surfaces NICU cubicle C. This is an isolation unit for the NICU. The neonate in the unit was isolated due to sepsis due to XDR *Acinetobacter baumannii.*

3.3 Microbial bioburden and MDRO ESKAPE organisms

Table 3.11 Microbial bioburden of samples that isolated MDR ESKAPE organisms vs. samples that did not isolate MDR ESKAPE organism

MDRO isolated from the sample	Mean (cfu/cm^2)	Median (cfu/cm^2)	Range (cfu/cm^2)	Standard Deviation
MDRO isolated	1,23x10^3	1,55x10	1 – 3,42x10^5	± 5,43x10^3
No MDRO isolated	9,54x10^4	3,1x10	1 – 2,74x10^7	± 4,56x10^5

The microbiological bioburden aerobic plate count did not predict whether an MDR ESKAPE organism was more likely to be isolated after broth enrichment or not. Comparing microbial bioburden when an MDR ESKAPE organism was isolated to when an MDR ESKAPE organism was not isolated, the p-value of 0,22 was not significant.

Table 3.12 Microbial bioburden stratification and MDRO isolation

Microbial Bioburden	Number of MDRO isolated	Percentage of total MDRO isolated	Percentage of samples isolating MDRO
<1 cfu/ml	4	8%	36% (4/11)
Too numerous to count	3*	6%	25% (2/8)*
Contaminated count plates	2*	4%	20% (1/5)*
≤5 cfu/cm^2	10	19%	48% (10/21)
6-10 cfu/cm^2	7*	13%	85% (6/7)*
10-100 cfu/cm^2	15	29%	48% (15/31)
>100 cfu/cm^2	11*	21%	40% (10/25)*

*Number of MDRO isolated does not correlate to the number of samples because some samples grew more than one MDRO

It was not possible to determine an aerobic colony count from 12% (6/52) of the samples that isolated a MDR ESKAPE organism. This was due to 4 samples not growing any organisms on the aerobic colony count plates, 2 samples having a growth too numerous to count, and 1 sample on contaminated plates.

3.4 Clinical isolates

From 5 July 2021 to 30 July 2021, after removal of duplicate specimens, 27 blood cultures, each representing first-time isolation of an MDR ESKAPE+C organism in a neonate, were identified. Two cerebrospinal fluid isolates were also identified, but they were removed as duplicates of blood culture isolates in both cases. Isolates were collected over one month to ensure a sufficient number of isolates were

collected and assessed for any changes that may develop over time in the isolates on macro-restriction analysis. Date of isolation and species of MDR ESKAPE+C organism are represented graphically in Figure 3.2 and tabulated in Table 3.13 below.

Figure 3.2 Line diagram showing date of collection of clinical isolates

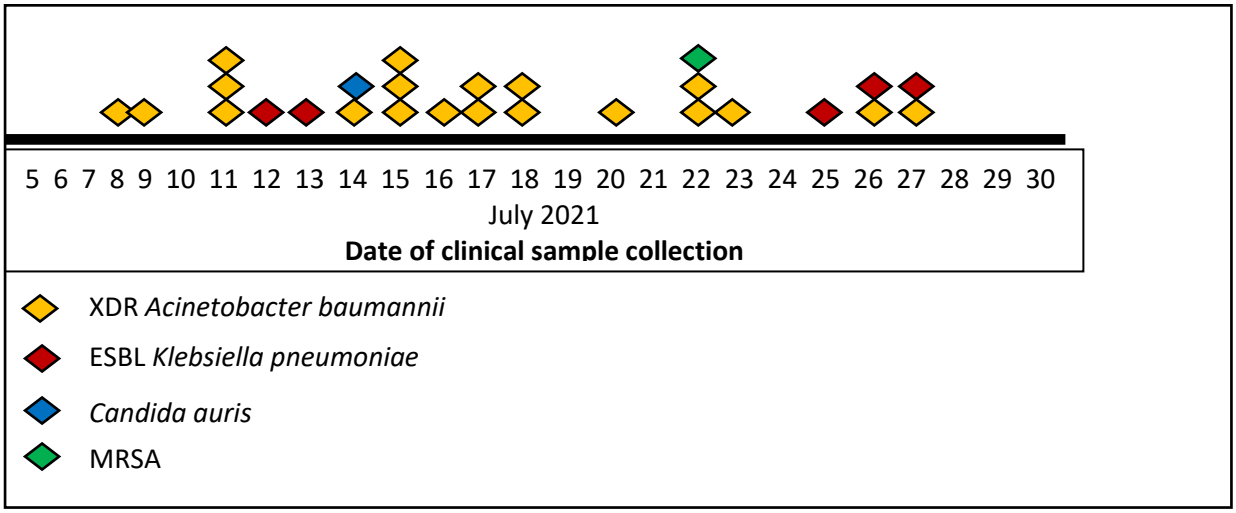


Table 3.13 List of MDRO clinical isolates

Specimen number	Date of specimen collection	Organism isolated	Area patient cared for
1	08-Jul	*XDR Acinetobacter baumannii*	NICU
2	09-Jul	*XDR Acinetobacter baumannii*	TICU
3	11-Jul	*XDR Acinetobacter baumannii*	TICU
4	11-Jul	*XDR Acinetobacter baumannii*	NICU
5	11-Jul	*XDR Acinetobacter baumannii*	TICU
6	12-Jul	ESBL *Klebsiella pneumoniae*	TICU
7	13-Jul	ESBL *Klebsiella pneumoniae*	TICU
8	14-Jul	*XDR Acinetobacter baumannii*	TICU
9	14-Jul	*Candida auris*	TICU
10	15-Jul	*XDR Acinetobacter baumannii*	TICU
11	15-Jul	*XDR Acinetobacter baumannii*	TICU
12	15-Jul	*XDR Acinetobacter baumannii*	TICU
13	16-Jul	*XDR Acinetobacter baumannii*	TICU
14	17-Jul	*XDR Acinetobacter baumannii*	NICU
15	17-Jul	*XDR Acinetobacter baumannii*	TICU
16	18-Jul	*XDR Acinetobacter baumannii*	TICU
17	18-Jul	*XDR Acinetobacter baumannii*	NICU
18	20-Jul	*XDR Acinetobacter baumannii*	NICU
19	22-Jul	MRSA	TICU
20	22-Jul	*XDR Acinetobacter baumannii*	TICU
21	22-Jul	*XDR Acinetobacter baumannii*	NICU
22	23-Jul	*XDR Acinetobacter baumannii*	NICU
23	25-Jul	ESBL *Klebsiella pneumoniae*	NICU
24	26-Jul	*XDR Acinetobacter baumannii*	NICU
25	26-Jul	ESBL *Klebsiella pneumoniae*	TICU
26	27-Jul	*XDR Acinetobacter baumannii*	TICU
27	27-Jul	ESBL *Klebsiella pneumoniae*	NICU

XDR *Acinetobacter baumannii* made up most, 20/27 (74% of the clinical isolates. There were 5/27 (19%) *Klebsiella pneumoniae* with ESBL resistance susceptibility pattern. There was one isolate of *Candia auris* and one methicillin-resistant *Staphylococcus aureus.*

3.5 Macro restriction analysis

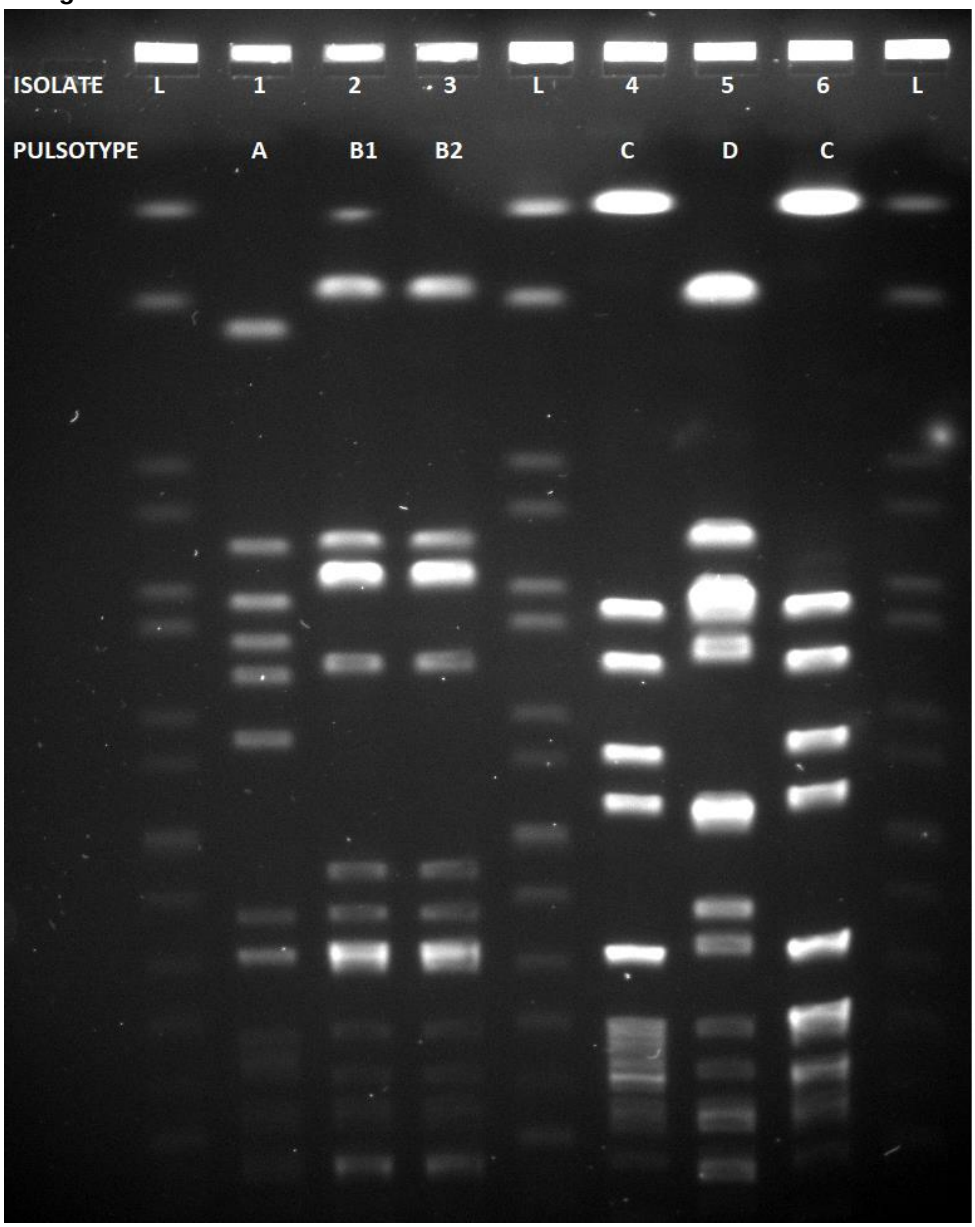
3.5.1 Methicillin-resistant *Staphylococcus aureus*

There was one clinical isolate of MRSA, isolated from a neonatal blood culture in TICU and 4 MRSA environmental isolates. The environmental isolate from the TICU D trolley lost viability prior to PFGE and could not be processed for PFGE analysis.

Table 3.14 List of PFGE isolates for MRSA isolates

Lane number	Identification	Date of collection	Location	Comments	Pulsotype
L	Ladder	N/A	N/A	*Salmonella* Braenderup H9812	
1	Control isolate	N/A	N/A	EQA MRSA sample from CAP	A
2	Clinical isolate	22 July 2021	TICU		B1
3	Environmental isolate 40	29 July 2021	TICU isolation – composite high-touch surfaces		B2
4	Environmental isolate 53	29 July 2021	TICU A – composite high-touch surfaces bed 1		C
5	Environmental isolate 55	29 July 2021	TICU A – composite high-touch surfaces bed 3		D
6	Environmental isolate 110	29 July 2021	TICU D – composite high-touch surfaces bed 1		C

Image 3.1 MRSA PFGE Gel



All the isolates were successfully typed. The lanes labelled “L” are representative of the ladder used. From the external quality control program of the College of American Pathologists, the control isolate in lane 1 showed a fingerprinting pattern (>9 bands) different to most of the isolates, indicating that the technique was suitably discriminatory.

Three clusters were identified. Isolate 1 had pulsotype B1 (clinical isolate) and isolate 2 had pulsotype B2 (TICU isolation – composite high-touch surfaces) and had one fragment difference between each other, indicating that they are most probably related.

Isolate 4 (TICU A – composite high-touch surfaces bed 1) and Isolate 6 (TICU D – composite high-touch surfaces bed 1 had the same pulsotype – type C and were indistinguishable from each other.

Isolate 5 (TICU A – composite high-touch surfaces bed 3) had a separate pulsotype – type D.

Pulsotypes B, C and D had greater than 7 fragment differences and were not related.

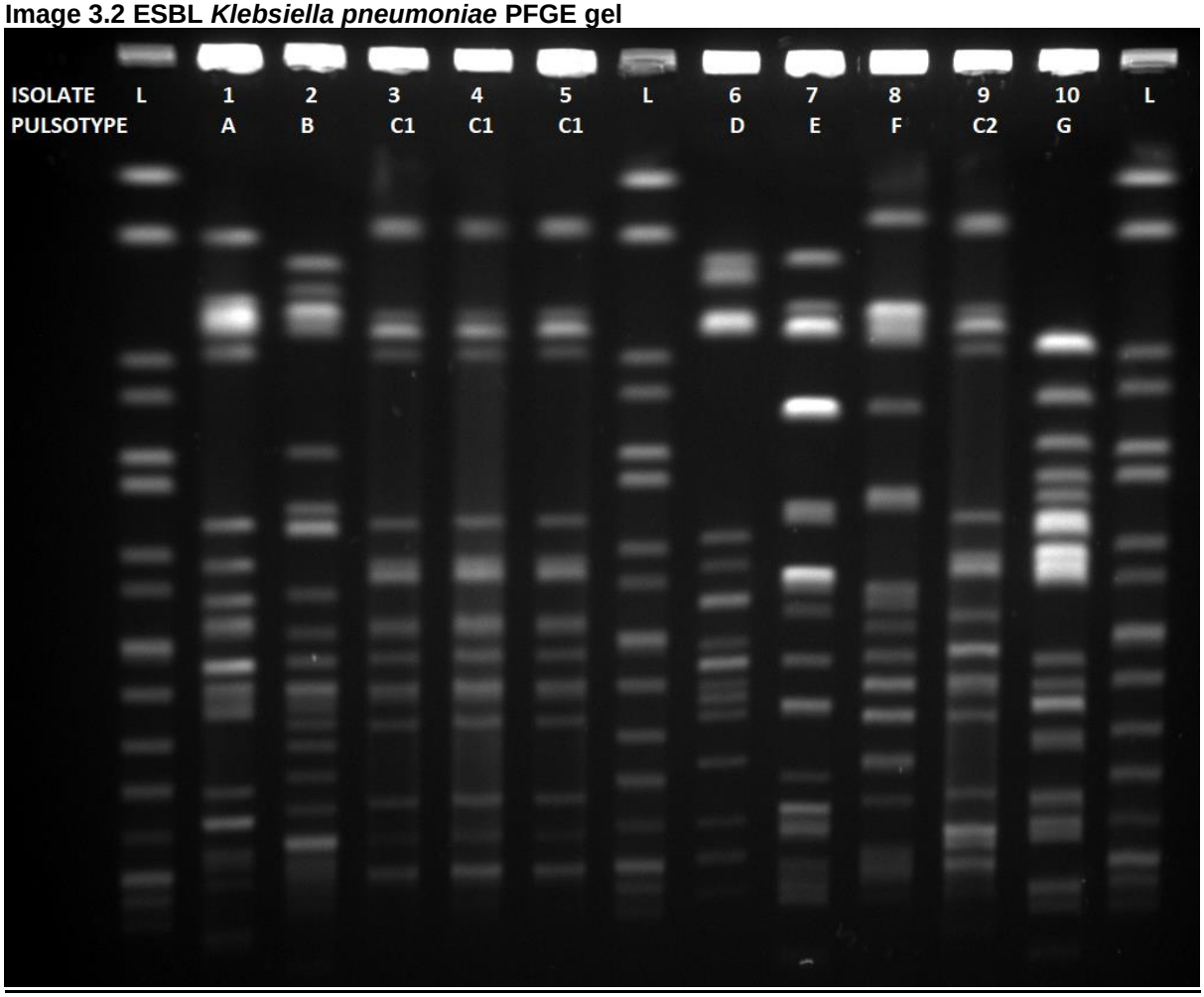
3.5.2 Extended-spectrum beta-lactamase producing *Klebsiella pneumoniae*

There were 8 isolates for comparison. Three environmental isolates from TICU cubicle D and 5 clinical isolates.

Table 3.15 List of PFGE isolates for ESBL *Klebsiella pneumoniae*

Lane number	Identification	Date of collection	Location	Comments	Pulsotype
L	Ladder	N/A	N/A	*Salmonella* Braenderup H9812	
1	Control isolate	N/A	N/A	CMJAH, adult ICU	A
2	Control isolate	N/A	N/A	Edenvale Hospital, adult ICU	B
3	Environmental isolate 100	29 July 2021	TICU D- handwash bottle		C1
4	Environmental isolate 103	29 July 2021	TICU D – composite high-touch surfaces – bed 3		C1
5	Environmental isolate 104	29 July 2021	TICU D – Weigh station		C1
6	Clinical isolate	12 July 2021	TICU		D
7	Clinical isolate	13 July 2021	TICU		E
8	Clinical isolate	25 July 2021	NICU		F
9	Clinical isolate	26 July 2021	TICU		C2
10	Clinical isolate	27 July 2021	NICU		G

Image 3.2 ESBL *Klebsiella pneumoniae* PFGE gel



All the isolates were successfully typed. The lanes labelled “L” were representative of the ladder used. The control isolates, lane 1 (from CMJAH adult ICU, stored May 2021) and lane 2 (from Edenvale Hospital, adult ICU, stored June 2021), showed fingerprinting patterns (>9 bands) different to the isolates indicating that the technique was suitably discriminatory.

One cluster was identified. The three environmental isolates, isolates 3,4 and 5, were indistinguishable. One clinical isolate, isolate 9 had three fragment differences from the environmental isolates and is probably related to the environmental isolates. The 3 environmental isolates were all isolated from TICU cubicle D and were not distributed within the entire unit.

The rest of the clinical isolates (isolates 6,7,8, and 10) had greater than 7 fragments differences from the environmental isolates and each other and were not related.

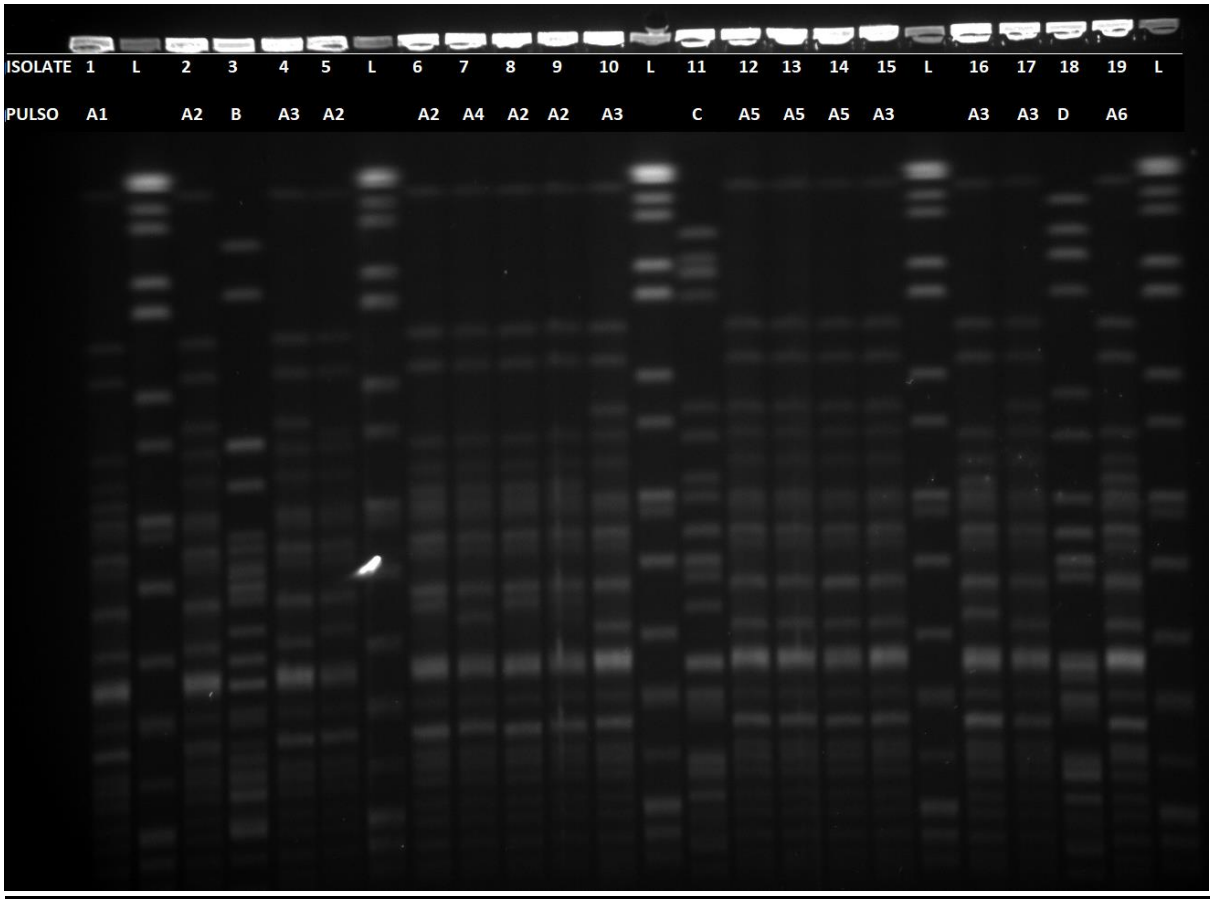
3.5.3 Extensively drug-resistant *Acinetobacter baumannii*

Due to the large number of XDR *Acinetobacter baumannii* isolates from both the environmental (n=24) and clinical samples (n=20), a representative sample of 8 environmental samples and 8 clinical samples were chosen. It would have been ideal to process all the isolates for macro-restriction analysis, but cost constraints and availability of the PFGE equipment made this impossible. The environmental samples chosen to be processed were selected to represent each cubicle (n=7), representing the spatial distribution of isolates throughout the unit and the isolate from the glucose meter (n=1). The clinical isolates were chosen to be representative of the period of collection. Two environmental isolates stored from a previously identified *Acinetobacter baumannii* outbreak in October 2020 were included to evaluate for persistence in the environment.

Table 3.16 List of PFGE isolates for XDR *Acinetobacter baumannii*

Lane number	Identification	Date of collection	Location	Comments	Pulsotype
L	Ladder	N/A	N/A	*Salmonella* Braenderup H9812	
1	Control isolate	N/A	N/A	CMJAH, Neonatal ICU	A1
2	Environmental isolate 14	N/A	Glucose monitor		A2
3	Environmental isolate 18	29 July 2021	NICU 1 – composite high-touch surfaces – bed 3		B
4	Environmental isolate 32	29 July 2021	NICU isolation 2 – medication trolley	Isolation cubicle for *A. baumannii*	A3
5	Environmental isolate 40	29 July 2021	TICU isolation – composite high-touch surfaces bed 1	Isolation cubicle for *A. baumannii*	A2
6	Environmental isolate 54	29 July 2021	TICU A– composite high-touch surfaces bed 2		A2
7	Environmental isolate 74	29 July 2021	TICU D– composite high-touch surfaces bed 8		A4
8	Environmental isolate 76	29 July 2021	TICU B– composite high-touch surfaces bed 8		A2
9	Environmental isolate 87	29 July 2021	TICU C - composite high-touch surfaces bed 3		A2
10	Clinical isolate	8 July 2021	NICU		A3
11	Clinical isolate	11 July 2021	TICU		C
12	Clinical isolate	15 July 2021	TICU		A5
13	Clinical isolate	15 July 2021	TICU		A5
14	Clinical isolate	17 July 2021	TICU		A5
15	Clinical isolate	18 July 2021	NICU		A3
16	Clinical isolate	22 July 2021	NICU		A3
17	Clinical isolate	26 July 2021	NICU		A3
18	Environmental isolate	Oct 2020	TICU		D
19	Environmental isolate	Oct 2020	TICU		A6

Image 3.3 XDR *Acinetobacter baumannii* PFGE Gel



All the isolates were successfully typed. The lanes labelled “L” were representative of the ladder used. The control isolate (CMJAH neonatal ICU clinical isolate) was closely related to isolate 2 (differing by 3 fragments), suggesting interhospital transfer. Isolate 3 and 18 showed fingerprinting patterns (>9 bands) different to the other isolates indicating that the technique was suitably discriminatory. The second ladder had a fluorescent artefact, but this did not impair the interpretation of the gel.

One cluster with 6 pulsotypes was found. Environmental isolates in lanes 2,5,6,8, and 9 were indistinguishable from each other and designated pulsotype A2. They were isolated from the glucose monitor and the TICU. Pulsotype A3 was found in environmental isolate lane 4 (NICU isolation 2 – medication trolley) and clinical samples 10,15,16,17 from neonates in the NICU. Only one isolate had pulsotype A4 from an environmental sample in TICU cubicle D. Pulsotype A5 was isolated from 3 clinical samples in the TICU. Pulsotype A6 was from an environmental sample taken in October 2020 and only differentiated from pulsotype A2 by two fragments. Between pulsotype A1 – A6, there were only 3-4 fragment differences, the multiple

pulsotypes arising because the fragment differences were in different positions. With these few differences in pulsotypes, A1- A6 are probably to possibly related, forming an outbreak cluster. This shows spatial distribution within the unit (and to other referral hospitals) and temporal distribution over the period of blood culture collection and back to October 2020, when isolate 19 was sampled in the unit's environment.

Isolate 3, an environmental sample from NICU 1 – composite high-touch surfaces – bed 3 showed greater than 7 fragment differences being unrelated to cluster A and isolate 18 sampled from the environment in October 2020 also had greater than 7 fragment differences. There was no relation between isolate 3 and isolate 18.

4. DISCUSSION

4.1 Limit of detection from environmental sampling methods

The preliminary evaluation of the sampling method demonstrated that even though MDRO organisms can be identified through broth enrichment at concentrations of 10 to 100 cfu/ml, this did not result in the growth of colonies for counting by the spread plate method. Environmental sampling tends to underestimate the microbiological bioburden present on a surface. This may be due to not removing the organisms from the surface when sampling, poor recovery of organisms from the sampling swab or sponge into the diluent broth, or not surviving the transport from sampling location to the laboratory. Steps taken to minimise these limitations included: performing environmental sampling according to a standardised protocol based on available manuals and manufacturer instructions, vortexing the swabs and stomaching the sponges to improve recovery of organisms, and prompt transport to the laboratory and prompt processing of specimens.

With the addition of nutrient broth media for growth, organisms present in low numbers could be identified and characterised. The sampling methods applied has a higher cfu/ml threshold for identifying *Candida auris* compared to bacteria. Increased growth requirements and time to grow may result in *Candida auris* being outcompeted by bacterial growth for identification. This occurs even when selective media is used to isolate *Candida* spp. The method used could identify MDR ESKAPE organisms, even in low amounts. The method most likely under-represents the microbial bioburden and may miss *Candida* spp that may be present on surfaces.

4.2 Environmental Sampling

A composite sponge sample was used to target high-touch surfaces in proximity to the neonate. This composite sampling evaluated for the potential transfer of microbes from the neonate to proximal high-touch surfaces and the potential for transfer from the surfaces to the neonate. The composite high-touch surface samples represented the majority of MDR ESKAPE organisms isolated, representing the potential transfer from and to health care worker hands before the opportunity to wash hands after interacting with the neonate and immediate surroundings. In a review of evidence that contaminated surfaces contribute to the transmission of HAI, Otter *et al* demonstrated that microbiological studies, epidemiological studies, intervention studies and outbreaks reports show evidence of contaminated surfaces contributing to HAI.(14) Surfaces may remain contaminated, despite multiple appropriate rounds of disinfection.(14) In a study of patient safety related to microbiological contamination of the environment, it was demonstrated that although the majority of positive environmental samples isolated non-pathogenic microorganisms, 20,8% isolated potentially pathogenic microorganisms.(19)

Microbial bioburden was highest when there was associated water, such as the water basins in the unit. This finding of higher microbial bioburden associated with water sourced is congruent with the literature of Walker, Anaissie and Umezawa.(24,26,40) This provides a more favourable environment for bacterial growth. In this study MDR ESKAPE+C organisms were not found on sampling the basins, however from 6 basins CRE *Serratia marcescens* was isolated. *S. marcescens* is a known hospital environmental pathogen and the archetypal HAI pathogen. This organism would be challenging to treat with the CRE resistance mechanism, as this *S. marcescens* is intrinsically resistant to colistin. Colistin is the antibiotic of choice for the treatment of multidrug-resistant CRE organisms in the unit. These CRE *Serratia marcescens* isolates can potentially transfer resistant determinants to other Enterobacterales in the unit. The microbial bioburden of the water basin, taps and faucets was dynamic and showed inconsistency of microbial burden on these surfaces.

Surfaces that were grossly contaminated with blood had a higher microbial burden. Organic secretions provide nutrients and a favourable environment for bacteria to grow and survive. Organic secretions decrease the effectiveness of disinfecting

solutions. Prior cleaning is mandatory to ensure subsequent disinfection is effective.(13)

The microbial bioburden was unevenly distributed in the unit, influenced by overcrowding in certain areas, staff practices, contamination with biological fluids and cleaning practices. The unit had microbial bioburden values above the tentatively suggested cut-off by Dancer of 5 cfu/cm^2.(18) The NICU, with lower patient burden, higher staff-to-patient ratios, less staff movement and dedicated equipment to each neonate, and a cleaning and decontamination procedure after a patient's discharge, had lower microbial bioburdens, with recorded a mean microbial bioburden of 5,6 cfu/cm^2. The TICU showed much variation, but only TICU cubicle A showed a microbial bioburden less than 100 cfu/cm^2. Areas not involved with direct patient care and more distant from the neonates (nursing station, pharmacy preparation area) showed a lower microbial bioburden with a mean of 7,5 cfu/cm^2. This may be due to increased surface cleaning and disinfection and the distance from the patient treatment areas preventing excessive microbial contamination. The flow of medication from the pharmacy area is unidirectional towards the patient only.

The terminally disinfected incubators that were used for a new admission, had low microbial bioburden counts of ≤3 cfu/cm^2. There was no isolation of MDR ESKAPE+C organisms from the incubators tested in this study. The terminally disinfected incubators were not shown to harbour any MDR ESKAPE+C organisms.

ESKAPE organisms were isolated from 49,1% of the environmental samples, of which 70,3% of the ESKAPE organisms showed acquired resistance in their antimicrobial susceptibility patterns. There were no isolates of *Candida auris* nor any other *Candida* spp. This may be due to the requirement for a higher fungal load to identify yeast organisms or suboptimal incubation time to allow the yeast to grow, even in nutrient broth and on selective yeast media. *Candida auris* has similar growth patterns to *Candida albicans,* reaching stationary phase in 20 hours and having a doubling time of 105 minutes in broth culture.(41) Growth should be visible on Sabouraud dextrose agar within 24 hours, depending on inoculum.(41) The high bacterial bioburden may outcompete yeast organisms, decreasing potential yield. Welsh *et al* demonstrated a protocol for enhanced isolation of *Candida auris* using an enriched broth consisting of 10% salt Sabouraud dextrose broth.(42) *Candida auris* can survive in high saline conditions, whereas most other organisms fail to grow.

The addition of antibiotics can further reduce bacterial growth.(42) In retrospect it would have potentially increased the yield of *Candida auris* if this protocol was incorporated into the specimen processing of this study.

There were no isolates of *Pseudomonas aeruginosa* from the environmental samples. *Pseudomonas aeruginosa* is a known hospital contaminant, particular in water sources, and is not a fastidious organism. It was expected that there would be some isolates of *Pseudomonas aeruginosa* identified. The lack of identification may be due to bacterial overgrowth of competing organisms or *Pseudomonas aeruginosa* present in the environment in form that is not easy to sample. *Pseudomonas aeruginosa* was specifically sought on selective and differential MacConkey agar. The lack of isolation of *Pseudomonas aeruginosa* does not mean it is not present in the environment, as organisms in biofilms are not always isolated using methods used during this study

The lack of isolation of the two target organisms, *Pseudomonas aeruginosa* and *Candida auris,* may also have been influenced by the unit's closure for terminal environmental decontamination due to an outbreak of *Candida auris* in the unit, 8 months prior to sampling. This decontamination may have been effective in clearing the unit environment of *Candida auris.* It is not possible to determine if these organisms were not present in the environment or if the environmental sampling and processing were not selective enough to isolate these organisms.

None of the *Enterococcus* spp isolated demonstrated vancomycin resistance. This is in keeping with the local epidemiology of low vancomycin resistance rates and the absence of VRE clinical infection in the unit (unpublished data, internal NHLS laboratory surveillance data 2020-2021). In contrast, only one Gram negative MDRO did not show evidence of resistance, a single *Enterobacter cloacae* isolate. This is in keeping with high rates of MDRO Gram negative infections in the unit (unpublished data, internal NHLS laboratory surveillance data 2020-2021).

Four carbapenem-resistant *Klebsiella pneumoniae* were isolated. All four were negative on the lateral flow assay on the RESIST-4 O.N.K.V (Coris bioConcept, Gembloux, Belgium). This may represent carbapenem resistance by a mechanism not detected by the assay or non-expression of the enzymes for detection. Testing was done from isolates grown on 5% blood agar plates, and microbiological identification and susceptibility testing were done on Vitek 2. Carbapenemases may

not have been adequately expressed for their detection without antibiotic exposure to induce the carbapenemase enzyme on the 5% blood agar plate. Comparison with a molecular PCR test would have been helpful to resolve this issue but, unfortunately, the study was not designed or funded for this extra testing. Most clinical isolates that were carbapenem-resistant tested positive for OXA-48 or NDM carbapenemases. MIC results from antibiotic susceptibility testing indicate high-level resistance to most antibiotics tested. The CRE *Klebsiella pneumoniae* isolates were susceptible to amikacin.

Enterobacter cloacae are known to harbour an inducible chromosomal AmpC beta-lactamase. The *Enterobacter cloacae* isolates met the criteria for ESBL set in this study by being resistant to cefotaxime/ceftriaxone and/or ceftazidime. Still, the antibiotic susceptibility profile of being ampicillin, co-amoxiclav, first and second-generation cephalosporin and cefoxitin resistant, while susceptible to cefepime and piperacillin-tazobactam, suggests a derepressed AmpC being the mechanism of resistance. Distinguishing between these two mechanisms requires molecular testing with either PCR probes, gene amplification and sequencing or line probe assays for ESBL or AmpC enzymes/genes, which were not available during the study period.

4.2.1 Distribution of MDRO isolates

Consistent with the literature, the majority of MDR ESKAPE organisms were found in proximity to the neonates on high-touch surfaces. Using whole genome sequencing Smibert *et al* demonstrated that *S. aureus* found on surfaces, such as keyboards and mobile phones, not in the patients proximity, were not molecularly related to clinical isolates from colonised/infected patients.(43)

Healthcare worker hands most likely act as the mode of transmission between the neonate and the closely located high-touch surfaces. Healthcare worker hands can transfer organisms from colonised/infected neonates to the environment and from the environment to the neonate, establishing an infection/colonisation loop.

Frequently touched equipment in the unit or within a cubicle was colonised with MDR ESKAPE organisms. If not cleaned and disinfected properly, equipment and environmental surfaces can act as reservoirs and serve as vehicles for transmitting organisms around the ward via healthcare worker hands. The only glucose meter

used throughout the unit was contaminated with XDR *Acinetobacter baumannii*. This device is taken from neonate to neonate to test for blood sugar levels and comes into close, sometimes direct contact with the neonate, acting itself as a vehicle for transmitting MDR organisms. The XDR *Acinetobacter baumannii* isolate from the glucose meter was indistinguishable from 4 environmental isolates of XDR *Acinetobacter baumannii* from the TICU. It was part of the outbreak cluster identified on PFGE.

Other high-touch surfaces that can act as a reservoir or vehicle for transmission within the cubicles that demonstrated contamination with MDR ESKAPE organisms include the medication station in each cubicle. XDR *Acinetobacter baumannii* was isolated from the medication stations in NICU cubicles A, C (isolation) and D. It was also isolated from the medication stations in TICU cubicles A and C. These isolates were not included in the PFGE analysis and cannot be determined if they are part of the outbreak cluster identified. The medication station in cubicle B grew an ESBL *Enterobacter cloacae,* which was the predominant MDRO isolated from this cubicle. The medication station is where ivi medications are prepared, and if this contaminates intravenous medication administered it can act as a very efficient transmission route for infection. The multifunctional trolleys in the TICU cubicles are used for various functions, such as note-taking, preparing procedure equipment, and placement for milk bottles before individual feeding had MDRO ESKAPE organisms, reflecting the dominant organism present in the cubicle. This is a vehicle for colonising healthcare workers' hands and equipment used for blood sampling, ivi cannulation, airway manipulation and feeding. These surfaces were not observed to be cleaned before or after use for new activity and were wheeled in the unit between neonates. The weigh station that was available for sampling in TICU D grew an XDR *Acinetobacter baumannii* and an ESBL *Klebsiella pneumoniae.* The ESBL *Klebsiella pneumoniae* was part of the ESBL *Klebsiella pneumonia* environmental cluster that was indistinguishable on PFGE.

Alcoholic hand rub was inadequate in the unit. There were one or two bottles in each cubicle. There should be a bottle or dispenser at each neonate bed to allow easy hand sanitisation before and after contact with the neonate and the surroundings. One sanitiser bottle itself in TICU cubicle D was externally contaminated with ESBL *Klebsiella pneumoniae* and XDR *Acinetobacter baumannii*, acting as a possible vehicle for infection/colonisation transmission if hand hygiene is inadequate after

handing the bottle (inadequate amount of hand rub used or too short a period of application). The handwash basins were not shown to harbour MDR ESKAPE organisms during this study. The literature states that the aerolisation of water during hand washing and basin use can spread HAI pathogens.(24,26) The basin drains grew *Serratia marcescens*, but there were no clinical samples with this organism during the period of collection of clinical samples. Discussion with the head of the units indicated that *Serratia marcescens* was not a frequently isolated organism from clinical specimens in the unit. There was good awareness that the basins should not be used to clean, wash, or dispose of any clinical equipment or patient fluids.

Acinetobacter baumannii was found throughout the unit. *Acinetobacter baumannii* is known to be a hospital environmental contaminant, and this finding was not unexpected. During an ICU outbreak of *Acinetobacter baumannii* in Malaysia, Ng *et* al conducting a point prevalence surveillance investigation of the environment and found extensive environmental contamination of the environment.(22) They found that the environmental isolates were genetically related using WGS, however they were unable to prove a link between environmental isolates and clinical isolates of *Acinetobacter baumannii* and advised further investigation into the epidemiological and clinical risk factors for HAI with *Acinetobacter baumannii*.(22) The frequency of isolation from the environment increased from the NICU to TICU, where cleaning seemed to be less robust, spacing was less between the neonates, and the cubicles were more overcrowded. *Enterobacter cloacae* and *Klebsiella pneumoniae* were found in specific cubicles. This may represent contamination of the environment from a colonised neonate in those cubicles or the staff allocated to those cubicles. Although ESBL *Enterobacter cloacae* was isolated from the environment in TICU cubicles A and B, there were no clinical isolates with this organism during the time of the study. Empiric carbapenem antibiotic therapy, possibly started prior to blood culture collection, could lower the blood culture yield of this organism. Empiric carbapenem therapy can result in selection bias for extensively resistant bacteria, as these remain the only culturable organisms after the commencement of empiric antibiotic treatment.

Isolation of *Enterobacter cloacae* on the TICU nursing station may indicate poor hand hygiene techniques or the movement of used equipment or milk bottles in this area. Patient notes and folders that are stored in the incubator and then taken to the

nursing station to make notes may also act as a vehicle for transmission over such a large distance. There were too few isolates of MRSA to comment on unit distribution.

4.2.2 Microbial bioburden and MDRO ESKAPE organisms

This study did not show an association between the microbial bioburden and the likelihood of isolating a MDR ESKAPE organism. In this study and this environment, high microbial bioburden could not be used as a proxy to suggest further investigation or laboratory workup to investigate MDR ESKAPE organisms and a low or non-detectable microbial bioburden does not indicate that there are no MDR ESKAPE organisms present in the environment. Walker et al found a poor correlation between aerobic colony counts and the isolation of *Pseudomonas aeruginosa* during hospital plumbing sampling.(26) It is uncertain if certain communities of environmental bacteria prevent the establishment of an environmental reservoir of HAI organisms by outcompeting them for resources. Twelve percent (6/52) of the aerobic colony counts from which a MDR ESKAPE organism was isolated were not available to be assessed, reducing the ability to evaluate for an association between microbial bioburden and the isolation of MDR ESKAPE organisms.

4.3 Macro-restriction analysis

4.3.1 Methicillin-resistant *Staphylococcus aureus*

MRSA from a blood culture is probably related to the environmental isolate of MRSA isolated from the TICU isolation room, as indicated by a single fragment difference on PFGE. The blood culture did not state which cubicle in TICU the neonate was in, and one cannot say whether these isolates were from the same cubicle, even if it seems likely. There were seven days between the collection of the clinical sample and environmental sampling. This may represent the environmental persistence of the MRSA isolate in the environment after contamination from the infant. There is no relation between the clinical isolate and the 3 other environmental isolates of MRSA. The two environmental MRSA isolates (isolates 4 and 5) from TICU cubicle A were not related to each other, even though they were isolated one bed apart. Isolate 4 from TICU cubicle A and Isolate 6 from TICU cubicle D are indistinguishable from each other, despite being isolated from essentially opposite ends of the unit. This

suggests transmission via a health care worker over this long distance, and inadequate hand hygiene or transfer of a colonised infant from cubicle A (high care) to cubicle D (step down care). There is insufficient data to implicate the environment in the transmission of MRSA hospital-acquired infection in this study, but the literature has demonstrated this phenomenon.(44,45)

4.3.2 Extended-spectrum beta-lactamase producing *Klebsiella pneumoniae*

The three environmental samples from TICU cubicle D were indistinguishable from each other and likely represented local contamination. Of the 5 clinical isolates, only one was probably related to the environmental cluster with only 3 fragment differences from the environmental isolates. This clinical isolate was sampled from an infant in the TICU (cubicle not recorded) 3 days before the environmental sampling occurred. The other clinical isolates were not related to the environmental cluster based on the PFGE performed and were not related to each other. The clinical isolates were spread throughout the unit and over the time frame that clinical isolates were collected. The non-relatedness of the clinical isolates may be representative of acquiring infection before arrival in the NICU/TICU unit, possibly representative of colonising bacteria from the mother during childbirth or from the labour ward at the time of delivery. During the period of collection of clinical and environmental samples, this study demonstrated minimal evidence for ESBL *Klebsiella pneumoniae* in the environment acting as a reservoir/source of infection of neonates within the unit.

4.3.3 Extensively drug-resistant *Acinetobacter baumannii*

Seven of the 8 environmental isolates and 7 of the clinical isolates were shown to be part of the same outbreak cluster. There were 6 pulsotypes identified within this cluster. Each pulsotype of the outbreak cluster was spatially distributed in the unit. Pulsotype A2 was isolated from environmental samples in TICU, identified in four of the five cubicles of the TICU, cubicle A, cubicle B, cubicle C and the isolation cubicle. This pulsotype was also isolated from the only glucose monitor in the combined NICU/TICU unit.

The 3 clinical blood culture isolates from patients in TICU, sampled from15-17 July 2021, were all pulsotype A5, which differed from pulsotype A2 by 2 fragments,

indicating probable relation and persistence in the outbreak strain from the clinical sample to the environment over 10 days.

Pulsotype A3 was isolated from the NICU environment, from isolation cubicle 2 on the medication trolley and all the blood culture isolates (n=4) that were taken from neonates in the NICU. The NICU blood culture isolates were taken over a date range from 8 July to 22 July, indicating possible persistence in the unit over this period.

The outbreak cluster showed environmental persistence over at least 8 months. As demonstrated by the environmental isolate from Oct 2020 (from previous environmental sampling during an outbreak) with pulsotype A6, also being part of the outbreak cluster. *Acinetobacter baumannii* may persist in the environment over time and still cause infective outbreaks. The control sample used was isolated from a clinical sample from a neonate in a neonatal unit located in a different hospital in the same province. It was an unexpected result that it had a pulsotype (pulsotype A1) probably related to the outbreak strain. This most likely demonstrates inter-facility spread. These different hospital neonatal units often transfer patients between each other depending on the clinical needs and services offered at the respective units. Baang *et al* demonstrated environmental persistence of an *Acinetobacter baumannii* outbreak strain in the hospital environment over three years, despite aggressive infection control procedures.(46) The geographical clustering between two closely interlinked hospitals is unsurprising as Hamidian and Negro have demonstrated the global distribution of *Acinetobacter baumannii* sequence type (ST) 1 and ST2 based on multi-locus sequence typing (MLST) v2.16.1 (https://github.com/tseemann/abricate).(47) They analysed 3575 sequenced *Acinetobacter baumannii* genomes downloaded from the GenBank database and found that globally 66% of sequenced clinical isolates were in ST1 and ST2, with 59% being ST2. The majority of sequenced genomes came from the USA, China, Thailand, Australia and Pakistan, but did include genomes from all over the world. (46) This demonstrates outbreaks occurring due to two global clones. PFGE is more discriminatory than MLST, but evaluation by WGS would provide more discrete discrimination of the clones. PCR and sequencing or WGS were not performed as this was not within the scope of the study and would not have been affordable due to funding constraints.

Different pulsotypes (A1-A6) were identified within the XDR *Acinetobacter baumannii* outbreak cluster. PFGE allows for the identification of pulsotype variation within the different areas of the unit. Pulsotypes A2, A4 and A5 were only isolated from the TICU. Pulsotype A2 (n=5) and A4 (n=1) were isolated from the TICU environment A5 (n=3) from clinical isolates originating from the TICU. Pulsotype A3 (n=5) was identified from clinical and environmental isolates from the NICU. Localising of different pulsotypes to different areas of the unit may be due to the relative separation of staff between the NICU section and the TICU section. This may also be biased due to sampling the environment once, providing a static representation of the environmental XDR *Acinetobacter baumannii* distribution. Repeat sampling of the environment would provide further information on the dynamics of environmental pulsotypes. Performing PFGE only on a representative selection of the large number of XDR *Acinetobacter baumannii* isolates available limited the information obtainable from the macro-restriction analysis. There is evidence of *Acinetobacter baumannii* strains not related to the outbreak strain in the environment, as evidenced by the isolation of unrelated pulsotypes, pulsotype B from an environmental sample and pulsotype C from a clinical sample from a neonate from the NICU.

4.4 Limitations

Limitations identified in this study are:

- Single time point sampling of the environment provides a single snapshot of the organisms present in the environment and limits the evaluation of temporal evolution of environmental organisms.
- Performing environmental sampling at the end of the collection period of the clinical samples, instead of at the mid-way point, may have influenced findings of clonal transmission of environmental bacterial isolates and clinical isolates.
- Performing environmental sampling during the second wave of the COVID-19 outbreak may have influenced findings. Increased infection prevention control measures were instituted at the hospital and unit entrance. The staffing and maternal movement were changed. There were fewer people (mothers and family) allowed in the unit. Health care workers may have had enhanced hand hygiene adherence, lowering spread via health care worker hands.

Alternatively, COVID 19 may have resulted in health care worker absenteeism or redeployment, exacerbating poor staff-patient ratios.

- Enrichment by nutrient broth for the growth and processing of stressed and MDR ESKAPE organisms present in low numbers precludes the possibility of MDR ESKAPE quantification.
- Lack of information about the specific cubicle neonates were in at the time of blood culture collection limits correlation with the environmental isolates in specific cubicles.
- Lack of monitoring for colonisation in the unit prevents assessing colonised infants as a source of MDR organisms.
- Lack of funding prevented accessing newer whole genome sequencing that provides more discriminatory ability than PFGE.

5. CONCLUSION

Microbiological cleanliness of the hospital environment is an important and often underappreciated factor in preventing HAI. Environmental sampling can provide valuable information about the environmental bioburden composition and the distribution of organisms.

The study determined the microbial bioburden and distribution of MDR ESKAPE organisms in the unit. The majority of MDR ESKAPE organisms were isolated from high-touch surfaces. This study did not demonstrate a correlation between the level of the environmental microbial bioburden in cfu/cm^2 and the isolation of MDR ESKAPE organisms.

The association of MDR ESKAPE pathogens in clinical samples and environmental samples related by macro-restriction analysis underscores the importance of the environment in the acquisition of HAI. Temporal persistence demonstrated by macro-restriction analysis of *Acinetobacter baumannii* isolates suggests the environment may act as a reservoir for HAI organisms.

In the context of persistent infection within the neonatal unit with MDRO ESKAPE organisms, these findings emphasise the importance of infection prevention and control practices and interventions aimed at environmental cleanliness and disinfection. These interventions should be continuously emphasised and targeted as the need arises.

6. APPENDICES

Appendix 1 – Ethics clearance letter

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr Michel Le Grange

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M200228

NAME: (Principal Investigator)	Dr Michel Le Grange
DEPARTMENT:	Clinical Microbiology and Infectious Diseases Chris Hani Baragwanath Academic Hospital National Health Laboratory Service
PROJECT TITLE:	Characterisation of common environmental healthcare associated infection pathogens and their genetic relatedness to isolates causing severe infection in a neonatal care unit at a tertiary level hospital in Soweto
DATE CONSIDERED:	28/02/2020
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Andriano Duse
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	03/08/2020

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

Appendix 2 Environmental Sampling Taking Log

Date:_____

Sampler:_____

SAMPLE METHOD	AREA	CUBICLE	SITE SAMPLED	SAMPLE EPISODE	DATE PROCESSED

Appendix 3 – Quality control template and expected outcomes

Catalase Exp Date:	**Reaction**
Staphylococcus aureus ATCC2593	**Positive - bubbles seen**
Enterococcus faecalis ATCC 29212	**Negative - No bubbles**
Oxidase Exp Date:	**Reaction**
Pseudomonas aeruginosa ATCC27853	**Positive - Blue/Purple colour in 10 seconds**
Escherichia coli ATCC 25922	**Negative - No colour change**
Rapid Indole Exp Date:	**Reaction**
Escherichia coli ATCC 25922	**Positive - Green colour in 30 seconds**
Pseudomonas aeruginosa ATCC27853	**Negative - pink colour**
Staphurex Exp Date:	**Reaction**
Batch Number:	
Staphylococcus aureus ATCC2593	**Positive - Agglutination**
Staphylococcus epidermidis ATCC12228	**Negative - No Agglutination**
Latex Control	**Agglutination**
PYR Exp Date:	**Reaction**
Batch Number:	
Escherichia coli ATCC 25922	**Negative - No reaction**
Enterococcus faecalis ATCC 25923	**Positive – Pink colour in 1 minute**
Colourex Staph Exp Date:	**Reaction**
Batch Number:	
Staphylococcus aureus ATCC2593	**Growth good to excellent, lilac-coloured colonies**
Escherichia coli ATCC 25922	**No Growth**
Staphylococcus epidermidis ATCC12228	**No growth to fair growth; green to blue colonies**
Uninoculated	**Yellowish to light brownish, opaque**
MacConkey Agar Exp Date:	**Reaction**
Batch Number:	
Staphylococcus aureus ATCC2593	**Inhibition, partial to complete**
Proteus mirabilis ATCC1253	**Growth, colourless colonies, no swarming**
Escherichia coli ATCC 25922	**Growth, pink colonies, +- bile precipitant**
Enterococcus faecalis ATCC 25923	**Inhibition, partial to complete**
Uninoculated	**Light Pink, translucent**
CNA Agar Exp Date:	**Reaction**
Batch Number:	
Staphylococcus aureus ATCC2593	**Good to excellent growth, may be beta-haemolytic**
Escherichia coli ATCC 25922	**Inhibition partial to complete**
Streptococcus pyogenes ATCC 19615	**Good to excellent growth, beta haemolysis**
Enterococcus faecalis ATCC 25923	**Good to excellent growth, variable haemolysis**
Uninoculated	**Reb (blood) colour**
Sabouraud Detrose Agar Exp Date:	**Reaction**
Batch Number:	
Candida albicans ATCC 10231	**Growth, Creamy white colonies**
Uninoculated	**Straw coloured**

Appendix 4 - Standard operating procedure – Pulsed-field electrophoresis for MRSA

Macro-restriction analysis of Staphylococci for epidemiological analysis

Purpose

The purpose of this method is to determine the degree of relatedness (if any) between Staphylococci isolates for epidemiological analyses.

Principle

Single colonies from blood agar plates are inoculated into BHI broth and incubated overnight at 36℃. DNA is then extracted within agarose blocks and subsequently macro-restricted using *SmaI* restriction enzyme. Fragments are separated through pulsed-field gel electrophoresis (PFGE) and relatedness established by comparing the number of band differences.

Method

The method is based in principle on that published by McDougal et al. (2003). The protocols have been optimised using the Bio-Rad Chef DRII and DRIII system.

Equipment

- 36℃ incubator
- Spectrophotometer (600nm)
- Benchtop centrifuge
- 37℃ heating block
- 50℃ water bath
- 56℃ shaking incubator
- Bio-Rad DRIII system
- Bio-Rad agarose block molds
- Bio-Rad Gel Doc
- Microwave
- Shaker
- 1000µl, 100µl and 10µl pipette
- Timer
- cuvettes

Reagents required

- DNA isolation:
    - EET buffer: 100 mM EDTA, 10 mM Tris-HCl pH 8
    - PFGE storage buffer: 10mM EDTA, 10mM Tris-HCl pH 8.0
    - 5 mg/ml lysostaphin
    - 20 mg/ml proteinase K (prepare aliquots and store at -20°C)
    - 10% SDS
- Digestion with restriction enzyme:
    - *SmaI* restriction endonuclease
    - *XbaI* restriction endonuclease for ladder
- 400 mM PMSF (made up in ethanol), stored at -20□C
- PFGE storage buffer: 10 mM EDTA, 10 mM Tris-HCl pH 8.0 (500ml)
- 10 mM Tris-HCl pH 8.0 (500ml)
- Pulsed-field gel electrophoresis:
- 0.5X TBE buffer: 45mM Tris-Borate, pH 8.0; 1mM EDTA (200ml 10X TBE in 4
    - liters)
- Ethidium Bromide (10mg/ml)

DNA isolation

Extraction of DNA is carried out within agarose blocks to prevent shearing of DNA.
Subculture a single colony from the blood agar plate into a BHI broth. Subculture a single colony of Salmonella ser. Braenderup H9812 into BHI broth. Incubate overnight at 36C +-1C. NOTE: Cell suspension and casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of 10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches

- Aliquot 1.9 ml into 2ml eppendorf tube and centrifuge at 8000 rpm for 2 min.
- Remove supernatant and resuspend in 500 mcl EET buffer.
- Warm to 37C in heating block.
- Add 500mcl 1.5% agarose in EET buffer (0.3g in 20ml), melted and cooled to approximately 50C.
- Mix quickly and pour into Block moulds (supplied by Bio-Rad).
- Allow to set for 15 min.
- Press out blocks into 2ml eppendorf tube.
- Calculate amount of EET buffer needed for number of tubes used (2 ml per tube).
- Add 4 mcl lysostaphin per 2 ml EET buffer.
- Add 2 ml EET containing 5 mcg/ml lysostaphin to each tube.
- Incubate at 30C for 5 hours with gentle shaking.
- Calculate amount of EET buffer needed for number of tubes used.
- Add 100 mcl Proteinase K and 200mcl 10% SDS per 2 ml EET buffer.
- Replace EET buffer containing lysostaphin with 2 ml EET buffer containing proteinase K and incubate overnight at 37C with gentle shaking.
- Replace EET buffer with PFGE storage buffer and store at 2-8C.

Digestion of DNA with restriction endonucleases

- Cut agarose block containing DNA to a volume of 30 □l (one third) and place in 2ml Eppendorf tube.
- Wash block twice in 2ml storage buffer containing 1mM PMSF for 40 min at room temperature each time (add 25 mcl 400mM PMSF to 10 ml storage buffer).
- Wash block 3 times in 2ml 10 mM Tris buffer, pH 8 for 20 min at room temperature.
- Remove excess liquid and add 70 mcl solution containing 10 mcl 10X reaction buffer, 10 mcl 0.01% BSA, and 30 units SmaI restriction endonuclease (3 mcl of 10 U/mcl stock, 0.3 U/□mc final concentration).
- Incubate for 2 hours at 30C.
- Perform PFGE immediately after digestion.

PFGE

- Pour 1% Pulse field certified agarose gel using TBE buffer. For a standard sized gel use 1g agarose in100 ml of TBE buffer. If more than 20 wells are needed, use a large gel. For large gels, use 1.6g in 160ml of TBE buffer.
- Allow to set for 1 hour.
- Pour 2l TBE buffer into electrophoresis chamber and switch on peristaltic pump followed by the cooling unit set to 14C, at least 1 hour before digest is complete.
- Allow buffer to reach 14C.
- Insert agarose blocks into wells in the gel.
- Fix blocks in place using molten 1% agarose.
- Place gel in electrophoresis chamber.

- Set current-switching parameters of apparatus for optimal separation of DNA fragments.
- The parameters for Staphylococci digested with *SmaI* have been optimised at: 6V/cm; 20 hours running time; 5 sec initial switch time; 50 sec final switch time.
- Operate the Chef DRII electrophoresis apparatus according to SOP NJHF0093.
- Remove gel from apparatus and stain in water containing 0.5mcg/ml ethidium bromide for 30 min
- Destain in water for 20 min.
- View using a UV transilluminator.
- Capture the image on the gel doc system (SOP NJHF0093).

Interpretation of results

Results are interpreted according to Tenover criteria. Briefly, the banding patterns of isolates are compared, and the number of band differences counted. The number of band differences is used to indicate whether isolates are related and to what degree they are related.

Controls

Reference strains should be included to verify the discriminatory power of the technique. i.e., Reference strains, where possible, or epidemiologically unrelated strains, would be expected to have different patterns to the isolates under investigation. If this is not the case the discriminatory power of the technique should be questioned.

Appendix 5 – Standard operating procedure – Pulsed-field electrophoresis for Enterobacterales

Macro-restriction analysis of Gram-negative Enterobacteraciae organisms for epidemiological analysis

Purpose

The purpose of this method is to determine the degree of relatedness (if any) between Gram-negative organisms for epidemiological analyses.

Principle

Single colonies from blood agar plates are inoculated into BHI broth and incubated overnight at 36℃. DNA is then extracted within agarose blocks and subsequently macro-restricted using *XbaI* restriction enzyme. Fragments are separated through pulsed-field gel electrophoresis (PFGE) and relatedness established by comparing the number of band differences. The following protocols have been optimised for Enterobacter cloacae, Serratia odorifera, P.aeruginosa and K. pneumoniae. Use of different organisms will require individual optimisation by trial and error.

Method

The protocols have been optimised using the Bio-Rad Chef DRIII systems. This method is based in principle on the reference by Gautom (1997) with some modifications.

Equipment

- 36℃ incubator
- Spectrophotometer (600nm)
- Benchtop centrifuge
- 37℃ heating block
- 50℃ water bath
- 56℃ shaking incubator
- Bio-Rad DRIII system
- Bio-Rad agarose block molds
- Bio-Rad Gel Doc
- Microwave
- Shaker
- 1000µl, 100µl and 10µl pipette
- Timer
- cuvettes

Reagents required

- DNA isolation:
    - PFGE lysis buffer: 0.1M EDTA, 10 mM Tris-HCl pH 8.0, 1% sarcosyl
    - 20 mg/ml proteinase K
    - TE buffer: 1mM EDTA, 10mM Tris-HCl pH 8.0
    - PFGE storage buffer: 10mM EDTA, 10mM Tris-HCl pH 8.0
- Digestion with restriction enzyme:
    - *XbaI* restriction endonuclease
    - 400 mM PMSF (made up in ethanol), stored at –20C
    - PFGE storage buffer: 10 mM EDTA, 10 mM Tris-HCl pH 8.0
    - 10 mM Tris-HCl pH 8.0
- Pulsed-field gel electrophoresis:
    - 0.5X TBE buffer: 45mM Tris-Borate, pH 8.0; 1mM EDTA

DNA isolation

Extraction of DNA is carried out within agarose blocks to prevent shearing of DNA.

Subculture a single colony from a blood agar plate into a BHI broth. Incubate overnight at 36C.

NOTE: Cell suspension and casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. If large numbers of samples are being prepared, it is recommended that they be processed in batches of around 10 samples at a time. Once the first batch of isolates are in the cell lysis incubation, then start preparing the cells suspensions the next group samples, and so on. All batches can be lysed and washed together, since additional lysis time will not affect the initial batches Measure the OD at 600nm.

- Normalise the samples, by aliquoting that volume of sample that would expect to give an OD 0f 0.9, into a 2ml microfuge tube
- Centrifuge at 10 000rpm for 2 min.
- Remove supernatant and resuspend the pellet in 500 mcll TE buffer.
- Warm tubes to 37 in a heating block.
- Add 500mcl 1.5% agarose in TE buffer, melted and cooled to approximately 50C in a water bath.
- Mix quickly and pipette into block molds (supplied by Bio-Rad).
- Allow to set for 15 min.
- Press out blocks into 10 or 15ml centrifuge tube.
- Calculate amount of PFGE lysis buffer needed for number of tubes used.
- Add 60 mcl of 20 mg/ml Proteinase K per 6 ml PFGE lysis buffer.
- Add 6 ml PFGE lysis buffer to each tube.
- Incubate for 5 hours at 36C with gentle shaking.
- Replace lysis buffer with 6 ml fresh PFGE lysis buffer (with proteinase K added) and incubate overnight at 50C.
- Transfer blocks to 2 ml microfuge tube, add 2 ml PFGE storage buffer and store at 2-8C.

Digestion of DNA with restriction endonucleases

- Cut agarose block containing DNA to a volume of 30 mcl (one third) and place in 2ml microfuge tube.
- Wash block twice in 2 ml storage buffer containing 1 mM PMSF for 40 min at room temperature each time (add 5mcll of 400mM PMSF to 2 ml storage buffer).
- Wash block 3 times in 2 ml 10 mM Tris buffer, pH 8 for 20 min at room temperature.
- Remove excess liquid and add 70 mcl mixture containing 10 mcll 10X reaction buffer, 10 mcll 0.01% BSA, and 30 units *XbaI* restriction endonuclease
- Incubate for recommended time for *Klebsiella pneumoniae* for 3 hours

PFGE

- Pour 1% Pulse field certified agarose gel using TBE buffer. For a standard sized gel use 1g agarose in100 ml of TBE buffer. If more than 20 wells are needed, use a large gel. For large gels, use 1.6g in 160ml of TBE buffer.
- Allow to set for 1 hour.
- Pour 2l TBE buffer into electrophoresis chamber and switch on peristaltic pump followed by the cooling unit set to 14C, at least 1 hour before digest is complete.
- Allow buffer to reach 14C.
- Insert agarose blocks into wells in the gel.
- Fix blocks in place using molten 1% agarose.
- Place gel in electrophoresis chamber.
- Set current-switching parameters of apparatus for optimal separation of DNA for *Klebsiella pneumoniae* 6V/cm; 18 hours running time; 5 sec initial switch time; 50 sec final switch time.

- Operate the Chef DRII electrophoresis apparatus according to SOP NJHF0093.
- Remove gel from apparatus and stain in water containing 0.5mcg/ml ethidium bromide for 30 min
- Destain in water for 20 min.
- View using a UV transilluminator.
- Capture the image on the gel doc system (SOP NJHF0093).

Interpretation of results

Results are interpreted according to Tenover criteria. Briefly, the banding patterns of isolates are compared, and the number of band differences counted. The number of band differences is used to indicate whether isolates are related and to what degree they are related.

Controls

Reference strains should be included to verify the discriminatory power of the technique. i.e., Reference strains, where possible, or epidemiologically unrelated strains, would be expected to have different patterns to the isolates under investigation. If this is not the case the discriminatory power of the technique should be questioned.

Appendix 6 – Standard operating procedure – Pulsed-field electrophoresis for *Acinetobacter baumannii*

Macro-restriction analysis of Acinetobacter baumannii for epidemiological analysis

Purpose

The purpose of this method is to determine the degree of relatedness (if any) between clinical *Acinetobacter baumannii* isolates for epidemiological analyses.

Principle

Single colonies from blood agar plates are inoculated into BHI or tryptic soy broth and incubated overnight at 36℃. DNA is then extracted within agarose blocks and subsequently macro-restricted using *ApaI* restriction enzyme. Fragments are separated through pulsed-field gel electrophoresis (PFGE) and relatedness established by comparing the number of band differences.

Method

The protocols have been optimised using the Bio-Rad DRIII systems. The method used is adapted from those by Seifert et al. (1994) and Sader et al. (1996).

Equipment

- 36℃ incubator
- Spectrophotometer (600nm)
- Benchtop centrifuge
- 37℃ heating block
- 50℃ water bath
- 56℃ shaking incubator
- Bio-Rad DRIII system
- Bio-Rad agarose block molds
- Bio-Rad Gel Doc
- Microwave
- Shaker
- 1000µl, 100µl and 10µl pipette
- Timer
- cuvettes

Reagents required

- DNA isolation:
  - SE buffer: 75mM NaCl-25mM EDTA, pH 7.4 (500ml)
  - Lysis buffer: 50mM Tris-50mM EDTA (pH 8.0) containing 1% N-lauryl sarcosine
  - 20 mg/ml proteinase K (prepare aliquots and store in -20°C)
  - PFGE storage buffer: 10mM EDTA, 10mM Tris-HCl pH 8.0 (500ml)
  - Salmonella ser. Braenderup H9812 culture standards for ladder
  - PFGE agarose
- Digestion using restriction enzymes:
  - *ApaI* restriction enzyme
  - *XbaI* restriction enzyme for ladder
  - 400 mM PMSF (made up in ethanol), stored at -20C
  - PFGE storage buffer: 10 mM EDTA, 10 mM Tris-HCl pH 8.0
  - 10 mM Tris-HCl pH 8.0 (500ml)
- Pulsed-field gel electrophoresis:
  - 0.5X TBE buffer: 45mM Tris-Borate, pH 8.0; 1mM EDTA pH 8.0 (200ml 10 XTBE in 4L)
  - PFGE agarose

- 10mg/mg Ethidium Bromide

DNA isolation

Extraction of DNA is carried out within agarose blocks to prevent shearing of DNA.
Subculture a single colony from the blood agar plate into a BHI or tryptic soy broth.
Incubate overnight at 36C

- Turn on Heating block (37°C ± 0.5°C), Shaker incubator (56°C ± 1°C), Water baths (60°C) and Spectrophotometer @ 600nm.
- Thaw proteinase K aliquots
- Normalise the amount of bacteria used per block by measuring the optical density at 600nm.
- Aliquot that amount of culture that corresponds to an OD of 1 into a 2ml eppendorf tube and centrifuge at 8 000 rpm for 2 min on a benchtop centrifuge.
- Remove the supernatant and resuspend in 500mcl SE buffer. Do not vortex
- Warm to 37C in heating block.
- Add 500mcll 2% PFGE agarose in SE buffer (2g in 100ml), melted and cooled to approximately 60C in the water bath.
- Mix quickly and pipette into Block molds (supplied by Bio-Rad). Over-pipetting can cause DNA shearing. Do not allow bubbles to form.
- Allow to set for □15 min.
- Trim excess agarose from top of plugs with scalpel, razor blade or similar instrument. Press out blocks into 15ml disposable tube.
- Calculate amount of lysis buffer needed for number of tubes used (5ml per tube).
- Add 250mcl 20mg/ml proteinase K per 5 ml lysis buffer.
- Add 5 ml lysis buffer containing the 1mg/ml proteinase K to each tube.
- Incubate at 56C for 18 hours in shaking incubator at ± 100rpm.
- Replace lysis buffer with 2ml PFGE storage buffer and store at 2-8□C for up to 1 year.

Digestion of DNA with restriction endonucleases

Prepare the following:

- Thaw 10X buffers for ApaI and XbaI, BSA 10mg/ml, and PMSF aliquots
- Keep vial of restriction enzyme on ice or in insulated storage box (-20°C) at all times.
- Switch on 37□C incubator

- Cut agarose block containing DNA to a volume of 30 □l (one third or 2-2.5mm thick slice) and place in 2ml eppendorf tube.
- Cut 2.0-2.5mm wide slices of the appropriate number of S. ser. Braenderup H9812 Standard DNA ladder (3 for 10 well gel; 4 for 15 well gel), with a single edge scalpel blade
- Wash block twice in 2 ml storage buffer containing 1 mM PMSF for 40 min at room temperature each time (add 25 mcl 400mM PMSF to 10 ml storage buffer).
- Wash block 3 times in 2 ml 10 mM Tris buffer, pH 8 for 20 min at room temperature.
- Be careful not to damage the plug slice with pipette.
- Prepare a separate master mix for the samples and the ladder

For Samples:

- Remove excess liquid and add 70 mcl solution containing 10 mcl 10X reaction buffer, 10 mcl 0.1% BSA, and 20 units *ApaI* restriction endonuclease (0.2 U/mcl final concentration). Be sure the plug slices are covered by the solution
- Incubate for 4 hours at 37C.
- Perform PFGE after digestion

PFGE

- Pour 1% Pulse field certified agarose gel using TBE buffer. For a standard sized gel use 1g agarose in100 ml of TBE buffer. If more than 20 wells are needed, use a large gel. For large gels, use 1.6g in 160ml of TBE buffer.
- Allow to set for 1 hour.
- Pour 2l TBE buffer into electrophoresis chamber and switch on peristaltic pump followed by the cooling unit set to 14C, at least 1 hour before digest is complete.
- Allow buffer to reach 14C.
- Insert agarose blocks into wells in the gel.
- Fix blocks in place using molten 1% agarose.
- Place gel in electrophoresis chamber.
- Set current-switching parameters of apparatus for optimal separation of DNA for *Acinetobacter baumannii* 6V/cm; 23 hours running time; 5 sec initial switch time; 25 sec final switch time.
- Operate the Chef DRII electrophoresis apparatus according to SOP NJHF0093.
- Remove gel from apparatus and stain in water containing 0.5mcg/ml ethidium bromide for 30 min
- Destain in water for 20 min.
- View using a UV transilluminator.
- Capture the image on the gel doc system (SOP NJHF0093).

Interpretation of results

Results are interpreted according to Tenover criteria. Briefly, the banding patterns of isolates are compared, and the number of band differences counted. The number of band differences is used to indicate whether isolates are related and to what degree they are related.

Controls

Reference strains should be included to verify the discriminatory power of the technique. i.e., Reference strains, where possible, or epidemiologically unrelated strains, would be expected to have different patterns to the isolates under investigation. If this is not the case the discriminatory power of the technique should be questioned.

Appendix 7 – Consolidated data sheet

Sample ID	Area	Cubicle	Site	Method	Surface area (cm^2)	CFU/ml	CFU/cm2	ESKAPE ISOLATED						Other Organism
								Organism1	RP	Organism 2	RP	Organism 3	RP	
ENV0001	Pharmacy		Medication Prep	Sponge	20 000	No Growth	NG							
ENV0002	Entrance		Door handle	sponge	200	<30	<1							Bacillus spp
ENV0003	Control Swab			Swab		No Growth	NG							
ENV0004	Entrance		Basin Drain	Swab	25	innumerable	innumerable							Serratia marcescens CRE
ENV0005	Entrance		Tap faucet	Swab	5	No growth	NG							
ENV0006	Entrance		Tap handles	Swab	20	<30	50							Bacillus spp
ENV0007	Entrance		U/S probes	swab	25	550	110							Bacillus spp, CNS
ENV0008	NICU	Nursing Station	Phone	Sponge	300	No Growth	NG							
ENV0009	NICU	Nursing Station		Sponge	40 000	<30	<1							Bacillus spp, CNS
ENV0010	NICU	A	Basin Drain	Swab	25	370	74							Bacillus spp, Micrococcus, Serattia marcesens CRE
ENV0011	NICU	A	Tap faucet	Swab	5	280000	280000							Baccilus, micrococcus
ENV0012	NICU	A	Tap handles	Swab	20	450	112							Bacillus spp, CNS
ENV0013	NICU	A	Medication Trolley	Sponge	500	2100	42	Acinetobacter baumannii	XDR					CNS
ENV0014	NICU	A	Glucose meeter	Swab	25	<30	<1	Acinetobacter baumannii	XDR					CNS
ENV0015	NICU	A	Handwash bottle	Sponge	200	620	31							Bacillus, CNS,micrococcus
ENV0016	NICU	A	High touch bed 1 (ventilator, infusion pump, monitor)	Sponge	5700	930	2	Acinetobacter baumannii	XDR					CNS
ENV0017	NICU	A	High touch bed 2 (ventilator, infusion pump, monitor)	Sponge	5700	570	1	Enterococcus faecium	nil					CNS
ENV0018	NICU	A	High touch bed 3 (ventilator, infusion pump, monitor)	Sponge	5700	600	1	Acinetobacter baumannii	XDR					Bacillus, CNS
ENV0019	NICU	A	(ventilator, infusion pump, monitor, trolley)	Sponge	5700	1200	2							Baciliu, CNS
ENV0020	NICU	A	High touch bed 5 (ventilator, infusion pump, monitor)	Sponge	5700	<30	<1							Bacillus spp, CNS
ENV0021	NICU	A	High touch bed 6 (ventilator, infusion pump, monitor)	Sponge	700	<30	<1	Acinetobacter baumannii	XDR					Bacillus spp, CNS
ENV0022	NICU	Isolation 1	Basin Drain	Swab	25	74000	2960							Serratia marcescens CRE
ENV0023	NICU	Isolation 1	Tap faucet	Swab	5	TNTC	TNTC							Bacillus spp, micrococcus
ENV0024	NICU	Isolation 1	Tap handles	Swab	20	<30	<1							Bacillus spp
ENV0025	NICU	Isolation 1	Medication Table	Sponge	500	700	14							CNS
ENV0026	Pharmacy		Delivery table	Sponge	500	No Growth								
ENV0027	NICU	Isolation 1	Handwash bottle	Sponge	200	No Growth								Bacillus, CNS
ENV0028	NICU	Isolation 1	High touch bed 7 (ventilator, infusion pumps, monitor, trolley)	Sponge	700	<30	<1	Enterococcus faecium	R ampi					CNS
ENV0029	NICU	Isolation 2	Basin Drain	Swab	25	TNTC	TNTC							Serattia marcesens CRE
ENV0030	NICU	Isolation 2	Tap faucet	Swab	5	contaminated	contaminated							Bacillus spp
ENV0031	NICU	Isolation 2	Tap handles	Swab	20	<30	25							Bacillus spp, micrococcus, CNS, mould
ENV0032	NICU	Isolation 2	Medication table	Sponge	500	690	14	Enterococcus faecium	R ampi	Acinetobacter baumannii	XDR			
ENV0033	TICU	Isolatio E	High Touch 1 (Monitor)	Sponge	200	<30	10							CNS, Strept spp
ENV0034	NICU	Isolation 2	Handwash bottle	Sponge	200	300	15							Micrococcus, CNS
ENV0035	NICU	Isolation 2	High touch bed 8 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	370	1	Enterococcus faecium	R ampi	Klebsiella pneumoniae	CRE (Enzyme not identified)			Mould, CNS
ENV0036	NICU	D	Basin Drain	Swab	25	2500	500							Serratia marcescens CRE
ENV0037	NICU	D	Tap faucet	Swab	5	810	8100							Bacillus spp
ENV0038	NICU	D	Tap handles	Swab	20	<30	<1	Acinetobacter baumannii	XDR					
ENV0039	NICU	D	Medication table	Sponge	500	860	17	Acinetobacter baumannii	XDR					CNS

ENV0040	TICU	Isolation E	(CPAP, infusion, monitor)	Sponge	500	contaminated	contaminated	Staphylococcus aureus	MRSA	Acinetobacter baumannii	XDR			
ENV0041	NICU	D	Handwash bottle	Sponge	200	<30	4	Enterococcus faecium	R ampi					CNS
ENV0042	NICU	D	High touch bed 9 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	2200	4	Acinetobacter baumannii	XDR					CNS
ENV0043	NICU	D	High touch bed 10 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	860	2	K. pnemoniae	CRE (enzyme not identified)					CNS
ENV0044	TICU	D	High touch bed 7 (infusion pump)	Sponge	200	890	45	Enterococcus faecium	R ampi	Klebsiella pneumoniae	CRE(enzyme not identified			Bacillus, CNS
ENV0045	NICU	D	High touch bed 12 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	<30	3	Enterococcus faecium	R ampi	Acinetobacter baumannii	XDR			Bacillus, CNS
ENV 0046	TICU	Nursing Station		Sponge	40 000	620	<1	Enterococcus faecium	R ampi	Enterobacter cloacae	ESBL			CNS
ENV0047	TICU	A	Basin Drain	Swab	25	970	194							Bacillus, CNS, Serattia marcescens CRE
ENV0048	TICU	A	Tap faucet	Swab	5	No Growth	NG							
ENV0049	TICU	A	Tap handles	Swab	20	<30	35							Bacillus, CNS
ENV0050	TICU	A	Medication Table	Sponge	500	640	13	Acinetobacter baumannii	XDR					CNS
ENV0051	TICU	A	Trolleys	Sponge	6000	300	1	Enterococcus faecium	R ampi	Acinetobacter baumannii	XDR			CNS
ENV0052	TICU	A	handwash bottle	sponge	200	<30	5							CNS
ENV0053	TICU	A	High touch bed 1 (CPAP, monitor, infusion pump)	Sponge	700	400	6	Enterococcus faecium	R ampi	Staphylococcus aureus	MRSA			CNS
ENV0054	TICU	A	High touch bed 2 (CPAP, monitor, infusion pump)	Sponge	700	1200	18	Acinetobacter baumannii	XDR					Bacillus, CNS
ENV0055	TICU	A	High touch bed 3 (CPAP, monitor, infusion pump)	Sponge	700	580	8	Staphylococcus aureus	MRSA	Enterobacter cloacae	ESBL			CNS
ENV0056	TICU	A	High touch bed 4 (CPAP, monitor, infusion pump)	Sponge	700	6000	85	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS, Pantoa spp
ENV0057	TICU	A	High touch bed 5 (CPAP, monitor, infusion pump)	Sponge	700	<30	2	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS
ENV0058	TICU	A	High touch bed 6 (CPAP, monitor, infusion pump)	Sponge	700	<30	3	Enterobacter cloacae	ESBL					CNS
ENV0059	TICU	A	High touch bed 7 (CPAP, monitor, infusion pump)	Sponge	700	440	6	Enterobacter cloacae	ESBL					CNS
ENV0060	TICU	A	High touch bed 8 (CPAP, monitor, infusion pump)	Sponge	700	620	9	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS
ENV0061	TICU	A	High touch bed 9 (CPAP, monitor, infusion pump)	Sponge	700	<30	2	Enterobacter cloacae	ESBL					CNS
ENV0062	TICU	A	High touch bed 10 (CPAP, monitor, infusion pump)	Sponge	700	830	12							Bacillus, CNS
ENV0063	TICU	B	Basin Drain	Swab	25	<30	22							Bacillus, Sphingomonas paucimobilis R carbapenems
ENV0064	TICU	B	Tap handles	Swab	20	400	200	Enterobacter cloacae	ESBL					Bacillus
ENV0065	TICU	B	Tap faucet	Swab	5	innumerable	innumerable							Bacillus
ENV0066	TICU	B	Medication table	Sponge	500	6300	34200	Enterococcus gallinarum		Enterobacter cloacae	ESBL			CNS
ENV0067	TICU	B	Trolleys	Sponge	9000	520	22	Enterobacter cloacae	ESBL					CNS
ENV0068	TICU	B	Handwash bottle	Sponge	200	460	26							CNS, Bacillus, Pantoa spp
ENV0069	TICU	B	High touch bed 1 (monitor)	Sponge	200	<30	3							CNS

ENV0040	TICU	Isolation E	(CPAP, infusion, monitor)	Sponge	500	contaminated	contaminated	Staphylococcus aureus	MRSA	Acinetobacter baumannii	XDR			
ENV0041	NICU	D	Handwash bottle	Sponge	200	<30	4	Enterococcus faecium	R ampi					CNS
ENV0042	NICU	D	High touch bed 9 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	2200	4	Acinetobacter baumannii	XDR					CNS
ENV0043	NICU	D	High touch bed 10 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	860	2	K. pnemoniae	CRE (enzyme not identified)					CNS
ENV0044	TICU	D	High touch bed 7 (infusion pump)	Sponge	200	890	45	Enterococcus faecium	R ampi	Klebsiella pneumoniae	CRE(enzyme not identified			Bacillus, CNS
ENV0045	NICU	D	High touch bed 12 (ventilator, infusion pump, monitor, trolley)	Sponge	5700	<30	3	Enterococcus faecium	R ampi	Acinetobacter baumannii	XDR			Bacillus, CNS
ENV 0046	TICU	Nursing Station		Sponge	40 000	620	<1	Enterococcus faecium	R ampi	Enterobacter cloacae	ESBL			CNS
ENV0047	TICU	A	Basin Drain	Swab	25	970	194							Bacillus, CNS, Serattia marcescens CRE
ENV0048	TICU	A	Tap faucet	Swab	5	No Growth	NG							
ENV0049	TICU	A	Tap handles	Swab	20	<30	35							Bacillus, CNS
ENV0050	TICU	A	Medication Table	Sponge	500	640	13	Acinetobacter baumannii	XDR					CNS
ENV0051	TICU	A	Trolleys	Sponge	6000	300	1	Enterococcus faecium	R ampi	Acinetobacter baumannii	XDR			CNS
ENV0052	TICU	A	handwash bottle	sponge	200	<30	5							CNS
ENV0053	TICU	A	High touch bed 1 (CPAP, monitor, infusion pump)	Sponge	700	400	6	Enterococcus faecium	R ampi	Staphylococcus aureus	MRSA			CNS
ENV0054	TICU	A	High touch bed 2 (CPAP, monitor, infusion pump)	Sponge	700	1200	18	Acinetobacter baumannii	XDR					Bacillus, CNS
ENV0055	TICU	A	High touch bed 3 (CPAP, monitor, infusion pump)	Sponge	700	580	8	Staphylococcus aureus	MRSA	Enterobacter cloacae	ESBL			CNS
ENV0056	TICU	A	High touch bed 4 (CPAP, monitor, infusion pump)	Sponge	700	6000	85	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS, Pantoa spp
ENV0057	TICU	A	High touch bed 5 (CPAP, monitor, infusion pump)	Sponge	700	<30	2	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS
ENV0058	TICU	A	High touch bed 6 (CPAP, monitor, infusion pump)	Sponge	700	<30	3	Enterobacter cloacae	ESBL					CNS
ENV0059	TICU	A	High touch bed 7 (CPAP, monitor, infusion pump)	Sponge	700	440	6	Enterobacter cloacae	ESBL					CNS
ENV0060	TICU	A	High touch bed 8 (CPAP, monitor, infusion pump)	Sponge	700	620	9	Enterococcus faecalis		Enterobacter cloacae	ESBL			CNS
ENV0061	TICU	A	High touch bed 9 (CPAP, monitor, infusion pump)	Sponge	700	<30	2	Enterobacter cloacae	ESBL					CNS
ENV0062	TICU	A	High touch bed 10 (CPAP, monitor, infusion pump)	Sponge	700	830	12							Bacillus, CNS
ENV0063	TICU	B	Basin Drain	Swab	25	<30	22							Bacillus, Sphingomonas paucimobilis R carbapenems
ENV0064	TICU	B	Tap handles	Swab	20	400	200	Enterobacter cloacae	ESBL					Bacillus
ENV0065	TICU	B	Tap faucet	Swab	5	innumerable	innumerable							Bacillus
ENV0066	TICU	B	Medication table	Sponge	500	6300	34200	Enterococcus gallinarum		Enterobacter cloacae	ESBL			CNS
ENV0067	TICU	B	Trolleys	Sponge	9000	520	22	Enterobacter cloacae	ESBL					CNS
ENV0068	TICU	B	Handwash bottle	Sponge	200	460	26							CNS, Bacillus, Pantoa spp
ENV0069	TICU	B	High touch bed 1 (monitor)	Sponge	200	<30	3							CNS

ENV0102	TICU	D	High Touch Bed 2 (Monitor, infusion pump)	Sponge	400	890	1975							CNS
ENV0103	TICU	D	High Touch Bed 3 (Monitor, infusion pump)	Sponge	400	TNTC	TNTC	Enterococcus faecium	R ampi	Klebsiella pneumoniae	ESBL			CNS
ENV0104	TICU	D	Weigh Station	Sponge	600	TNTC	TNTC	Klebsiella pneumoniae	ESBL	Acinetobacter baumannii	XDR			Bacillus, CNS
ENV0105	TICU	D	High touch bed 5(CPAP, monitor, Infusion pump)	Sponge	700	140000	2000	Staphylococcus aureus	MRSA					CNS
ENV0106	TICU	D	High Touch Bed 6 (Monitor, infusion pump)	Sponge	400	contaminated	contaminated							Bacillus, CNS
ENV0107	TICU	Isolation E	Drain	Swab	25	TNTC	TNTC							Bacillus, CNS, Delftia acidovorans, Serratia marcescens CRE
ENV0108	TICU	Isolation E	Tap faucet	Swab	5	No Growth	NG							
ENV0109	TICU	Isolation E	Medicaion Table	Sponge	500	8300	1660							CNS
ENV0110	TICU	Isolation E	Trolleys	Sponge	3000	19000	64	Staphylococcus aureus	MRSA					CNS
ENV0111	Clean incubator area		Clean open Incubator	Sponge	6000	<30	1							Bacillus
ENV0112	Clean incubator area		clean open incubator s	Sponge	10 000	<30	<1							Bacillus, CNS
ENV0113	Clean incubator area		Closed incubator	Sponge	1500	<30	2							Coagulase negative Staphylococci
ENV0114	Clean incubator area		Closed incubator	Sponge	1500	<30	3							Bacillus spp, CNS
ENV0115	Clean incubator area		Closed incubator	Sponge	1500	360	24							Bacillus spp, CNS

Appendix 8 - Working card for processing environmental sample

WORKING CARD FOR PROCESSING ENVIRONMENTAL SAMPLE

SAMPLE EPISODE: _____ DATE SAMPLED:_____ DATE PROCESSED:_____

AREA SAMPLED:_____CUBICLE:_____SITE:_____

SAMPLE TYPE: SWAB / SPONGE /WIPE DISSASOCIATION METHOD: VORTEX / STOMACHER

DILUENT ADDED TO BHI BROTH: YES / NO TIME:_____

QUANTITATIVE CULTURE

	AGAR SPREAD COUNTING				
		UNDILUTED	1:10	1:100	1:1000
		Place 0.1ml of each dilution to 5% blood agar			
24 hour count					
48 hour count					
CFU		COLONIES COUNTED	VOLUME	DILUTION	
	CFU = ÷ X =				

QUALITATIVE CULTURE

BHI EVALUATION		
24 HOURS	CLEAR / TURBID	REINCUBATED / PLATED OUT
48 HOURS	CLEAR / TURBID	REINCUBATED / PLATED OUT

GRAM STAIN:_____ DATE:_____ PLAN:_____

MEDIA	COLONIAL MORPHOLOGY	RAPID TESTS	PLAN
COLOREX STAPH		CATALASE: STAPHUREX:	5% BLOOD PLATE □ VITEK ID_____ VITEK ATTACHED□
5% BLOOD NALI PLATE		CATALASE: STREPTEX:	MS ID_____ VITEK ATTACHED □
MACONKEY AGAR	LF □ NLF□	OXIDASE: RAPID INDOLE:	5% BLOOD PLATE □ VITEK ID_____ VITEK ID_____ VITEK ID_____ VITEK ATTACHED □
SABOURAUD DEXTROSE		GERM TUBE	VITEK ID_____ VITEK ATTACHED □

IF VITEK 2 SENSES FAIL - DISC DIFFUSION SESNSES:

GRAM NEGATIVE ORGANISMS	TS\SXT	AMP	AUG	CXM	CZ/KZ	CTX	CAZ	FEP	PIPTAZ	ESBL	CIP	GEN	TOB	AMI	ERT	IMI	MER	IPM	CRE	FOX	Chloramp	COL		INITIAL	DATE

GRAM POSITIVE ORGANISMS	AMP	OXA	CD	E	TS/ SXT	VA	LZD	TET	CTX	CIP	RIF	FOX	SYNERCI	BLAC	D-ZONE	INITIAL	DATE

MDRO ORGANISMS

ORGANISM	MDRO PHENOTYPE	FURTHER TEST
Staphylococcus aureus	MRSA	
Enterococcus spp	VRE:	VancoEtest:_____ TeicplaninEtest:_____ VRE Xpert:_____
Klebsiella pneumoniae	ESBL: CRE:	Resist 4:_____
Enterobacter spp	ESBL: CRE:	Resist 4:_____
Acinetobacter baumannii	MDR XDR	
Pseudomonas aeruginosa	MDR XDR	
Candida auris		Amphotericin B etest:_____

SIGNATURE

DAY 1	DATE:	SIGN/INITIAL:
DAY 2	DATE:	SIGN/INITIAL:
DAY 3	DATE:	SIGN/INITIAL:
DAY 4	DATE:	SIGN/INITIAL:
DAY 5	DATE:	SIGN/INITIAL:
REVIEW	DATE:	SIGN/INITIAL:

7. REFERENCES

1. Allegranzi B, Nejad SB, Combescure C, Graafmans W, Attar H, Donaldson L, et al. Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. The Lancet. 2011 Jan;377(9761):228–41.

2. Aiken AM, Mturi N, Njuguna P, Mohammed S, Berkley JA, Mwangi I, et al. Risk and causes of paediatric hospital-acquired bacteraemia in Kilifi District Hospital, Kenya: a prospective cohort study. The Lancet. 2011 Dec;378(9808):2021–7.

3. Chen LF, Knelson LP, Gergen MF, Better OM, Nicholson BP, Woods CW, et al. A prospective study of transmission of Multidrug-Resistant Organisms (MDROs) between environmental sites and hospitalized patients—the TransFER study. Infect Control Hosp Epidemiol. 2019 Jan;40(1):47–52.

4. Ramsamy Y, Essack SY, Sartorius B, Patel M, Mlisana KP. Antibiotic resistance trends of ESKAPE pathogens in Kwazulu-Natal, South Africa: A five-year retrospective analysis. Afr J Lab Med [Internet]. 2018 Dec 6 [cited 2020 Feb 4];7(2). Available from: http://www.ajlmonline.org/index.php/AJLM/article/view/887

5. Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268–81.

6. Founou RC, Founou LL, Essack SY. Extended spectrum beta-lactamase mediated resistance in carriage and clinical gram-negative ESKAPE bacteria: a comparative study between a district and tertiary hospital in South Africa. Antimicrob Resist Infect Control. 2018 Dec;7(1):134.

7. GERMS-SA Annual Report 2019. Available from: http://www.nicd.ac.za/index.php/publications/germs-annual-reports/

8. Vallabhaneni S. Investigation of the First Seven Reported Cases of Candida auris, a Globally Emerging Invasive, Multidrug-Resistant Fungus — United States, May 2013–August 2016. MMWR Morb Mortal Wkly Rep [Internet]. 2016 [cited 2020 Feb 4];65. Available from: https://www.cdc.gov/mmwr/volumes/65/wr/mm6544e1.htm

9. Govender NP, Magobo RE, Mpembe R, Mhlanga M, Matlapeng P, Corcoran C, et al. *Candida auris* in South Africa, 2012–2016. Emerg Infect Dis. 2018 Nov;24(11):2036–40.

10. van Schalkwyk E, Mpembe RS, Thomas J, Shuping L, Ismail H, Lowman W, et al. Epidemiologic Shift in Candidemia Driven by *Candida auris* , South Africa, 2016–20171. Emerg Infect Dis. 2019 Sep;25(9):1698–707.

11. Magobo RE, Naicker SD, Wadula J, Nchabeleng M, Coovadia Y, Hoosen A, et al. Detection of neonatal unit clusters of *Candida parapsilosis* fungaemia by

microsatellite genotyping: Results from laboratory-based sentinel surveillance, South Africa, 2009-2010. Mycoses. 2017 May;60(5):320–7.

12. Kumar J, Eilertson B, Cadnum JL, Whitlow CS, Jencson AL, Safdar N, et al. Environmental Contamination with Candida Species in Multiple Hospitals Including a Tertiary Care Hospital with a Candida auris Outbreak. Pathog Immun. 2019 Oct 28;4(2):260–70.

13. Dusé AG. Infection control in developing countries with particular emphasis on South Africa. South Afr J Epidemiol Infect. 2005 Jan;20(2):37–41.

14. Otter JA, Yezli S, Salkeld JAG, French GL. Evidence that contaminated surfaces contribute to the transmission of hospital pathogens and an overview of strategies to address contaminated surfaces in hospital settings. Am J Infect Control. 2013 May;41(5):S6–11.

15. Guidelines for Environmental Infection Control in Health-Care Facilities: (545922006-001) [Internet]. Centers for Disease Control; 2003 [cited 2020 Feb 4]. Available from: http://doi.apa.org/get-pe-doi.cfm?doi=10.1037/e545922006-001

16. Dancer SJ. Controlling Hospital-Acquired Infection: Focus on the Role of the Environment and New Technologies for Decontamination. Clin Microbiol Rev. 2014 Oct;27(4):665–90.

17. Otter JA, Yezli S, French GL. The Role Played by Contaminated Surfaces in the Transmission of Nosocomial Pathogens. Infect Control Hosp Epidemiol. 2011 Jul;32(7):687–99.

18. Dancer SJ. How do we assess hospital cleaning? A proposal for microbiological standards for surface hygiene in hospitals. J Hosp Infect. 2004 Jan;56(1):10–5.

19. Robakowska M, Bronk M, Tyrańska-Fobke A, Ślęzak D, Kraszewski J, Balwicki Ł. Patient Safety Related to Microbiological Contamination of the Environment of a Multi-Profile Clinical Hospital. Int J Environ Res Public Health. 2021 Apr 6;18(7):3844.

20. Shams AM, Rose LJ, Edwards JR, Cali S, Harris AD, Jacob JT, et al. Assessment of the Overall and Multidrug-Resistant Organism Bioburden on Environmental Surfaces in Healthcare Facilities. Infect Control Hosp Epidemiol. 2016 Dec;37(12):1426–32.

21. Phoon HYP, Hussin H, Hussain BM, Lim SY, Woon JJ, Er YX, et al. Distribution, genetic diversity and antimicrobial resistance of clinically important bacteria from the environment of a tertiary hospital in Malaysia. J Glob Antimicrob Resist. 2018 Sep;14:132–40.

22. Ng DHL, Marimuthu K, Lee JJ, Khong WX, Ng OT, Zhang W, et al. Environmental colonization and onward clonal transmission of carbapenem-resistant

Acinetobacter baumannii (CRAB) in a medical intensive care unit: the case for environmental hygiene. Antimicrob Resist Infect Control. 2018 Dec;7(1):51.

23. Jain M, Sharma A, Sen MK, Rani V, Gaind R, Suri JC. Phenotypic and molecular characterization of Acinetobacter baumannii isolates causing lower respiratory infections among ICU patients. Microb Pathog. 2019 Mar;128:75–81.

24. Anaissie EJ, Penzak SR, Dignani MC. The Hospital Water Supply as a Source of Nosocomial Infections: A Plea for Action. Arch Intern Med. 2002 Jul 8;162(13):1483.

25. Hopman J, Tostmann A, Wertheim H, Bos M, Kolwijck E, Akkermans R, et al. Reduced rate of intensive care unit acquired gram-negative bacilli after removal of sinks and introduction of 'water-free' patient care. Antimicrob Resist Infect Control. 2017 Dec;6(1):59.

26. Walker JT, Jhutty A, Parks S, Willis C, Copley V, Turton JF, et al. Investigation of healthcare-acquired infections associated with Pseudomonas aeruginosa biofilms in taps in neonatal units in Northern Ireland. J Hosp Infect. 2014 Jan;86(1):16–23.

27. Hopman J, Meijer C, Kenters N, Coolen JPM, Ghamati MR, Mehtar S, et al. Risk Assessment After a Severe Hospital-Acquired Infection Associated With Carbapenemase-Producing *Pseudomonas aeruginosa*. JAMA Netw Open. 2019 Feb 15;2(2):e187665.

28. Rawlinson S, Ciric L, Cloutman-Green E. How to carry out microbiological sampling of healthcare environment surfaces? A review of current evidence. J Hosp Infect. 2019 Dec;103(4):363–74.

29. Jansson L, Akel Y, Eriksson R, Lavander M, Hedman J. Impact of swab material on microbial surface sampling. J Microbiol Methods. 2020 Sep;176:106006.

30. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995 Sep;33(9):2233–9.

31. Sharma-Kuinkel BK, Rude TH, Fowler VG. Pulse Field Gel Electrophoresis. Methods Mol Biol Clifton NJ. 2016;1373:117–30.

32. Herschleb J, Ananiev G, Schwartz DC. Pulsed-field gel electrophoresis. Nat Protoc. 2007 Mar;2(3):677–84.

33. Goering RV. Pulsed field gel electrophoresis: A review of application and interpretation in the molecular epidemiology of infectious disease. Infect Genet Evol. 2010 Oct;10(7):866–75.

34. Ranjbar R, Karami A, Farshad S, Giammanco GM, Mammina C. Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide. New Microbiologica 2014, 37:1-15:

35. McFarland Standard [Internet]. [cited 2021 Dec 3]. Available from: http://www.dalynn.com/dyn/ck_assets/files/tech/TM53.pdf

36. Pfaller MA, Diekerma MJ. Section 13: Epidemiologic and Infection Control Microbiology. In: Leber AL editor. ASM Clinical Microbiology Procedures Handbook. Washington, D.C:ASM Press, 2016; p. 13.3.4.1-13.3.5.1

37. Public Health England (2017) Detection and Enumeration of Bacteria in Swabs and other Environmental Samples. National Infection Service, Food, Water & Environmental Microbiology Standard Method FNES4 (E1); Version 4. .

38. Excision_SOP.pdf [Internet]. [cited 2021 Dec 3]. Available from: https://www.teagasc.ie/media/website/publications/2008/Excision_SOP.pdf

39. CLSI. Performance Standards of Antimicrobial Susceptibility testing. 31st ed. CLSI supplement M100. Clinical Laboratory Standards institute; 2021.

40. Smibert OC, Aung AK, Woolnough E, Carter GP, Schultz MB, Howden BP, et al. Mobile phones and computer keyboards: unlikely reservoirs of multidrug-resistant organisms in the tertiary intensive care unit. J Hosp Infect. 2018 Jul;99(3):295–8.

41. Larkin E, Hager C, Chandra J, Mukherjee PK, Retuerto M, Salem I, et al. The Emerging Pathogen Candida auris: Growth Phenotype, Virulence Factors, Activity of Antifungals, and Effect of SCY-078, a Novel Glucan Synthesis Inhibitor, on Growth Morphology and Biofilm Formation. Antimicrob Agents Chemother [Internet]. 2017 May [cited 2022 Jan 21];61(5). Available from: https://journals.asm.org/doi/10.1128/AAC.02396-16

42. Welsh RM, Bentz ML, Shams A, Houston H, Lyons A, Rose LJ, et al. Survival, Persistence, and Isolation of the Emerging Multidrug-Resistant Pathogenic Yeast Candida auris on a Plastic Health Care Surface. Diekema DJ, editor. J Clin Microbiol. 2017 Oct;55(10):2996–3005.

43. Mkhize S, Amoako DG, Shobo CO, Zishiri OT, Bester LA. Genotypic and Phenotypic Characterizations of Methicillin-Resistant Staphylococcus aureus (MRSA) on Frequently Touched Sites from Public Hospitals in South Africa. Falkinham J, editor. Int J Microbiol. 2021 Oct 23;2021:1–9.

44. Plipat N, Spicknall IH, Koopman JS, Eisenberg JN. The dynamics of methicillin-resistant Staphylococcus aureus exposure in a hospital model and the potential for environmental intervention. BMC Infect Dis. 2013 Dec;13(1):595.

45. Baang JH, Axelrod P, Decker BK, Hujer AM, Dash G, Truant AR, et al. Longitudinal epidemiology of multidrug-resistant (MDR) Acinetobacter species in a tertiary care hospital. Am J Infect Control. 2012 Mar;40(2):134–7.

46. Hamidian M, Nigro SJ. Emergence, molecular mechanisms and global spread of carbapenem-resistant Acinetobacter baumannii. Microb Genomics [Internet]. 2019 Oct 1 [cited 2021 Dec 21];5(10). Available from: https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000306