Extended characterization of multi-drug resistant organisms colonising neonates at a tertiary hospital in Johannesburg, South Africa.

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DECLARATION

I Nonkululeko Marcia Mntla declare that this Research Report is my own, unaided work. It is being submitted for the Degree of [name of degree] at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____ (Signature of candidate)

3rd day of April 2023 in Johannesburg

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, [Nonkululeko Marcia Mntla], student number [456149], declare that this Research Report submitted in the "submissible format" is my own work and that I contributed adequately towards research findings in the article for submission to the journal: *Epidemiology and Infection*. This is included in my Research Report.

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Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of her Research Report. N. Mntla was involved in conceptualization, and performed data curation, methodology statistical analysis and prepared the manuscript.

Article 1: Title: Prevalence of multi-drug resistant organisms colonizing neonates at a tertiary hospital in Johannesburg, South Africa.

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ABSTRACT

Neonatal deaths remain high globally, particularly in sub-Saharan Africa. A third of deaths are due to infections, often secondary to multi-drug resistant (MDR) organisms. The purpose of this study was to investigate the prevalence of MDR ESKAPE+ *C. auris* colonisation amongst hospitalised neonates, to determine risk factors associated with MDR colonisation, and perform antimicrobial resistance characterization of these isolates. Two hundred and fifty-eight swabs were collected from 86 hospitalised neonates at a tertiary South African hospital between November and December 2020. A total of 135 ESKAPE+ *C. auris* isolates were identified; 68% were MDR. Majority of neonates (51,2%) were colonised with extended spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae*, followed by extensively-drug resistant (XDR) *Acinetobacter baumannii*. New Delhi metallo-beta-lactamase (NDM) producing *A. baumannii* were more prevalent than carbapenemase producing Enterobacterales (CPE). A prolonged hospital stay, median=14 days (p<.001), was identified as a risk factor for MDR organism colonisation. The high prevalence of MDR ESKAPE+ *C. auris* colonisation supports the use of non-invasive samples to determine colonisation prevalence. More data are needed to develop improved surveillance systems which should incorporate colonisation swabs and clinical biomarkers in neonates with independently established risk factors.

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Nomenclature

Abbreviations and acronyms

ACIBA:	*Acinetobacter baumannii*
AMR:	Antimicrobial resistance
CANAU:	*Candida auris*
CDC:	Centre for Disease Prevention and Control
CPE:	Carbapenemase producing Enterobacterales
CRAB:	Carbapenem resistant Acinetobacter baumannii
CRE:	Carbapenem resistant Enterobacterales
ENTCL:	*Enterobacter cloacae complex*
EOS:	Early onset sepsis
ESBL:	Extended Spectrum Beta-lactamase
GNB:	Gram negative bacilli
GPC:	Gram positive cocci
HAI:	Hospital acquired infection
KLEPN:	*Klebsiella pneumoniae*
LOS:	Late onset sepsis
MDR:	Multi Drug Resistant/Multi Drug Resistance
MDRO:	Multi Drug Resistant Organism
MRSA:	Methicillin Resistant *Staphylococcus aureus*
MSSA:	Methicillin susceptible *Staphylococcus aureus*
NDM:	New Delhi Metallo-beta-lactamase-1
NHSN:	National Healthcare Safety Network
SOP:	Standard operating procedure
WHO:	World Health Organisation
VRE:	Vancomycin Resistant Enterococci
XDR:	Extensively Drug Resistant

INTRODUCTION

The neonatal period contributes greatly to under-5 mortality worldwide, despite the decline in childhood mortality (1). In 2019, almost one million neonatal deaths occurred within their first 24 hours of life (1,2). Neonatal infection accounts for a third of these deaths which may be attributable to a premature immune system permitting bacterial, fungal and viral infections (1,2). Low-to-middle income countries (LMICs) have a high neonatal mortality rate, more so in Sub-Saharan Africa (3–6). Timeous recognition, diagnosis and treatment of infection may be challenging in these settings and hence prevention of infection is important.

The relationship between perinatal colonisation and early neonatal sepsis (EOS) (neonatal infection occurring before and up to 72 hours of life) is well described and is used to develop prenatal screening and vaccination programmes (7,8). This distinction of EOS from late onset neonatal sepsis (LOS) (infection following 72 hours of life) is exploited to ascertain the presumed aetiology and selection of empiric antimicrobials, for instance in hospitalised neonates in whom LOS is thought to be healthcare associated (HAI) with nosocomial organisms (3,5–8).

Although Gram positive organisms have historically been associated with most neonatal infections, there has been an alarming rise in Gram negative sepsis. In South Africa, the incidence risk of neonatal infections established by Baby GERMS-SA was reported to have gradually increased from 2014 - 2019 (5). This report along with previous data from neonatal units in South Africa (3,4) show a predominance of LOS with a majority of Gram negative invasive infections being caused by multi-drug resistant organisms (MDRO) i.e. non-susceptibility to one or more agents in three antibiotic classes (9).

In the past decade, a group of micro-organisms commonly associated with HAI have been identified as the 'ESKAPE' pathogens, namely *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii complex, Pseudomonas aeruginosa* and *Enterobacter* species (10). These highly transmissible organisms are infamous for their multi-drug resistant phenotypes namely: vancomycin resistant *E. faecium* (VRE); methicillin resistant *S. aureus* (MRSA); carbapenem resistant *K. pneumoniae* and *Enterobacter* species (denoted as carbapenem resistant Enterobacterales (CRE) or carbapenemase producing Enterobacterales (CPE) and their extended spectrum beta-lactamase producing (ESBL) phenotypes; multi-, extensive- and pan-drug resistance amongst *A. baumannii* and *P. aeruginosa*. *Candida* species have also gained notoriety. In 2019 *C. auris* was listed as an urgent threat on the Centre of Disease Prevention Control (CDC) antibiotic resistance threats list (11). Subsequently, multiple outbreaks and significant mortality have ensued.

ESKAPE + *C. auris* organisms have recently been categorised as critical and high priority pathogens by the WHO and CDC (11,12). Neonates are especially at risk of invasive infections due to the nature of their skin and mucosal barriers that allow for transient colonisation that may lead to opportunistic infection (7,12–15). Reliance on identification of MDROs only from invasive infections results in underestimation of the burden of MDROs in a setting, as asymptomatic colonisation levels exceed those of invasive infections.

The correlation between colonisation and subsequent clinical infection is not clearly established. Although some studies have failed to show a significant consequential development of infection with the same organism following colonisation (13,16,17), data show that under certain conditions, gut colonisation with ESBL and CREs may result in invasive disease; the mechanisms thereof remains unclear (18,19). Consequently, neonatal units have used screening swabs for MDROs in an attempt to prevent horizontal transmission and outbreaks (15,18,20). In LMICs however, prevalence studies concerning MDRO colonisation are limited, and very few studies in South Africa have investigated the epidemiology of colonisation of MDROs, identifying ESBL and CRE producing Enterobacterales primarily (15). It is with this in mind that this study aimed to determine colonisation prevalence, risk factors associated with colonisation and characterization of multi-drug resistant (MDR) ESKAPE+ *C. auris* organisms, from hospitalised neonates at the largest tertiary South African hospital.

METHODOLOGY/ MATERIALS

This study is a sub-study of a larger, externally funded research project which was conducted concurrently. The main project is entitled: “Microbiological Surveillance of hospital environmental surfaces, medical equipment, healthcare workers, patients and patients’ parents in a tertiary hospital in Soweto, South Africa”; hereafter referred to as the primary investigation.

The principal investigator for this study was a member of the research team for the primary investigation; however, this sub-study focused on data and samples collected from neonates.

2.1. STUDY DESIGN:

A prospective survey was conducted in four wards at the Chris Hani Baragwanath Academic Hospital (CHBAH) neonatal unit. Sampling and data collection in the intensive care section (ICU) took place between 11 and 18 November, and between 13 and 15 December 2020 in the non-intensive care section of the neonatal unit (non-ICU).

2.2 PARTICIPANTS:

i. Study setting:

CHBAH is a tertiary-level hospital which caters to ~8000 caesarean section deliveries and 19 000 live births annually (21). The neonatal unit at CHBAH consists of a subdivided intensive care unit with total occupancy of ~45 beds, and the non-intensive neonatal care wards with total occupancy of ~96 beds.

ii. Study population:

The study population included neonates admitted in the ward on the day of sample collection. For the purpose of this study, a neonate was defined as an infant who was 28 days old or younger on the day of admission in the unit. Neonates were stratified into the 2 groups based on a hospitalization period reported to distinguish EOS and LOS i.e. 72 hours.

Neonates were excluded from the study if they had congenital malformations such as imperforate anus, gastroschisis, choanal atresia or cleft palate, which would hinder sampling, if they were transferred in from other hospitals within 48 hours of sampling, if they had undergone surgical intervention, and if they were nasally intubated as this precluded nasal sampling.

2.3 DATA COLLECTION

2.3.1. SAMPLE COLLECTION

Three screening swabs were collected from randomly selected neonates according to the standard operating procedures (SOP) [appendix 4]. These included one rectal swab, one skin swab (umbilicus, both axillae and the groin area sampled with the same swab), and one nasal swab. All swabs were labelled with the patient's unique study number, and transported to the processing laboratory in Cary Blair transport medium at ± 4°C along with a data collection sheet [appendix 4]

2.3.2. LABORATORY PROCEDURES

The screening swabs were placed into Brain Heart Infusion (BHI) enrichment media (Diagnostic Media Products (DMP), National Health Laboratory Service (NHLS, South Africa) and incubated aerobically overnight at 35ºC. Following 16-18hours of incubation, 10µl of each turbid sample was sub-cultured onto six agar plates as per the study – approved laboratory processing SOP [appendix 4].

The agar plates were reviewed following 16-18-hour incubation for growth, and a presumptive identification was made using phenotypic methods i.e., by employing the colony morphology on selective agar and rapid biochemical tests performed as per SOP [supplemental material]. If the presumptive identification suggested an ESKAPE+ *C. auris* organism, identification was confirmed using the VITEK® -MS MALDI-TOF system (bioMérieux, France) or the VITEK® 2 (bioMérieux, France). Antimicrobial susceptibility testing (AST) was performed on confirmed ESKAPE + *C. auris* organisms on the VITEK® 2 or by Kirby Bauer disk diffusion depending on availability. This was interpreted according to Clinical and Laboratory Standards Institute (CLSI) M100 guidelines, and MDROs were categorised as ESBL, MRSA or CRE/CPE based on CLSI definitions (23). The definitions for MDR and XDR definitions for *P. aeruginosa* and *A. baumannii* were based on those outlined by Magiorakos et al (9). All *C. auris* isolates were presumed to be azole resistant and were categorised as MDR based on local published data (24).

2.3.3. EXTENDED CHARACTERIZATION

Further characterization included determining the presence of carbapenemases in CRE and *A. baumannii* isolates using a lateral flow assay (RESIST- 4.O.K.N.V., Coris) which tests for OXA-48, KPC, NDM and VIM carbapenemases. We also performed susceptibility testing with a vancomycin gradient strip (Etest) for MRSA, amphotericin B and micafungin Etests for *C. auris*, and colistin broth microdilution for XDR *A. baumannii* isolates.

A subset of carbapenem resistant *K. pneumoniae* (KLEPN) isolates was further tested using Pulse-Field Gel Electrophoresis (BIORAD CHER-DRII/III electrophoresis system) to establish their genetic relatedness. This was based on 2019 microbiology laboratory data, where *Klebsiella* spp was the second most prevalent organism from positive blood cultures, and constituted approximately 20% of all blood culture isolates (unpublished). Testing included comparing fragmented DNA on an agarose gel from the isolated CRE KLEPN and clinical CRE KLEPN isolates from two other institutions.

2.4 VARIABLES AND BIAS

Each neonate who was in every alternate bed was randomly sampled and the following variables were assessed: organism identified, pattern of multi-drug resistance and phenotypic determinants of carbapenem resistance in *K. pneumoniae*, *E. cloacae and A. baumannii*. Independent variables included antimicrobial therapy and duration of hospitalization prior to sampling.

2.5 STATISTICAL ANALYSIS

Data were compiled and analysed with Microsoft Excel 2019 and Graphpad Software version 9.3.1. Descriptive statistical analysis was used to analyse the data. Categorical data were recorded as proportions of the overall data in frequencies and percentages. Comparison between categorical variables was performed using Fisher's exact test. Continuous variables were reported as median and interquartile range, and analysed using the Mann-Whitney U test.

Each neonate was included once for enumeration of MDROs, although they may have been colonised with the same pathogen at different body sites. Each ESKAPE + *C. auris* isolate was included in the data analysis. Where more than one MDR phenotype of the same organism was isolated (e.g. both carbapenem susceptible and non-susceptible strain), each strain was analysed separately.

For risk factor identification, the neonates were divided into two groups: Group 1: Neonates colonised with MDR ESKAPE + *C. auris* and Group 2: Neonates with no growth on their swabs, non-ESKAPE + *C. auris* colonised and neonates with non-MDR ESKAPE colonisation i.e. susceptible isolates. To identify risk factors for MDRO colonisation, neonatal characteristics were described for each patient and the following factors were analysed: the length of hospitalization (days) prior to the day of sampling, antimicrobial exposure prior to sampling and the day of life on which sampling was conducted. A two-tail P value of $< .05$ was considered to be statistically significant.

2.6 ETHICAL CONSIDERATION

This study received approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (clearance certificate number M200583). (Appendix 1)

RESULTS

Eight-six neonates were sampled, 43 in the intensive care setting (ICU) and 43 in the general neonatal wards (non-ICU), and a total of 258 swabs were collected. An equal number of rectal, skin and nasal swabs were collected from which significant and non-significant (normal skin flora) colonisers were isolated. Figures 1 and 2 display the prevalence of MDR ESKAPE+ *C. auris* organisms identified amongst neonates:

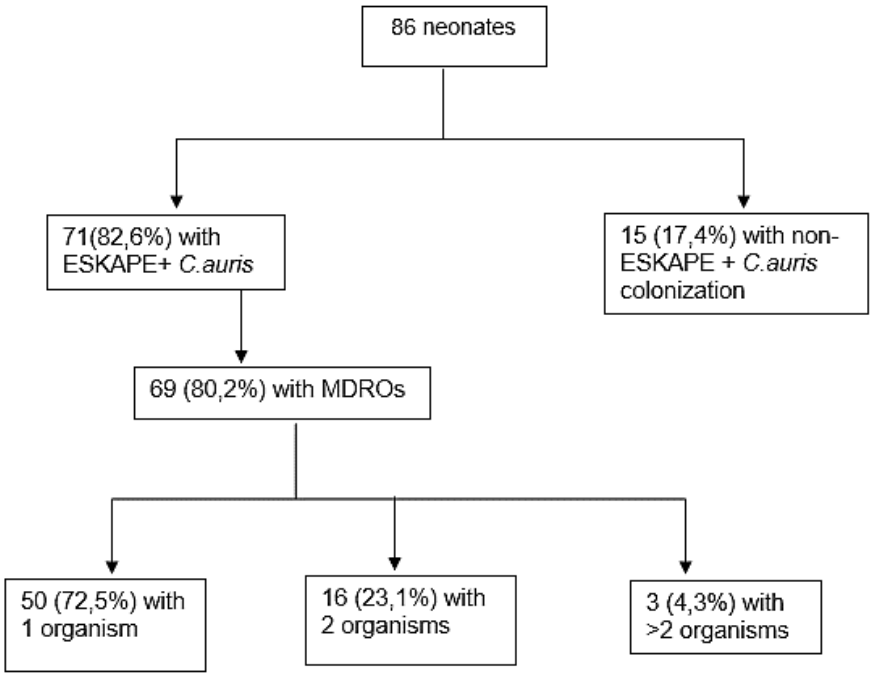

FIGURE 1: PREVALENCE OF ESKAPE + *C. auris* COLONISATION AMONGST NEONATES

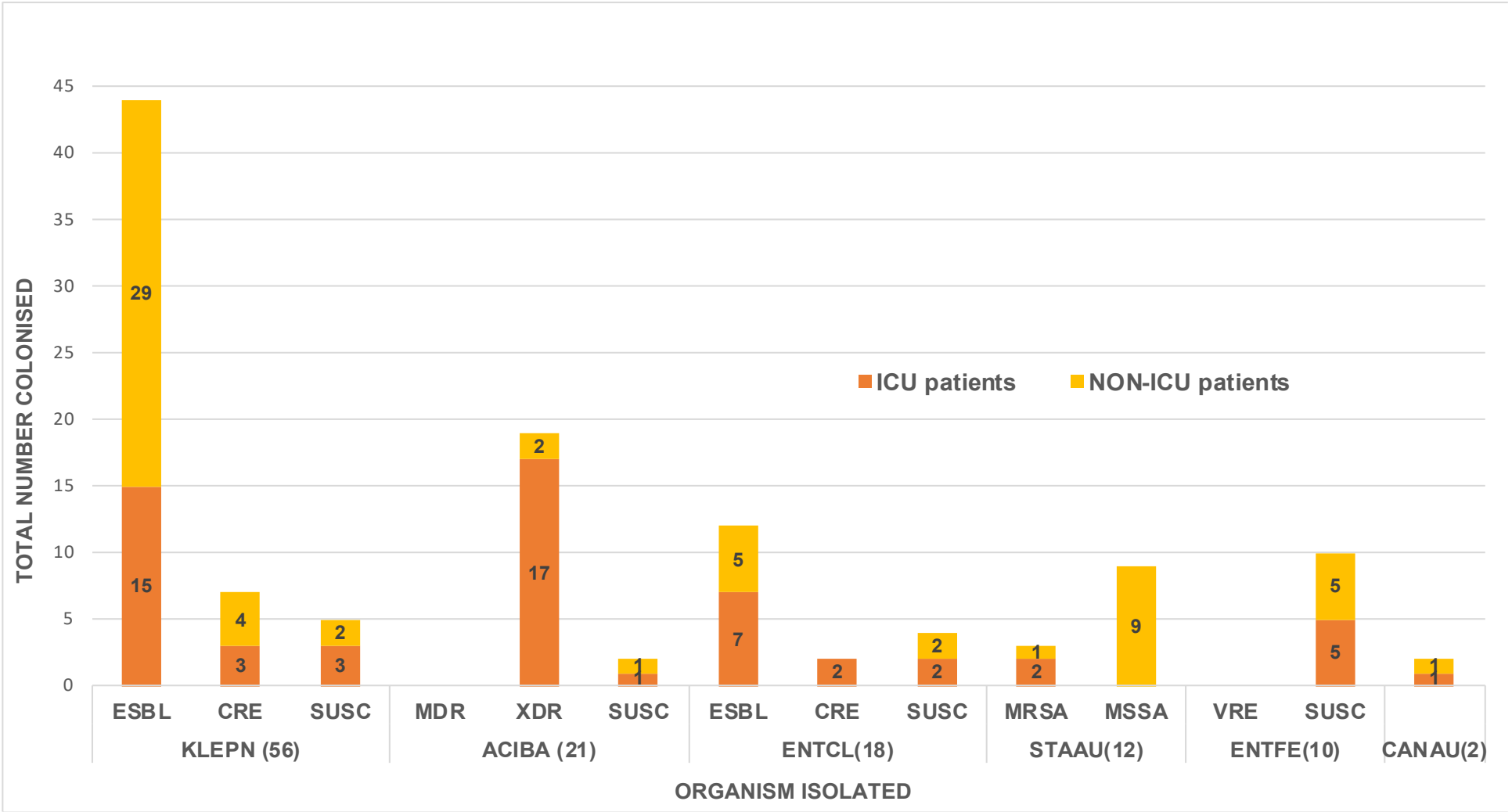

FIGURE 2: CUMULATIVE PREVALENCE OF ESKAPE + *C. auris* ORGANISMS AMONGST NEONATES STRATIFIED BY SITE (ICU VS NON-ICU). ICU: intensive care unit, SUSC: susceptible, CANAU: *C.auris*

A total of 135 ESKAPE+ *C. auris* isolates were isolated from the 258 swabs collected. Fifty-six (41,8%) isolates were ESBL producing Enterobacterales, and nine (22%) were CREs (of which two, tested positive for OXA-48 carbapenemase production). In contrast, amongst carbapenem resistant *A. baumannii* isolates (CRAB), 5/19 (26%), tested positive for New Delhi Metallo-beta-lactamase-1 (NDM) carbapenemase production. Further characterization of ESKAPE+ *C. auris* isolates and the respective susceptibility profiles are discussed in Table 1 and Table 2 (23,25):

TABLE 1: PREVALENCE OF ESKAPE+ *C. auris* ISOLATES AMONGST SWABS COLLECTED

	n (%) of total isolates (135)
MDR ESKAPE+ *C. auris* isolates	91(67,4)
MRSA	3(2,2)
	2(1,5)
ESBL *K. pneumoniae* and *E. cloacae*	56(41,5)
CRE/CPE *K. pneumoniae* and *E. cloacae*	9(6,7)
MDR *A. baumannii*	2(1,5)
XDR *A. baumannii*	19(14,1)
(Col R)	3/19(15 ,8)
Susceptible ESKAPE+ *C. auris* isolates	44(32,6)

Col R: Colistin resistant

TABLE 2: CUMULATIVE ANTIMICROBIAL SUSCEPTIBILITY REPORT – ANTIMICROBIAL AGENTS LISTED BY CLASS

			% OF TOTAL STRAINS							% Susceptibility to Routinely Used Antimicrbials for Empiric therapy											
											β-lactams					Aminoglycosides		Polymixins	Glycopeptides	Polyenes	Echinocandins
	Organisms	Total number of strains	Non-resistant	ESBL Isolates (%)	CRE Isolates (%)	MDR (%)	XDR(%)	MRSA (%)	Carbapenamases detected	Phenotype	Ampicillin	Cloxacillin	Pip-taz	Cefotaxime	Meropenem	Getamicin	Amikacin	Colistin (BMD) (MIC ≤2)*	Vancomycin (MIC ≤4)*	Amphotericin B (MIC ≤1) †	Micafungin (MIC ≤2) †
GNB																					
1	*Klebsiella pneumoniae*	64	13	44	7	-	-	-	OXA 48+v (2)§	ESBL	IR	-	16	2	100	0	100	-	-	-	-
				68,8%	11%					CRE	IR	-	0	0	29	29	86	-	-	-	-
										non-resistant	IR	-	100	100	100	100	100	-	-	-	-
2	*Acinetobacter baumannii complex*	27	6	-	-	2	19	-	NDM (5)§	MDR	-	-	0	IR	50	50	100	-	-	-	-
						7,4%	70,4%			XDR	-	-	0	IR	26	26	47	84	-	-	-
										non-resistant	-	-	100	IR	100	83	100	-	-	-	-
3	*Enterobacter cloacae complex*	19	5	12	2	-	-	-	Nil	ESBL	IR	-	23	0	100	46	100	-	-	-	-
				63,2%	10,5%					CRE	IR	-	0	0	0	0	100	-	-	-	-
										non-resistant	IR	-	31	100	100	100	100	-	-	-	-
GPC																					
5	*Staphylcoccus aureus*	13	10	-	-	-	-	3	-	MRSA	IR	-	-	-	-	33	-	-	100	-	-
								23,1%		non-resistant	IR	100	-	-	-	100	-	-	100	-	-
6	*Enterococcus faecium*	10	10	-	-	-	-	-	-	non-resistant	10	-	-	IR	-	-	-	-	100	-	-
			100%																		
Yeast																					
7	*C. auris*	2							-	All isolates	-	-	-	-	-	-	-	-	-	100	100

§: Number of carbapenemase producing strains
IR: Intrinsically resistant
↕: Interpretation according the CDC tentative break points (25)
*: Interpretation according to CLSI (23)
-: Not routinely tested for in this organism

Neonates with MDRO colonisation [Group 1] were hospitalised longer than their non-MDRO colonised [Group 2] counterparts (14 vs 2,5 days, $p < .001$), and a comparison of Group 1 vs Group 2 in each section (i.e. ICU vs non-ICU) revealed no increased risk for colonisation in ICU regardless of hospitalization period. This is depicted by a median length of hospitalization (MLOH) for Group 1 vs 2 (14 vs 6 days in ICU) and (14 vs 2 days in non-ICU) ($p = 0.3$). The neonatal characteristics assessed for association with MDRO colonisation are summarised in Table 3.

TABLE 3: CHARACTERISTICS ASSOCIATED WITH MDRO COLONISATION

Neonatal characteristics	Group 1 (n[#]= 67)	Group 2 (n[#]=16)	*p*-value
Days of life at time of sampling			
Median (IQR)	16(6-28)	3(1-6,5)	0.0001
⩽3 days of life	6	9	
>3 days of life	61	7	
Hospitalization period			
Median (IQR)	14 (5-25)	2,5 (1-6,5)	0.0001
⩽3days	6	9	
>3days	61	7	
ICU admission[##]	34/69 (49,3%)	9/17 (52,9%)	1
Over-all antimicrobial exposure	52	13	1
No antimicrobial exposure	15	3	
Beta- lactams:	48	13	0.58
a) Ampicillin	30	8	0.8
b) Carbapenems	13	5	0.3
c) Cephalosporins	1	0	1
d) Piperacillin-tazobactam	13	0	0.06
Aminoglycosides	39	7	0.4
Colistin	6	0	0.6
Vancomycin	4	6	0.0028
Fluconazole	4	1	1
Amphotericin B	4	0	1
Micafungin	0	0	-

Group 1: Colonised with MDRO ESKAPE+ *C. auris*
Group 2: Not colonised with MDRO ESKAPE+ *C. auris*
[#]: "n" represents the number of neonates for whom data was available. Statistical analysis was calculated on this number.
[##] Study numbers assigned according to area in which neonate was hospitalised during sample collection, therefore “ICU admission” refers to neonate’s samples overall
IQR: Inter quartile range
- : *p*-value not calculated

Further statistical analysis was not performed on the 'day of life' variable, as 77/83 (93%) of the neonates were admitted within 24 hours from birth to their respective sections, leading to comparable data with the 'length of hospitalization' variable. Of note, 61/65 (91%) of Group 1 neonates were found to be colonised after three days of life (*p*=.0001).

Although 60,1% of MDRO colonised neonates had increased antimicrobial exposure (52/67 vs 13/16), this was not a statistically significant risk factor (*p*=1). In fact, vancomycin exposure was significantly associated with the absence of MDRO colonisation (*p*=.0028). On sub analysis per MDRO type however, carbapenem exposure was significantly associated with XDR *A. baumannii* colonisation (10/19, *p*=.001). Two neonates were colonised with *C. auris*, of which one had been exposed to fluconazole and amphotericin B.

MDR ESKAPE+ *C. auris* isolation varied between the rectal, skin and nasal swabs. Statistically, rectal and skin swabs performed similarly for the culture of MDR GNB, despite rectal swabs yielding the highest number of GNBs overall. All MRSAs were isolated from nasal swabs, and all *C. auris* isolates were isolated from skin swabs (Table 4).

TABLE 4: YIELD OF VARIOUS MDROS FROM COLONISATION SWABS

Organism	Rectal swabs (n=86)		Skin swabs (n=86)		Nasal swabs (n=86)	
	MDRO	**Total**	**MDRO**	**Total**	**MDRO**	**Total**
Acinetobacter baumannii	MDR	0	MDR	0	MDR	2
	XDR	11	XDR	5	XDR	12
C. auris		0		2		0
Enterobacter cloacae complex	ESBL	3	ESBL	6	ESBL	4
	CRE	0	CRE	1	CRE	1
Klebsiella pneumoniae	ESBL	36	ESBL	38	ESBL	24
	CPE	2	CPE	0	CPE	0
	CRE	3	CRE	2	CRE	0
Staphylococcus aureus	MRSA	0	MRSA	0	MRSA	3
		55		**54**		**46**

Pulse Gel electrophoresis was attempted on the seven carbapenem resistant *K. pneumoniae* isolates multiple times, and although pulsotypes were comparable, the quality remained too poor to identify a trend

DISCUSSION

To our knowledge, this is one of the few studies reporting on colonisation of MDR ESKAPE + *C. auris* isolates from SA, and previously published South African studies have focused on a limited pathogen spectrum. In this study, over 80% of hospitalized neonates sampled were colonised with ESKAPE + *C. auris,* and the majority of these (97%) were MDR isolates. Furthermore 22% of neonates were colonised with multiple (>2) MDR isolates. This high prevalence of colonisation with MDR isolates in this study is consistent with published data on increasing clinical infections secondary to ESKAPE + *C. auris* isolates worldwide (4,5,12,18). Miliac et al. (2021) reported that 59% of hospitalised neonates had MDRO gut colonisation, and subsequent invasive infection with the same organism occurred in 8% (18). In comparison, Dias et al (2019), showed that only 13% of neonates sampled were colonised with MDROs and subsequent infection occurred in 7% with the same organism (13). These inconsistent findings illustrate the uncertainty of the utility of colonisation screening swabs in non-outbreak settings (13,26,27).

Multi-drug resistant GNB were predominant, and most commonly isolated from rectal and skin swabs. From the perspective of diagnostic stewardship and appropriate resource utilization, identifying the swab with the greatest yield of the targeted MDRO is a practically important consideration to minimise cost and optimise surveillance. The findings of our study suggest that in units with a similar epidemiology as the unit under investigation, either rectal or skin swabs can be used comparably to identify the prevalence of MDROs. This may differ in a setting with a higher prevalence of MRSA infections.

Data suggest that screening for MDROs may assist to identify high risk patients, and may inform empiric treatment and preventative measures such as decolonisation, which is hypothesized to prevent subsequent invasive infection. These data are however limited, and the independent use of colonisation is a poor marker for predicting neonatal sepsis (16,17,19,26). Hence screening is currently used to identify colonised cases and to implement adequate bundles of control measures rather than to predict sepsis or reduce case fatality during outbreaks. This has proven to be effective in restricting outbreaks (26,28)

The predominance of ESBL *K. pneumoniae* also mirrors data on invasive disease and colonisation from the same unit, and in other tertiary centres in South Africa (3,5,15). In fact, Ogunbosi et al. (2020) noted that 62,5% of ESBL producing organisms isolated on screening swabs from children in a tertiary hospital in Cape Town, South Africa were *K. pneumoniae* (15). This is of particular concern given the well described potential of ESBL *K. pneumoniae* to cause outbreaks. The low yield of CRE and CPE isolates was unexpected, but a study by Thomas et al. (2022) details how carbapenem resistance was greater amongst *A. baumannii* compared to Enterobacterales from blood and cerebrospinal fluid cultures in the same unit, and Ogunbosi et al. (2020) also reported a CRE prevalence as low as 0,5% amongst screening swabs collected from their participants (4,15).

A. baumannii was the second most commonly isolated organism in this study and was principally carbapenem resistant and XDR. This phenotype is described as a

leading cause of neonatal infection and death by Madhi et al. in the same neonatal unit, accounting for 52% of infection related deaths in a post-mortem study (29). Carbapenemase mediated resistance, namely NDM solitarily, accounted for a quarter of CRAB even amongst neonates without prior carbapenem exposure. This contradicts Lowe et al (2022) who report that NDM-producing *A. baumannii* isolates occur to a lesser degree in South Africa than OXA- type, despite 5/6 (83,3%) of their successfully sequenced CRAB isolates possessing a clonal bla_{NDM} gene. Similarly, Nogbou et al (2021) and Perovic et al (2022) describe a prevalence of bla_{NDM} possessing *A. baumanni* isolates of 58% and 79% respectively (30,31). The above authors hypothesize that *A. baumannii*'s ability to acquire resistant genes horizontally, has led to an increased prevalence and dissemination of the NDM carbapenemase.

Of interest, none of the neonates included within the first 24 hours of life were colonised with MDR ESKAPE+ *C. auris,* suggesting an unlikely role of vertical transmission in this group of patients. Similar to previous authors, a longer duration of hospitalization was significantly associated with MDRO colonisation in this study, with a period comparable to Leikin-Zach et al. in 2018 (18,32,33). This is partly attributable to the fact that hospitalised neonates, particularly when exposed to the hospital environment for prolonged periods, have a lower species diversity of commensals. This, in combination with colonisation pressure result in a higher rate of pathogenic colonisation (16). Folgori et al.(2019) have reported that in a non-outbreak setting, risk factors independently associated with ESBL colonisation include prematurity, low birth weight, invasive devices, antibiotic exposure (especially cephalosporins and carbapenems) and a lack of breastfeeding (19). However, like Miliac et al. and Leikin-Zach et al., not all the risk factors were found to be significant for MDRO colonisation in this study. For instance, admission in the ICU regardless of the duration and most antibiotic exposures were not found to be significant.

Risk factors found to be significant may represent underlying parameters which could not be defined individually because an adjusted and unadjusted logistic regression were not performed. Such parameters include: the initial reason for admission, presence of central lines and prolonged empiric antimicrobial therapy. Vancomycin was unexpectedly associated with the absence of MDRO colonisation; however due to the small number patients in the study, this would require further exploration in a larger sample size.

For this study, only *S. aureus* and *E. faecium* were the Gram positive organisms investigated, which may explain the overall low yield of GPCs (8,9%). This was unsurprising however, as Jimenez-Truque et al (2011) have described how neonatal *S. aureus* colonisation may be as low as 2,5% at birth and only peaks around 39% at two months of age, even amongst neonates in whom *S. aureus* colonisation has been demonstrated on their mother(17). The absence of *P. aeruginosa* was anticipated, given how uncommon it is as a cause of neonatal sepsis. In fact, Dias et al. describe a prevalence of only 0.3% amongst their colonisation swabs (20). Although, the prevalence of colistin resistance was 3/19 (15,8%) of XDR *A. baumannii* isolates*,* it is important to emphasise that only this phenotype has colistin susceptibility performed, and while Ramsamy et al. (2018) described an overall

colistin resistance rate of less than 5% for the period 2011-2015 more recent data has reported an increase in colistin resistance in South Africa (12,34,35).

The are several limitations to our study. Clinical data such as gender, Apgar scores and prematurity were not assessed, and correlation with clinical infection prior or following screening was not performed. These factors and the sample size were greatly influenced by the objectives, funding and laboratory processing in the primary investigation. This single centre study was cross sectional, and thus conclusions on the temporal progression of colonisation could not be made, nor can any broader conclusions be drawn. It was also performed at a tertiary facility, and thus findings may differ at other levels of care. The study was limited to neonates and therefore does not include data on maternal MDRO colonisation. Further characterization by whole genome sequencing would offer more information on genotypic relatedness and mechanism of colistin resistance, but could not be performed in the current study due to limited funding

The high burden of MDR ESKAPE+ *C. auris* colonisation in this unit is concerning, and although the role of colonisation data on its own in predicting infection is unclear, specific MDRO surveillance offers valuable information to identify colonised cases and inform on implementation of IPC measures. Molecular characterization to confirm the phenotypic characteristics documented in this study is required. In addition, further studies are required on the temporal characteristics of MDR colonisation, to review the overall duration of colonisation, individual risk factors associated with colonisation and to assess the long-term infective and non-infective consequences of MDR colonisation.

FUNDING
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CONFLICT OF INTEREST

The authors declare no conflict of interest

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article [and/or its supplementary materials].

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APPENDICES

APPENDIX 1: HREC APPROVAL

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr N Mntla

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M200583

NAME: (Principal Investigator)	Dr N Mntla
DEPARTMENT:	School of Patholgy Department of Clinical Microbiology and Infectious Diseases National Health Laboratory Service
PROJECT TITLE:	Extended characterisation and association between maternal/primary caregiver colonisation and neonatal colonisation by multi-drug resistant organisms at a tertiary hospital in Johannesburg, South Africa
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M191129
SUPERVISOR:	Drs V Chibabhai and T Nana
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2020/06/04

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **May** and will therefore reports and re-certification will be due early in the month of **May** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

30/03/2023
Date

bacterial, fungal and viral infections as a result of being immunocompromised from a premature immune system. It is thus very important to pre-empt the presence of infection in this group of patients, where timeous recognition, diagnosis and treatment may not always be easy.

Areas of the world associated with low to middle socio-economic status and high population densities, have a high prevalence of neonatal infections and fatality (3,4). The overall incidence of neonatal infections in South Africa from over a decade ago was reported as 8.5 - 10%, with late-onset sepsis accountingfor most of these infections (5). Unfortunately, no new national data have beenpublished. More than 50% of bacteraemias at a Johannesburg neonatal unit were found to be caused by multidrug resistant (non-susceptibility to one or more agent in three antibiotic classes) organisms (MDRO) in 2012, the majorityof which were extended beta-lactamase producers (4).

Despite the association of neonatal infections with high morbidity and mortality,not much research has been done to identify significant risk factors for maternally acquired neonatal infections, nor which route is more significant (3,4,6). Historically, neonatal infections have been categorised as early neonatal and late neonatal infections, and the presumed causative organisms and empiric antimicrobials have been based on that distinction (4,5).

For instance, the relationship between Group B Streptococci (GBS), and earlyneonatal disease and mortality is well documented. The establishment of its primary mode of transmission as being vertical, has allowed for implementationof protocols for antenatal screening and development of vaccines (3). Incontrast, the pathogenicity of hospital acquired infections, secondary to MDROs in particular is poorly understood. The rapidly increasing prevalence ofMDRO infection worldwide calls for the development of prevention strategies, which requires an understanding of factors influencing horizontal (from the environment or people) and vertical (in-utero or during delivery) acquisition (3,7)

The development of antimicrobial resistance is a predictable phenomenon; however, due to the inappropriate use of antimicrobials it has now become a global concern. It impacts markedly on mortality, with an estimated 700 000 deaths per year being attributed to antimicrobial resistance globally (8). In the last decade, a group of opportunistic micro-organisms commonly associated with hospital acquired infection have been identified as the ‘ESKAPE + C’ pathogens, and have been distinguished from commonly occurring MDROs, bythe use of multiple mechanisms of resistance and possessing high transmissionrates in hospital units (6,8).

This group of organisms includes, *Enterococcus faecium* (vancomycin resistant(VRE)), *Staphylococcus aureus* (methicillin-resistant) (MRSA), *Klebsiella pneumoniae* (Carbapenem Resistant Enterobacterales (CRE) or Carbapenemase Producing Enterobacterales (CPE)), *Acinetobacter baumannii*(multi-, extensively- and pan-drug resistant), *Pseudomonas aeruginosa* (multi-
, extensively- and pan-drug resistant), and *Enterobacter* species (carbapenem resistant(CPE/CRE) (8). Recently, the increasing drug resistance noted among *Candida* species has led to its further global awareness, and mention as a threat to public health as well.

Hospital-acquired infections with multi-drug resistant (MDR) pathogens, mostof which associated with hospitalisation in general, are on a rise (9). Of the WHO Priority Pathogens List of antibiotic-resistant bacteria, *K.pneumoniae* and *P.aeruginosa*, have been identified as critical priorities for research and development of novel treatment strategies. They account for a large proportion of hospital-acquired infections, and according to the WHO and CDC, are the most concerning with regards to new infections and spread of antibiotic resistance (9).

Amongst peri-partum mothers, a significant increase in colonisation in Enterobacterales is seen in the post-delivery period (10). Of interest , in a study conducted in 2018 in Sri Lanka, more than one quarter of the Enterbacterales identified were *Klebsiella* species; and although the post- delivery colonization rate with ESBL, CRE and MDR *Klebsiella* spp. or *Escherichia coli* was 3.1%, 0.8 % and 0.8% respectively, out of a cohort of 130, 23 (14.5%) of mother-baby pairs were colonised with the same organism that had the same antimicrobial susceptibility pattern; majority of which were *Klebsiella* spp. This was further confirmed by Randomly amplified polymorphicDNA (RAPD) analysis, that showed a similarity of more than 75% at a tolerance of 3.5 (10)

Colonisation is a naturally occurring process in the neonatal period (4,7,11). The foetal microbiome, previously considered a sterile site, has since been shown to encompass a variety of microbes suggesting that foetal microbial colonisation occurs in utero (12). Further colonisation by commensal bacteria occurs after delivery on the skin, mucosal membranes and within the gut, facilitated by contact with the mother and home environment, and subsequentfeeding practices; but neonates who remain in hospital are subject to antibiotic administration, invasive procedures and contact with the hospital environment, and become colonised with MDROs. Furthermore, these neonates are usually

premature and vulnerable, enabling colonisation to progress to invasive infection, more so when colonised by MRSA, vancomycin resistant *Entercoccus feacium* (VRE) and Extended Spectrum Beta-lactamase (ESBL) producing Gram negative bacilli (4,7,13)

Considering the above-mentioned health care associated risk factors, the effect of maternal-neonatal interaction in the healthcare environment on neonatal colonisation requires further investigation. A study done in 2015 showed how diligent skin-to-skin (sts) care by the mothers in neonatal intensive care units (ICU), benefited neonates in a variety of ways; including the development of diverse oral and gut microbiomes. The majority of organisms identified amongthe neonates who did not receive sts care, were associated with infectivity suchas *A.baumanii* and *Pseudomanas* spp, suggesting a beneficial displacement of organisms associated with hospital-acquired infection secondary to interactionwith the mother (12). In contrast, data from a study conducted in 2011, showedhow *S. aureus* infected neonates admitted from the community, were not infected by strains carried by their mothers but were infected with their own nasal strain (14). Similarly, in an investigation of a MRSA bacteraemia outbreakin a neonatal unit in a Gauteng province hospital, in which all the mothers andnursing staff, who were subsequently screened, were found to be negative forMRSA colonisation(15). This implies an alternative source for the hospital acquired pathogen.

Neonates may develop Gram negative sepsis, Gram positive sepsis (GPS) or fungaemia. South African data show a majority of Gram-positive sepsis (greaterthan two thirds of bacteraemias) being caused by GBS, Viridans group *Streptococci* and *Enterococcus* spp*,* and being associated with colonisation atbirth (16). Still, in view of nearly 30% of the adult population being colonised with *S. aureus* in their anterior nares, it is argued that horizontal transmission from their mothers is the primary mechanism for early infant staphylococcal colonisation (14).

Gram negative sepsis (GNS) is also of concern in the neonatal period, particularly with prematurity. Antimicrobial resistance patterns amongst Gram negative bacilli (GNB) with an increased prevalence, include: ESBL and CRE found in members of the Enterbacterales order, and carbapenem resistance amongst *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates occurring at 25% and 80%, respectively. Nearly two-thirds of *K. pneumoniae,* which was the most commonly isolated GNB between 2012 and 2017 in SouthAfrica, were ESBL; further complicated by the emergence of carbapenemase-producing *K. pneumoniae* strains that worsened carbapenem resistance in GNBs (4,17,18).

During the past 10 years, infection with carbapenem resistant *Klebsiella pneumoniae* (CRKP) have increased worldwide, due to the rapid spread of carbapenem-hydrolysing enzymes on mobile genetic elements (19). This hasnot only resulted in widespread outbreaks, but increased mortality with an estimated crude mortality rate secondary to CRKP infection of 30-80%, greater than that seen in the susceptible counterparts (19). A retrospective case–control study conducted over five years in neonatal and paediatric ICUs

identified a total of 3.03% of patients that were colonised with CRKP, and subsequent clinical infection occurred in 28.2% amongst those colonised. This resulted in a prolonged the length of stay and higher mortality rate (19).
Identifying colonised patients, may therefore assist with appropriate infection control measures, and implementing appropriate empiric therapy in suspectedsepsis.

The NICD reported in 2017 that *Candida auris* is the second most common cause of fungaemia both in the public and private sectors, secondary to *C. parapsilosis* (20). Although *C.auris* is not a common cause of neonatal fungaemia, outbreaks within neonatal intensive care units are frequently reported. This could be related to its ability to survive for weeks on hospital surfaces and for months as a coloniser after active infection. Furthermore, thelack of evidence to support the efficacy decolonisation practices such as chlorhexidine baths, may contribute to further transmission.

This further illustrates the need for MDRO surveillance systems, that will assistin recognising pathogens based on site of colonisation, to pre-empt action before development of disease and reduce colonisation rates.

It is with these factors in mind that this study is being undertaken. The aim of this study is to perform an extended characterisation, both a phenotypicdescription of the pattern of resistance, and genotypic determinants, of multi- drug resistant organisms colonising neonates and mothers/primary care givers.

It is also aimed at determining if there is a correlation between the multi-drug organisms colonising neonates and maternal/primary care givers without inferring

causality i.e. without necessarily proving that neonatal colonisation is as a direct consequence of exposure to the mother/primary care giver.

Study Objectives:

1. To perform extended characterisation of Multi-Drug Resistant (MDR) isolates colonising neonates at Chris Hani Baragwanath AcademicHospital (CHBAH) neonatal units, with primary consideration of ESKAPE + *C. auris* organisms.

- Specific parameters to be studied include:
  1A: Identification of the organism and antimicrobial susceptibility profile(performed as part of a larger study)
  1B: Determine the proportion of organisms that are multi-drug resistantfrom the cohort of neonates.
  1C: Identify the determinants of resistance of the multi-drug resistantorganisms

2. To establish the prevalence of multi-drug resistant micro-organisms colonising mothers/primary caregivers of neonates admitted at CHBAH neonatal units, with primary consideration of ESKAPE + *C. auris* organisms.

- Specific parameters to be studied include:
  2A: Identification of the organism and antimicrobial susceptibility profile(performed as part of a larger study)
  2B: Determine the proportion of organisms that are multi-drug resistant from the cohort of mothers/primary care givers.
  2C: Identify the determinants of resistance of the multi-drug resistant organisms

3. To determine whether phenotypic and genotypic correlation exists between MDR organisms isolated from neonates and mothers/primary care givers with laboratory proven colonisation
   3A: To analyse a subset of the most common organisms occurring in both cohorts, for correlation of the antimicrobial susceptibility profiles (ASP), and genotypic determinants of resistance.
   3B: To perform Pulse-Gel Electrophoresis (PFGE) on a subset of *K.pneumoniae* isolates to determine the genetic relatedness amongst isolates with the same ASP and determinants of resistance.

Methodology:

This study is a sub-study, of an approved research project which will be conducted concurrently. The project is entitled: “Microbiological Surveillance of hospital environmental surfaces, medical equipment, healthcare workers, patients and patients’ parents in a tertiary hospital in Soweto, South Africa.”.

I will be a member of the research team that will be sampling in the primary investigation; however, this sub-study will specifically focus on samples and data collected from neonates and mothers/primary care givers.

Study design:
A cross-sectional cohort study.

Source population:

The primary investigation involves neonates admitted, mothers/primary care givers and health care workers at the neonatal units at CHBAH.

Study population:

One hundred (100) neonates and one hundred matched (100) mothers/primarycare givers will be randomly selected for sampling.

The rationale of this sample size is based on:

a) Funding
b) Feasibility of processing samples
c) The likelihood of mothers/care givers being present during neonatalsampling.

a. Inclusion criteria

- All neonates present in the respective neonatal units on the days ofsampling.
- For the purpose of this study a neonate will be defined as being28 days old or younger
- All mothers of the neonates chosen for sampling will be included
- A primary care giver will be defined as any person who has the right to care for the neonate in the absence of the mother. This may include butis not limited to the father, next of kin or appointed guardian.

b. Exclusion criteria

- Congenital malformations that may cause hindrance in sampling such as imperforate anus, gastroschisis, choanal atresia or cleft palate
- Participants (neonates and mothers/primary care giver) that are transferred in from other hospitals
- Neonates that have undergone surgical intervention
- In cases of nasal intubation, the nostril in which the tube is, will not be swabbed.
- Mothers/primary care givers that are unable to consent
- Mothers who are non-ambulatory due a clinical condition that interruptstheir interaction with the neonate and/or are unable to perform sample collection themselves.

Data Collection

<u>Study Period and assigning of participants</u>

This study will be conducted in 2 phases because it involves data collected frommultiple areas within the hospital: Neonatal Intensive Care Unit (NICU), Transitional Intensive Care Unit (TICU), Ward 66 (general neonatal ward) andLabour Ward nursery.

The first phase of data collection will take place in NICU and TICU in November2020, during which I will randomly select one third of neonates and mothers/primary care givers being sampled in this phase, and then select the remaining two thirds during the second phase in December 2020. This secondphase will include data from Wards 66 and Labour ward nursery only.

The rationale behind this division is based the following factors:

1. Bed occupancy in each area: NICU and TICU have a combined capacityof ~45 beds, while W66 and LWN have a combined bed capacity of ~95beds; and are usually all occupied
2. Mothers/primary care giver of neonates in ward 66 and labour ward nursery will be easier to locate.

3. Given the disease profiles in each unit I anticipate prolonged lengths of stay in NICU/TICU, which would limit the number of new neonates fromwhich to collect data.

Sample collection Procedure

All swabs will be labelled with a unique study number prior clinical sampling, that discriminates participants according to ward only, and will be packaged together with a consent form (mothers/primary care giver) and data collection slip. Three screening swabs will be collected from each neonate, according to the standard operating procedure (SOP) described in Appendix 4. Two Rayon swabs (ChemLab supplies, South Africa) (5mm diameter) will be used to

perform a rectal swab and a skin swab of the umbilicus, both axilla and the groin area, respectively; and a nasopharyngeal swab (2mm diameter, rayon bud) will be used to perform a nasal swab. Each nostril will be swabbed up to the level of the inferior turbinate (~1cm depth) with the same swab. If the neonate has supplemental nasal prong oxygen, I will swab the individual prongs.

Mothers will be counselled about the purpose of the study as a group, and shown an educational video on how to perform the self-administered vaginal and rectal swabs, after-which we will distribute consent forms and data collection sheets to willing participants, in addition to the two rayon swabs (5mm diameter) for the self-administered swabs. We will begin with swabbing their hands on both palmar and dorsal surfaces, after which they will complete theirself-administered swabs.

Proposed sampling plan:

<table>
<tr><th></th><th>Maximum bed capacity in each ward</th><th>Sample size required in Phase 1</th><th>Week 1 (swabs)</th><th>Week 2 (swabs)</th><th>Week 3 (swabs)</th><th>Total swabs collected (per week)/in total</th></tr>
<tr><td>November 2020 (40%) (NICU+TICU)</td><td>12+ 35 = 47 neonates</td><td>40</td><td>14x3</td><td>13x3</td><td>13x3</td><td rowspan="2">(~84) /240</td></tr>
<tr><td>Matched number of mothers/Primary care givers</td><td></td><td>40</td><td>14x3</td><td>13x3</td><td>13x3</td></tr>
<tr><td rowspan="2">December 2020 (60%)

(W66 + Labour ward nursery)</td><td rowspan="2">90+5= 95 neonates</td><td>Sample size required in Phase 2</td><td>Week 1</td><td>Week 2</td><td>Week 3</td><td rowspan="3">(~120)/360</td></tr>
<tr><td>60</td><td>20x3</td><td>20x3</td><td>20x3</td></tr>
<tr><td>Matched number of mothers/Primary care givers</td><td></td><td>60</td><td>20x3</td><td>20x3</td><td>20x3</td></tr>
</table>

Each swab will be placed into Amies transport medium (ChemLab supplies, South Africa), and transported to the Infection, Prevention and Control laboratory at Charlotte Maxeke Academic Hospital at a temperature of 4°C Samples will be transported and processed by an appropriately allocated member of the research team within 24 hours of collection.

The medical technologists employed for the primary investigation will place each swab into Brain Heart Infusion (BHI) enrichment media (Diagnostic MediaProducts [DMP], National Health Laboratory Service [NHLS], South
Africa) and incubate aerobically overnight at 35 ° C for a better yield oforganisms. The BHI solution will be assessed after~18-24 hours for turbidity. Once turbid, using a 10 µl sterile metallic loop, the BHI solution will be sub- cultured the onto respective agar plates according to the SOP.

Both non-selective (5% sheep blood), and selective agar will be used to differentiate the organisms of interest (Diagnostic Media Products [DMP], National Health Laboratory Service [NHLS], South Africa) i.e. ESKAPE+ C*. auris* organisms. MacConkey agar (DMP, NHLS) used to select for Gram negative organisms, Cetrimide agar (DMP, NHLS) to select for *Pseudomonas aeruginosa;* Blood with nalidixic acid (DMP, NHLS) to select for *Enterococcus* species, Baird-Parker agar (DMP, NHLS) to select for *S. aureus* and Sabouraud dextrose agar (DMP, NHLS) with chloramphenicol to select for *Candida* species.

(Please see Appendix 4 for processing procedure and which agar will be usedfor each swab)

Plates will be reviewed after ~18-24 hours for growth, and organisms identifiedby phenotypic methods i.e. appearance on agar and rapid tests (Please see Appendix 4).

Once a preliminary identification has been made of ESKAPE+ *C. auris* organisms, identification will be confirmed using the automated VITEK-MS MALDI-TOF (bioMèrieux, France) system. This result will be available on the same day which will allow for automated AST to be performed only on confirmed ESKAPE + C organisms (VITEK 2 (bioMèrieux, France)). AST will be interpreted according to CLSI guidelines (2020)(21). If susceptibility testingfails on the VITEK 2, they will be repeated once. If a repeat test fails, antimicrobial susceptibility will be performed by the Kirby Bauer disk diffusion method for bacteria and E-test method for *C. auris.*

As this study will focus on the extended characterisation and correlation of multi-drug resistant isolates, only isolates meeting the following descriptions will be further characterised:

Organism isolated	**Pattern of resistance**	**Definition**	**Determinant of resistance available**	**Additional tests**
K. pneumoniae	**CRE**	non- susceptible (R/I) to ≥1carbapenem.	Carbapenemase production testing by RESIST-4.O.K.N.V assay (Coris®)	-Colistin Broth Micro Dilution (BMD) -Carbapenem Etests

	ESBL	Non- susceptible to ≥1 3rd generation cephalosporin	-	Carbapenem Etests
E. cloacae	**CRE**	non- susceptible (R/I) to ≥1carbapenem.	Carbapenemase production testing by RESIST-	-Carbapenem Etests -Colistin BMD
			4.O.K.N.V assay (Coris®)	
	ESBL	Non- susceptible to ≥1 3rd generation cephalosporin	-	Carbapenem Etests
P. aeruginosa	**XDR**	Non- susceptible (R/I) to ≥1 agent in all but ≤2 categories.	Carbapenemase production testing by RESIST-4.O.K.N.V assay (Coris®)	Colistin BMD
A. baumannii	**XDR**	non- susceptible (R/I) to ≥1 agent in all but ≤2 categories.	Carbapenemase production testing by RESIST-4.O.K.N.V assay (Coris®)	Colistin BMD
E. faecium	**VRE**	Vancomycin MIC > 4 µg/ml	Xpert VRE	Genotypic characterizationof resistance
S. aureus	**MRSA**	Oxacillin (MIC) ≥4 µg/ml		Perform Vancomycin Etest.
C. auris	**Azole resistance**	FluconazoleMIC ≥32µg/ml		Perform Amphotericin B Etest

Once multi drug resistant organisms have been identified, a subset of 10 *K.pneumoniae* isolates (based on cost and available funding) will be further tested using Pulse-Field Gel Electrophoresis to establish the geneticrelatedness of neonatal and maternal/primary care giver MDRO colonisers.

This subset is based on an average yield of ~20% of *Klebsiella* spp from positive blood cultures for the year 2019 from internal data, noted as the 2nd most prevalent organism; and an average prevalence of 6.2% amongst respiratory and blood specimens collected from healthy infants (13).

Three other *Klebsiella pneumoniae* strains, that are not part of the data set, will be used as controls to display unrelatedness. American type culture collection (ATCC) strains will be used as controls. Isolates will be run on theBIORAD CHER-DRII/III electrophoresis system, and the electrophoresis runaccording the parameters of *K. pneumoniae*. Samples will be analysed with the BioNumerics software.

All data will be documented in data collection sheets (see Appendix 5).Bias,

Variables and Limitations

A. Bias:

- Sampling will be random, by selecting every alternate neonate bed, and only encompass a cohort of participants, and not the entire population inthe units
- Of the mothers who are also admitted, only those well enough to be present in the neonatal units will be sampled, and not critically ill patientswho are bed bound and may have significant colonisation.

B. Variables:

- Temporal variation in colonisation due to sampling outside of visiting hours or sampling after chlorhexidine baths
- Study will be reflective of both early neonatal colonisation suggesting vertical transmission, and late neonatal colonisation (within 28 days of life) suggesting horizontal transmission.
- The mothers/primary care givers will be from different literarybackgrounds, and may speak a different language
- Given the current SARS-CoV2 pandemic and the nature of infection, prevention and control practices in the neonatal units, the mothers handswill be swabbed following hand washing or disinfection with alcohol which may affect findings.

C. Limitations:

- The accuracy of mother's/primary care givers' colonisation depends on their understanding of the self-administration procedure
- The desired sample size from mothers/primary care givers may not be achievable due their absence during times of sampling, or non-consentfor participation.

Data Analysis

Descriptive statistics will be used to analyse the data. Categorical data will be recorded as frequencies and percentages. Percentages will be used to determine the proportion of colonisation amongst the neonatal and maternal/care givers. Resistance to antimicrobials will also be recorded as percentages. Where possible, Pearson's correlation will be used to determine correlation of isolates between the neonate and mother/care giver. The following variables will be assessed: organism identified, pattern of multi-drug resistance and genotypic determinant thereof.

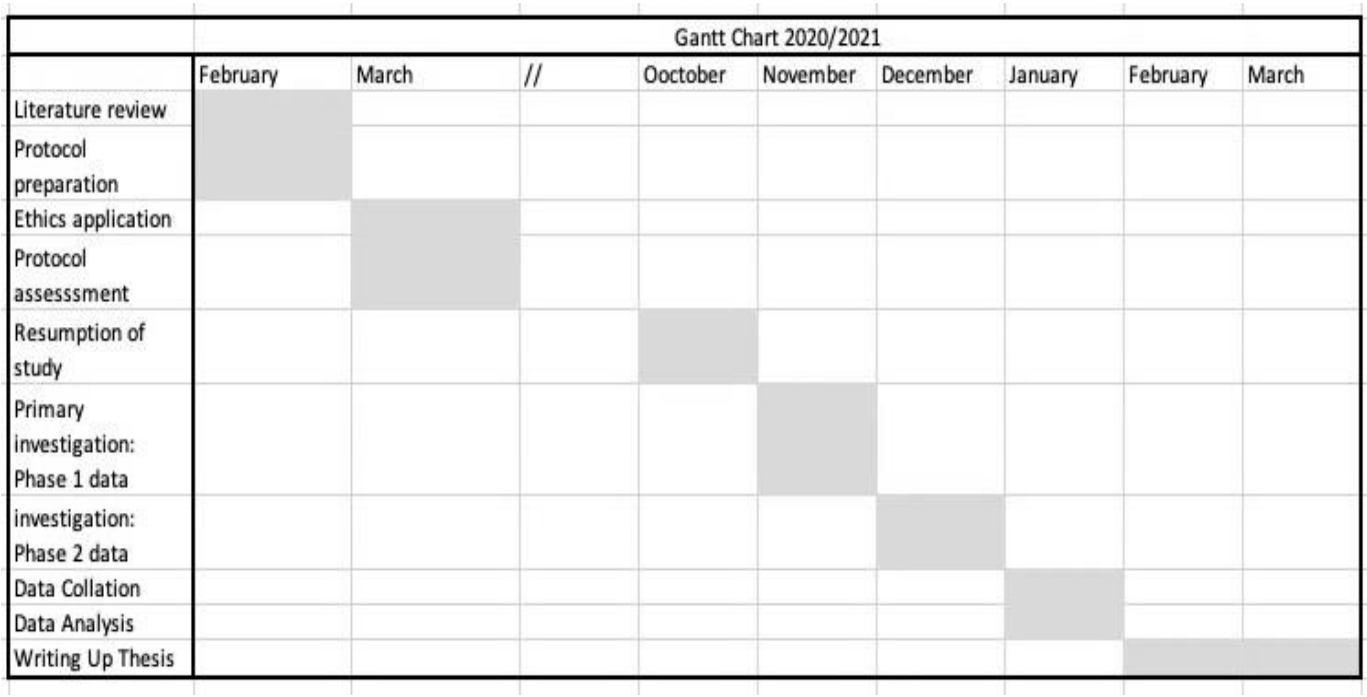
Ethical consideration

Ethics clearance has been granted for this investigation: clearance certificate number M200583, as well as the primary investigation (clearance certificate no:M191129). We

also have approval from the CEO of Chris Hani Baragwanath academic hospital to conduct the study (see Appendix 1). Regarding this study,patient anonymity will strictly be adhered to. Participants are identified by studynumber. Neonatal data collection sheets will be completed using clinical information volunteered by the mother/primary care giver or treating clinician,

and neonatal samples will be collected as routine clinical surveillance, the results of which will be available to clinicians.
Mothers/primary care givers will be shown an educational video prior to them being counselled and sampled in the same ward that their neonate is admittedin. This will explain the procedure of self-administered swabs. They will be counselled as a group, and then given individual consent forms and swabs to perform sample self-collection as described. Their data collection sheets are anonymised and will be included with the consent forms, which they can complete before/after sampling

Timing



Funding

- The primary investigation will be funded by a ~$100 000 grant awardedby The Bill and Melinda Gates Foundation
- A K grant of a minimum of R25 000 will be applied for further funding ofmolecular testing.
- Stationary costs and article publishing costs will be accessed throughCMID and Wits University Finance Office respectively
- All expenses (See Appendix 6)

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APPENDIX 3: CHANGE OF TITLE CONFIRMATION

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

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06 December 2021
Person No: 456149
TAA

Dr NM Mntla
P O Box 18300
Pretoria North
Annlin
0066
South Africa

Dear Dr Nonkululeko Mntla

Master of Medicine in Microbiological Pathology: Change of title of research

I am pleased to inform you that the following change in the title of your Research Report for the degree of **Master of Medicine in Microbiological Pathology** has been approved:

From:	**Extended characterisation and association between maternal/primary care giver colonisation and neonatal colonisation by multi-drug resistant organisms at a tertiary hospital in Johannesburg, South Africa.**
To:	**Extended characterisation of multi-drug resistant organisms colonising neonates at a tertiary hospital in Johannesburg, South Africa**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

APPENDIX 4: STANDARD OPERATING PROCEDURES

Standard operating procedure for the collection and processing of neonatal rectal swabs for the study: *Microbiological Surveillance of Hospital environmental surfaces, Medical equipment, Healthcare Workers, Patients and patients' parents in a tertiary hospital in Soweto, South Africa.*

1. **Purpose:**
   i. Provide detailed instruction on how to perform a rectal swab on neonates
   ii. Provide methodology for processing said clinical sample to identify patients colonized with carbapenem- resistant or carbapenemase – producing Enterobacterales/ Vancomycin Resistant Enterococcus faecium isolates in the intestinal tract
2. **Scope:**
   Performed by all competent staff trained in the collection and processing of clinical samples, this may include, but is not limited to: a CMID registrar, Medical technologist, Infection prevention and control personnel, and trained paediatric personnel.

3. **Responsibility:**
   All biomedical technologists, CMID registrars and IPC personnel involved in the collection of samples and processing.

4. **Definitions:**

   - CMID: Clinical Microbiology and Infectious Disease
   -CHBAH: Chris Hani Baragwanath Academic Hospital
   -CRE: Carbapenem Resistant Enterobacterale
   -VRE: Vancomycin resistant Enterococcus faecium
   -PPE: Personal protective equipment
   -IPC: Infection prevention and control
   -**ESKAPE-C pathogens**: Pathogens associated with nosocomial sepsis and drug resistance(34), namely:
   - *Enterococcus faecium*
   - *Staphylococcus aureus*
   - *Klebsiella pneumonia*
   - *Acinetobacter baumannii*
   - *Pseudomonas aeruginosa*
   - *Enterobacter species*
   - *Candida auris*

5. **Principle of procedure:**
   A cross sectional qualitative description of the micro-organisms neonates at CHBAH are colonized/infected by, primarily aimed at selective recovery of CRE/VREs.(35)

6. **Patient preparation and Specimen collection:**
   Specimens to be tested:

- Rectal swab only
    - No stool sample required

Transport requirement:

- To be placed in transport medium (Cary Blair medium); if possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium.

Patient Preparation:

- All swabs must be labelled prior to sampling

Rejection criteria:

- Un-labelled sample
- Sample incorrectly packaged
- Swab received beyond 24 hours after sampling and not placed on ice

7. Equipment and reagents:

	At Collection:	**For Processing:**
Equipment	PPE(non-sterile gloves and plastic apron)	Sterile scissors
	Rayon swabs (pre-moistened in Cary-Blair medium)	Sterile forceps
	Cary-Blair transport medium	Sterile loops 10µl
	Plastic sleeve	Bunsen burner
	Ice pack (maintain temp. 2-4◦C)	35◦C ± 2 aerobic incubator
	Cooler box	Vortex Mixer
		VITEK®-MS automated system
		VITEK® 2 automated system
Reagents and antimicrobials	Nil	Catalase reagent Rapid indole Oxidase
		Streptex kit
		5 ml Brain heart infusion broth(BHI)
		Vancomycin Etest Teicoplanin Etest Amphotericin B Etest
Agar plates	Nil	MacConkey agar plate (MAC)
		Cetrimide agar plate (C)
		Blood+ Nalidixic acid+ Colistin agar plates (BNC)
		Sabouraud dextrose agar (SDA)

8. Environmental and safety controls

At specimen collection:

- Restrict sampling to individual patient's cot
- Attempt to avoid contamination of the swab while sampling by removing any obstructive items

- Don appropriate PPE, which includes but is not limited to:
- Appropriately sized gloves
- Plastic apron
  - Seal the swab immediately after sampling in correct transport medium.

  <u>At specimen processing:</u>
  - A biological safety cabinet is not required for sampling of swabs
  - Material used for collection and or processing of samples is discarded into biohazard boxes lined with red plastic bags.
  - Needles and syringes should be disposed of into sharps containers.

9. Quality Control

Quality Control strains:	+	-
Catalase	*S. aureus* ATCC 25923	*Enterococcus faecalis ATCC 29212*
Aesculin bile agar/PYR	*Enterococcus faecalis ATCC 29212*	Streptococcus agalactiae ATCC10386
Rapid indole	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Oxidase	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922
Latex agglutination	*S. aureus* ATCC 25923	*Staphylococcus epidermidis ATCC 12228*

10. Procedural steps: For Specimen Collection(35,36)

(If possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium)

i. Wash hands and don appropriate PPE
ii. Expose the neonate
iii. Discard grossly contaminating stool with a wipe/ tissue paper
iv. Insert the premoistened rayon tipped swab 1-2cm into the anal canal
v. Rotate against rectal wall several times for 10 seconds
vi. Place the swab into transport media immediately, pushing it to the bottom of the tube.
vii. Tighten cap firmly. Transport swabs to the laboratory within the 24 hours after collection. Swab must be stored at 4 °C and below.

Procedure steps: For Swab processing (36–38)

i. Remove swab from packaging, and ensure correct swab (rectal) is selected
ii. Remove polyester-tipped/rayon-tipped swab from Cary Blair transport medium
iii. Place immediately in 5ml BHI and vortex for 15 seconds(39)
iv. Incubate aerobically overnight and examine for turbidity at 24 and 48 hours.

v. Sub-culture turbid BHI using a 10µl loop onto: MAC+BNC+SDA+C. Streak out for single colonies
vi. Perform presumptive identification on colony morphology and rapid reagent testing
vii. Further identification of suspicious colonies to take place on Vitek-MS(BioMèriuux)
viii. If target organisms are identified antimicrobial susceptibility testing will take place on VITEK® 2 or appropriate manual methods as per CLSI standards.
ix. All processing is to be recorded in detail on the working cards provided and signed.
x. All working cards must be reviewed and signed by a CMID registrar.

Morphological features of target organisms:

- *Enterococcus faecium*: Small off-white to grey round colonies with α-haemolysis or ϒ-haemolysis on 5% sheep blood agar.
- *Staphylococcus aureus*: Small round black colonies on Baird parker with a surrounding zone of clearing.
- *Klebsiella pneumonia*: Lactose fermenting on MacConkey agar, raised circular 2-3mm colonies with entire margins, often mucoid.
- *Acinetobacter baumannii*: Small 1-2mm round convex colonies with an entire margin that are non-lactose fermenting but light pink in colour and opaque on MacConkey agar.
- Pseudomonas aeruginosa: Non-lactose fermenting on MacConkey agar, flat spreading 2-3mm colonies with a wrinkled moist surface and a metallic sheen, oxidase Positive.
- *Enterobacter* spp: Grey to off-white spreading round colonies with entire to undulate margins on 5% blood agar.
- *Candida auris*: Large creamy whitish colonies on Sabouraud dextrose agar

MAC	Lactose-fermenting (pink red) colonies	Perform rapid indole test	If **positiv**e= finalise as **non-ESKAPE** pathogen
			If **negative**= continue to identify and perform susceptibility testing.
	Non-lactose fermenting colonies	Perform oxidase test	If **positive**, correlate with growth and pigmentation on CA plate_ presumptive *Pseudomonas aeruginosa*, but must confirm ID.

			Continue to ID **all** NLF isolates to species level, and only perform susceptibility testing on ESKAPE+ C organisms identified on the VITEK® 2 platform.
C (Correlate with growth on MAC)	Positive	Presence of growth on agar, with associated yellow-green to blue colour indicating the production of pyocyanin	Continue with ID, susceptibility only on *P. aeruginosa*
	Negative	No bacterial growth on agar plate	Finalise as non-ESKAPE pathogen
BNC	▪ **Streptococcal** morphology seen:	Perform Streptex	If agglutination for **group D** is present, suggests *Enterococcus spp.* Continue with ID an susceptibility from BNC Confirm Vancomycin and Teicoplanin MIC by Etest if resistant on VITEK® 2
			Agglutination with groups (A,B,C,F,G) or no reaction. Finalise as non-ESKAPE pathogen
	▪ **Staphylococcal** morphology seen	▪ Perform catalase test on similar sized colonies on MAC ▪ Perform Prolex latex Agglutination test	If **positive,** continue with ID. Perform susceptibility only on S. aureus isolates
			If **negative,** perform Streptex to exclude *Enterococcus* spp. If Negative, finalise as non-ESKAPE pathogen. If

			positive continue as mentioned above.
SDA	Assess for growth	Positive: White to cream coloured colonies	Continue with ID. Perform Amphotericin B E test only on *C. auris* isolates

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Compiled by Dr NM Mntla

Standard operating procedure for the collection and processing of neonatal nasal swabs for the study: *Microbiological Surveillance of Hospital environmental surfaces, Medical equipment, Healthcare Workers, Patients and patients' parents in a tertiary hospital in Soweto, South Africa.*

1. **Purpose:**
   i. Provide detailed instruction on how to perform a nasal swab on neonates
   ii. Provide methodology for processing abovementioned clinical sample to identify patients colonized with the ESKAPE + C pathogens.
2. **Scope:**
   Performed by all competent staff trained in the collection and processing of clinical samples, this may include, but is not limited to: a CMID registrar, Medical technologist, Infection prevention and control personnel, and trained paediatric personnel.

3. **Responsibility:**
   All biomedical technologists, CMID registrars and IPC personnel involved in the collection of samples and processing.

4. **Definitions:**
   - **CMID:** Clinical Microbiology and Infectious Disease
   - **CHBAH:** Chris Hani Baragwanath Academic Hospital
   - **PPE:** Personal protective equipment
   - **IPC:** Infection prevention and control
   - **MRSA:** Methicillin-resistant Staphylococcus aureus
   - **ET:** Endotracheal Tube
   - **ESKAPE+C pathogens**: Pathogens associated with nosocomial sepsis and drug resistance, namely(34):
   - *Enterococcus faecium*
   - *Staphylococcus aureus*
   - *Klebsiella pneumonia*
   - *Acinetobacter baumannii*
   - *Pseudomonas aeruginosa*
   - *Enterobacter species*
   - *Candida auris*

5. **Principle of procedure:**
   A cross sectional qualitative description of the multi-drug resistant organisms that neonates at CHBAH are colonized/infected by, primarily aimed at the selective recovery of MRSA(40) and other ESKAPE+ C organisms.

6. **Patient preparation and Specimen collection:**
   Specimens that may be used in this testing:
   Nasal swab only
   - In case of Nasal prong use: include swabbing over the surfaces of prongs from each nostril

- In case of intubation: include swabbing over ET and nasopharyngeal secretions

Transport requirement:

- To be placed in transport medium (Cary Blair medium); if possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium.

Patient Preparation:

- All swabs must be labelled prior to sampling

Rejection criteria:

- Un-labelled sample
- Sample incorrectly packaged
- Swab received beyond 24 hours after sampling and not placed on ice

7. Equipment and reagents:

	At Collection:	**For Processing:**
Equipment	PPE (non-sterile gloves and plastic apron)	Sterile scissors
	2mm Rayon swabs (pre-moistened in Cary-Blair medium)	Sterile forceps
	Cary-Blair transport medium	Sterile loops
	Plastic sleeve	Bunsen burner
	Ice pack (maintain temp. 2-4◦C)	35◦C ± 2C aerobic incubator
	Cooler box	Vortex Mixer
		VITEK® 2 automated system
		VITEK®-MS automated system
Reagents and antimicrobials	Nil	Catalase reagent Rapid indole Oxidase
		Streptex kit
		5 ml Brain heart infusion broth(BHI)
		Vancomycin Etest Teicoplanin Etest Amphotericin B Etest
Agar plates	Nil	Blood+ Nalidixic acid+ Colistin agar plates (BNC)
		Sabouraud dextrose agar (SDA)
		5% Sheep blood agar (BAP)
		Baird-Parker Agar (BP)

		MacConkey agar(MAC)
		Muller Hinton agar

8. Environmental and safety controls

At specimen collection:

- Restrict sampling to individual patient's cot
- Attempt to avoid contamination of the swab while sampling by removing any obstructive items
- Don appropriate PPE, which includes but is not limited to:

- Appropriately sized gloves
- Plastic apron

- Seal the swab immediately after sampling in correct transport medium.

At specimen processing:

- A biological safety cabinet is not required for sampling of swabs
- Material used for collection and or processing of samples is discarded into biohazard boxes lined with red plastic bags.
- Needles and syringes should be disposed of into sharps containers.

9. Quality Control

Quality Control strains:	+	-
Catalase	*S. aureus* ATCC 25923	*Enterococcus faecalis ATCC 29212*
Aesculin bile agar/PYR	*Enterococcus faecalis ATCC 29212*	Streptococcus agalactiae ATCC10386
Rapid indole	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Oxidase	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922
Prolex Latex agglutination	*S. aureus* ATCC 25923	*Staphylococcus epidermidis ATCC 12228*

10. Procedural steps: For Specimen Collection (41)

(If possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium)

i. Wash hands and don appropriate PPE
ii. Expose the neonate and rule out any nasal obstructions
iii. Insert a pre-moistened swab into each nostril at least 1cm deep or until resistance against the inferior turbinate is met (41)
iv. Rotate within the nostril and allow to remain in situ for at least 3 seconds to absorb
v. Using the same swab, repeat the process in the other nostril
    a. In the case of an infant on supplemental O2, include swab of nasal prongs

vi. Place the swab into transport media immediately, pushing it to the bottom of the tube.
vii. Tighten screw-cap firmly. Transport swabs to the laboratory within the 24 hours after collection, Swab must be stored at 4 °C and below.

Procedure steps: For Swab processing(38,42)

i. Remove swab from packaging, and ensure correct swab (nasal) is selected
ii. With sterile forceps, immediately place the nasal swab into the BHI liquid medium and vortex for 15 seconds
iii. Incubate overnight at 35 ± 2^0C under aerobic conditions (may require prolonged incubation if not turbid after 24 hours)
iv. Once solution has become turbid, vortex for 15 seconds.
v. Subculture using a 10µl loop onto: BAP+BNC+MAC+BP+SDA, and streak out for single colonies, incubate overnight at 35 ± 2^0C under aerobic conditions
vi. Perform presumptive identification on colony morphology and rapid reagent testing
vii. Further identification of suspicious colonies to take place on Vitek-MS(Biomerieux)
viii. If target organisms are identified antimicrobial susceptibility testing will take place on VITEK® 2 or appropriate manual methods as per CLSI standards.
ix. All processing is to be recorded in detail on the working cards provided and signed.
x. All working cards must be reviewed and signed by a CMID registrar.

Morphological features of target organisms:

- *Enterococcus faecium*: small off-white to grey round colonies with α-haemolysis or Ɣ-haemolysis on 5% sheep blood agar.
- *Staphylococcus aureus*: Small round black colonies on Baird parker with a surrounding zone of clearing.
- *Klebsiella pneumonia*: Lactose fermenting on MacConkey agar, raised circular 2-3mm colonies with entire margins, often mucoid.
- *Acinetobacter baumannii*: Small 1-2mm round convex colonies with an entire margin that are non-lactose fermenting but light pink in colour and opaque on MacConkey agar.
- Pseudomonas aeruginosa: non-lactose fermenting on MacConkey agar, flat spreading 2-3mm colonies with a wrinkled moist surface and a metallic sheen, oxidase Positive.
- *Enterobacter* spp: Grey to off-white spreading round colonies with entire to undulate margins on 5% blood agar.
- *Candida auris*: Large creamy whitish colonies on Sabouraud dextrose agar

BAP	**Staphylococcal colony** morphology seen (Correlate with growth on Baird Parker Agar)	▪ Perform catalase test on similar sized colonies on MAC or Baird Parker Agar	If **positive,** continue with formal ID. Perform susceptibility only on S. aureus isolates
			If **negative,** perform Streptex to exclude *Enterococcus* spp (agglutination with group D reagent). If Negative, finalise as non-ESKAPE pathogen. If positive continue with ID and perform susceptibility only on *E. faecium* isolates
	Streptococcal morphology seen	Perform Streptex	If agglutination for **group D** is present, suggests *Enterococcus spp.* Continue with ID a susceptibility from BNC Confirm Vancomycin and Teicoplanin MIC by Etest if resistant on VITEK® 2
			Agglutination with groups (A, B, C, F, G) or no reaction. Finalise as non-ESKAPE pathogen
	Yeast-like colony Morphology seen	▪ Perform a Gram to confirm	(See SDA)
	Lactose-fermenting (pink red) colonies	Perform rapid indole test	If **positiv**e= finalise as **non-ESKAPE** pathogen
			If **negative**= continue to identify and perform susceptibility testing.

MAC	Non-lactose fermenting colonies	Perform oxidase test	If **positive**, presumptive *Pseudomonas aeruginosa*, but must confirm ID.
			Continue to ID **all** NLF isolates to species level, and only perform susceptibility testing on ESKAPE+ C organisms identified
Baird Parker	Positive	Grey-black colonies with a halo ring	Continue with ID, susceptibility only on *S.aureus*
	Negative	No/other bacterial growth on plate	Finalise as non-ESKAPE pathogen
SDA	Assess for growth	Positive: White to cream coloured colonies	Continue with ID. Perform Amphotericin B E test only on *C.auris* isolates

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Compiled by Dr NM Mntla

Standard operating procedure for the collection and processing of neonatal skin (axillary/umbilical/groin)) swabs for the study: *Microbiological Surveillance of Hospital environmental surfaces, Medical equipment, Healthcare Workers, Patients and patients' parents in a tertiary hospital in Soweto, South Africa.*

1. **Purpose:**
   i. Provide detailed instruction on how to perform a screening skin swab on neonates
   ii. Provide methodology for processing the above-mentioned clinical sample to identify patients colonized with ESKAPE+C pathogens.
2. **Scope:**
   Performed by all competent staff trained in the collection and processing of clinical samples, this may include, but is not limited to: a CMID registrar, Medical technologist, Infection prevention and control personnel, and trained paediatric personnel.

3. **Responsibility:**
   All biomedical technologists, CMID registrars and IPC personnel involved in the collection of samples and processing.

4. **Definitions:**
   - **CMID:** Clinical Microbiology and Infectious Disease

   - **CHBAH:** Chris Hani Baragwanath Academic Hospital
   - **PPE:** Personal protective equipment
   - **IPC:** Infection prevention and control
   - **ESKAPE+C pathogens**: Pathogens associated with nosocomial sepsis and drug resistance, namely(34):
   - *Enterococcus faecium*
   - *Staphylococcus aureus*
   - *Klebsiella pneumonia*
   - *Acinetobacter baumannii*
   - *Pseudomonas aeruginosa*
   - *Enterobacter species*
   - *Candida auris*

5. **Principle of procedure:**

   A cross sectional qualitative description of the multi-drug resistant organisms neonates at CHBAH are colonizcolonised/infected by, primarily aimed at the selective recovery of *Candida auris, Pseudomonas aeruginosa* and *Acinetobacter baumannii* and other ESKAPE + C organisms
6. **Patient preparation and Specimen collection:**
   Specimens that may be used in this testing:
   - Skin swab only
     Areas of interest: Around the umbilicus, both axilla and the groin area(43)
   Transport requirement:

- To be placed in transport medium (Cary Blair medium); if possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium.

Patient Preparation:
- All swabs must be labelled prior to sampling
- Swab to be performed at least 48 hours after administration of topical antiseptic (e.g., chlorhexidine

Rejection criteria:
- Unlabelled sample
- Sample not in sterile packaging or contaminated
- Swab received beyond 24 hours after sampling, not placed on ice
- Non-patient specimen swabs.

7. Equipment and reagents:

	At Collection:	For Processing:
Equipment	PPE	Sterile scissors
	Standard Rayon swabs (pre-moistened in Cary-Blair medium)	Sterile forceps
	Cary-Blair transport medium	Sterile loops
	Plastic sleeve	Bunsen burner
	Ice pack (maintain temp. 2-4◦C)	35◦C ± 2◦C aerobic incubator
	Cooler box	Vortex Mixer
		VITEK®-MS utomated system
		VITEK® 2 automated system
Reagents and antimicrobials	Nil	Catalase reagent Rapid indole Oxidase
		Streptex kit
		5 ml Brain heart infusion broth(BHI)
		Vancomycin Etest Teicoplanin Etest Amphotericin B Etest
Agar plates	Nil	5% sheep blood agar (BAP)
		MacConkey agar plate (MAC)
		Sabouraud Dextrose Agar (SDA)
		Cetrimide Agar (C)

8. Environmental and safety controls

At specimen collection:

- Restrict sampling to individual patient's cot
- Attempt to avoid contamination of the swab while sampling by removing any obstructive items
- Don appropriate PPE, which includes but is not limited to:

- Appropriately sized gloves
- Plastic apron
  - Seal the swab immediately after sampling in correct transport medium to avoid contamination

At specimen processing:

- A biological safety cabinet is not required for processing of swabs; however, because *C. auris* may survive for weeks on plastic surfaces, with the potential to colonise healthy individuals, it is strongly advised that gloves, strong hand hygiene and decontamination with a disinfectant with sporociadal claim be used after work with *C. auris* cultures(44).
- Material used for collection and or processing of samples is discarded into biohazard boxes lined with red plastic bags.
- Needles and syringes should be disposed of into sharp's containers.

9. Quality Control

- All media and reagents in use should be examined for signs of contamination and expiration. Prior internal quality control testing will have to be conducted prior to use on clinical specimens to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction accordingly.
- All incubators in use need to have been recently serviced, and thermometers attached to observe the temperature.

Quality Control strains:	+	-
Catalase	*S. aureus* ATCC 25923	*Enterococcus faecalis ATCC 29212*
PYR	*Enterococcus faecalis ATCC 29212*	Streptococcus agalactiae ATCC10386
Latex agglutination test	*S. aureus* ATCC 25923	
Rapid indole	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Oxidase	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922

10. Procedural steps: For Specimen Collection (43)

(If possible, refrigerate Cary-Blair transport medium in advance, so that the swabs can be placed into a cool medium)

i. Wash hands and don appropriate PPE
ii. Expose the neonate
iii. Using a premoistened swab:
    a. Swab the umbilical stump in a linear direction, away from the stump opening
    b. Follow by swabbing with both the left axilla, and then right axilla concentrating on the creases, each for at least 3-5 times
    c. Using the same swab, rub across the left groin area, and then the right groin area, concentrating on the inguinal crease, swab in one direction at least 3-5 times
iv. Place the swab into Cary-blair transport media immediately
v. Transport swabs to the laboratory within the 24 hours after collection, Swab must be stored at 4 °C and below

Procedure steps: For Swab processing(38,43)

i. Remove swab from packaging, and ensure correct swab (skin) is selected
ii. With sterile forceps, Immediately place the skin swab into the BHI liquid medium and vortex for 15 seconds
iii. Incubate overnight at 35 ± 2^0C under aerobic conditions (may require prolonged incubation if not turbid after 24 hours)
iv. Once solution has become turbid, vortex for 15 seconds.
v. Subculture using a 10µl loop onto: BAP+C+MAC+SDA, and streak out for single colonies, incubate overnight at 35 ± 2^0C under aerobic conditions
vi. Perform presumptive identification on colony morphology and rapid reagent testing
vii. Further identification of suspicious colonies to take place on Vitek-MS(Biomerieux)
viii. If target organisms are identified antimicrobial susceptibility testing will take place on VITEK® 2 or appropriate manual methods as per CLSI standards.
ix. All processing is to be recorded in detail on the working cards provided and signed.
x. All working cards must be reviewed and signed by a CMID registrar.

Morphological features of target organisms:

- *Enterococcus faecium*: Small off-white to grey round colonies with α-haemolysis or ϒ-haemolysis on 5% sheep blood agar.
- *Staphylococcus aureus*: Small round black colonies on Baird parker with a surrounding zone of clearing.
- *Klebsiella pneumonia*: Lactose fermenting on MacConkey agar, raised circular 2-3mm colonies with entire margins, often mucoid.
- *Acinetobacter baumannii*: Small 1-2mm round convex colonies with an entire margin that are non-lactose fermenting but light pink in colour and opaque on MacConkey agar.

- Pseudomonas aeruginosa: Non-lactose fermenting on MacConkey agar, flat spreading 2-3mm colonies with a wrinkled moist surface and a metallic sheen, oxidase Positive.
- *Enterobacter* spp: Grey to off-white spreading round colonies with entire to undulate margins on 5% blood agar.
- *Candida auris*: Large creamy whitish colonies on Sabouraud dextrose agar

BAP	**Staphylococcal colony** morphology seen	▪ Perform catalase test on similar sized colonies on MAC ▪ Perform a latex agglutination test	If **positive,** continue with formal ID. Perform susceptibility only on S. aureus isolates
			If **negative,** perform PYR to exclude *Enterococcus* spp. If Negative, finalise as non-ESKAPE pathogen. If positive continue with ID and perform susceptibility only on *E. faecium* isolates
	Streptococcal colony morphology seen	Perform Streptex	If agglutination for **group D** is present, suggests *Enterococcus spp.* Continue with ID a susceptibility from BNC Confirm Vancomycin and Teicoplanin MIC by Etest if resistant on VITEK® 2
			Agglutination with groups (A,B,C,F,G) or no reaction. Finalise as non-ESKAPE pathogen
	Yeast-like colony Morphology seen	Perform a Gram to confirm	(See SDA)
		Perform rapid indole test	If **positiv**e= finalise as **non-ESKAPE** pathogen

MAC	Lactose-fermenting (pink red) colonies		If **negative**= continue to identify and perform susceptibility testing.
	Non-lactose fermenting colonies	Perform oxidase test	If **positive**, _ presumptive *Pseudomonas aeruginosa*, but must confirm ID.
			Continue to ID **all** NLF isolates to species level, and only perform susceptibility testing on ESKAPE+ C organisms identified on the VITEK® 2 platform.
SDA (correlate with BAP)	Assess for growth	Positive: White to cream coloured colonies	Continue with ID . Perform Amphoricin Etest on *C.auris* isolates only
C (Correlate with growth on MAC)	Positive	Presence of growth on agar, with associated yellow-green to blue colour	Continue with ID, susceptibility only on *P. aeruginosa*
	Negative	No bacterial growth on agar plate	Finalise as non-ESKAPE pathogen

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APPENDIX 4: LETTERS OF PERMISSION

Dr Fikile Mabena

Staff Number: A0025057
Phone: 082 500 2178

Human Research Ethics Committee

University of the Witwatersrand

2020/ 03 / 04

Dear Committee

Dr Nonkululeko Mntla will be performing her study:

Extended characterisation and association between maternal /primary care giver colonisation and neonatal colonisation by multi-drug resistant organisms at a tertiary hospital in Johannesburg, South Africa.
as a sub-study, using samples and data collected during the course of my study (primary study):

Microbiological surveillance of hospital environmental surfaces, medical equipment, healthcare workers, patients and patients' parents in a tertiary hospital in Soweto, South Africa.

Dr Mntla's study will incorporate findings from neonatal sampling and sampling from mothers/primary care givers. Funding for the sampling component is provided by a grant from the Bill and Melinda Gates foundation.

Kind Regards

Dr Fikile Mabena

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL MOTHER AND CHILD UNIT
DEPARTMENT OF PAEDIATRICS, DIVISION OF NEONATOLOGY

HREC (Wits)
Philip V Tobias Building
Faculty of Health Sciences
16 March 2020

Dear Sir/Madam

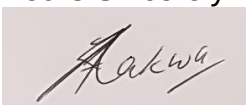
Re: PERMISSION TO CONDUCT RESEARCH

I hereby grant permission to **Dr Nonkululeko Mntla** student number **456149** to conduct research in the neonatal unit on:

EXTENDED CHARACTERIZATION AND ASSOCIATION BETWEEN MATERNAL /PRIMARY CARE GIVER COLONIZATION AND NEONATAL COLONIZATION BY MULTI-DRUG RESISTANT ORGANISMS AT A TERTIARY HOSPITAL IN JOHANNESBURG, SOUTH AFRICA

for the periods **March/April 2020 and at a second time point in 2020**. The investigator will collect and use the information from clinical swab samples from the neonates, primary caregivers, healthcare workers and the environment. The Wits Ethics approval number **M191129**

Yours Sincerely



Dr Firdose L. Nakwa
Head: Division of Neonatology
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Appendix 5: Plagiarism/turn-it in report

Turnitin Originality Report

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