MOLECULAR CHARACTERIZATION OF COAGULASE-NEGATIVE *STAPHYLOCOCCI* ISOLATED FROM NEONATES WITH SEPSIS AT MWANANYAMALA REGIONAL REFERRAL HOSPITAL

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By

Anjela J. Charles

A Dissertation Submitted in (Partial) Fulfilment of the Requirements for the Degree of Master of Science in (Microbiology and Immunology)

of the Muhimbili University of Health and Allied Sciences
October, 2024

CERTIFICATION

The undersigned certify that I have read and now recommend for acceptance by the Muhimbili University of Health and Allied Sciences a dissertation entitled "Molecular characterization of Coagulase-negative staphylococci isolated from neonates with sepsis at Mwananyamala Regional Referral Hospital" in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Immunology of the Muhimbili University of Health and Allied Sciences.

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DECLARATION AND COPYRIGHT

I, Anjela J Charles, hereby declare that this dissertation titled: Molecular Characterization of Coagulase-negative staphylococci isolated from neonates with sepsis at Mwananyamala Regional Referral Hospital, is my original work and that it has not been presented to any other university for similar or any other degree award.

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DEDICATION

I dedicate this dissertation to my lovely mother Modester Lukunja.

ABSTRACT

Background: Coagulase-negative staphylococci (CoNS) have emerged as pathogenic bacteria causing bloodstream infection (BSI), particularly in people with immature immune status such as neonates. **Single set of blood culture is collected in neonates hence becomes difficult to distinguish non-pathogenic and pathogenic CoNS**. Their capacity to form biofilm is regarded as the major virulence mechanism that facilitates pathogenicity, horizontal gene transfer and impairs penetration of antimicrobial agents and immunologic response.

Broad objective: To determine virulence genes (VGs) (*icaA*, *icaB*, *icaC*, and *icaD*) and antibiotic resistance genes (ARGs) (*blaZ*, *mecA*, and *aac(6')-Ie-aph(2'')-Ia*) expressed by CoNS isolated from neonates with sepsis at Mwananyamala Regional Referral Hospital (MRRH).

Methodology: A cross-sectional laboratory-based study was carried out from January to June 2024, using archived CoNS isolated from neonates with sepsis previously admitted at MRRH. VITEK® MS was used for speciation, and Polymerase Chain Reaction was performed for detection of VGs and ARGs. Amplicons were separated by Agarose gel electrophoresis and bands were visualized by Molecular Imager Gel Doc XR**.** Statistical analysis was performed using Statistical Package for Social Science software version 23.0. Descriptive statistics such as frequency tables were computed to describe categorical variables which were species, virulence genes, and ARGs distribution among the isolates.

Results: A total of 70 archived CoNS isolates were included in the study. Out of 70 CoNS six different CoNS species were identified with *S. epidermidis* 27 (36.8%) being the predominant isolate followed by *S. haemolyticus* 24 (34.3%). Virulence genes responsible for biofilm formation (*ica* genes) were detected, of which 90% of CoNS species harbored all *ica* genes (*icaADBC*) in the *ica* locus and were considered as pathogenic species. A high proportion 61 (87.1%) of CoNS isolates harbored all three ARGs against the commonly prescribed antibiotics, where *mecA* 69 (98.6%) was the most prevalent gene detected followed by *blaZ* 67 (95.7%).

Conclusion and recommendation: This study reveals CoNS species causing neonatal sepsis are predominantly *S. epidermidis* followed by *S. haemolyticus* and that most of them (90%) are pathogenic with a high carriage of ARGs *blaZ*, *mecA*, and *aac (6')-Ie-aph(2'')-*

Ia which confer resistance to the most commonly prescribed antibiotics including penicillin, methicillin, and gentamycin respectively.

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ABBREVIATION

AIDS	Acquired Immunodeficiency Syndrome
AMEs	Aminoglycoside modifying enzymes
ARGs	Antibiotic Resistance Genes
API	Analytical Profile Index
ATCC	American Type Culture Collection
BA	Blood Agar
BSIs	Blood stream infections
CAPD	Continuous Ambulatory Peritoneal Dialysis
CoNS	Coagulase-negative staphylococci
CRP	C - reactive protein
DNA	Deoxyribonucleic acid
EONS	Early onset neonatal sepsis
GPC	Gram positive cocci
HIV	Human Immunodeficiency Virus
LONS	Late onset neonatal sepsis
MALDI-TOF MS	Matrix associated laser desorption ionization time of flight mass spectrometer
MSCRAMMs	Microbial Surface Component Recognizing Adhesive Matrix Molecules
MDR	Multi-Drug Resistance
MRRH	Mwananyamala Regional Referral Hospital
MUHAS	The Muhimbili University of Health and Allied Science
NA	Nutrient agar
NICU	Neonatal intensive care unit
NPHL	National Public Health Laboratory
UTI	Urinary tract infections
PCR	Polymerase Chain Reaction
PDAIs	Prosthetic-device-associated infections
PIA	Polysaccharide intracellular adhesion
TSST-1	Toxic shock syndrome toxin-1

DEFINITION OF TERMS

Early-onset neonatal sepsis: refers to a type of bloodstream infection in newborn babies that occurs within or at 72 hours after birth.

Late-onset neonatal Sepsis: refers to a type of bloodstream infection in newborn babies that occurs 72 hours after birth.

Neonatal sepsis: refers to a clinical syndrome of bacteremia with systemic signs and symptoms of infection in newborn babies in their first 28 days of life after birth.

Neonates: a newborn baby particularly in the first 28 days of life after birth.

Pathogenic Coagulase-negative staphylococci (CoNS): is defined as CoNS isolated from neonates with clinical and laboratory indicators of sepsis with the presence of virulence genes *icaADB*C genes in *ica* operon responsible for biofilm formation.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Coagulase-negative staphylococci (CoNS) are normal commensal bacteria of the skin and mucous membranes and have emerged in recent years as causative agents of nosocomial infection (1). They are nowadays considered a common cause of healthcare-associated bloodstream infection (BSI) among immunocompromised patients including cancer, diabetes, hemodialysis, Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) patients, and individuals with immature immune status such as neonates (2,3). Implantation or indwelling of foreign polymer bodies like orthopedic devices, prosthetic heart valves, and cardiac pacemakers, along with intravascular catheters, critically ill and long-term hospitalized are risk factors for infections caused by CoNS (1,4).

There are about 41 species of CoNS that have been delineated to be pathogenic. Of this 18 species of pathogenic CoNS have been associated with human infections, where *Staphylococcus simulans*, *Staphylococcus epidermidis*, *Staphylococcus warneri*, *Staphylococcus lugdunensis*, *Staphylococcus capitis*, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus* are the most clinically relevant species (5,6). Apart from BSI, CoNS have been implicated in causing other infections like skin and soft-tissue infections, bacterial eye infections, central nervous system shunt infections, urinary tract infections (UTI), and biomaterial-based and prosthetic device infections(4). Globally, about 40% of all neonatal deaths which is approximately to 1.6 million neonatal deaths annually, are due to BSI (7). One-third of the neonatal mortality In sub-Saharan Africa results from septicemia, where CoNS accounts for 6%-32% of all positive blood cultures in sub-Saharan Africa (8). A study done in Mwananyamala Regional Referral Hospital (MRRH) showed about 81% of BSI in neonates were caused by gram-positive bacteria whereas CoNS were the most predominant bacterial (35%) (9). CoNS are more associated with late neonate's sepsis onset compared to early neonates' sepsis onset (9,10).

Challenges have been reported on the identification of CoNS using conventional methods on routine test for catalase test, misidentification depends on the storage and potency of hydrogen peroxide, old culture of more than 24 hours, the ability of some organisms to

possess peroxidase enzyme, picking components of blood agar media and uses of nichrome wire loop (11). Slide coagulase test can lead to false positive results in some species such as *S. ludgunensis* due to the presence of cell surface capsular polysaccharide hence needs a tube test as a confirmatory test. Results from several studies concluded that genotypic methods and proteomics, Matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) are superior to phenotypic identifications(12–15). The capacity of CoNS to form biofilm can be assessed by phenotypic methods: Congo red agar, tissue culture, and tube methods, genotypic methods such as polymerase chain reaction (PCR) are used to detect genes for biofilm formation (16,17).

Different virulence factors have been reported on CoNS and identified to be the major contribution to disease causation in humans. Biofilm formation is the prime virulence mechanism among CoNS and involves three stages which are adhesion, maturation, and dispersion(6). Biofilm formation contributes to the mechanism of bacteria to evade the body's immune response and resistance to antibiotics (9). The important element involved in biofilm formation is the *ica* operon found in bacterial chromosomes, which includes four genes *icaADBC* (18). Four genes (*icaADBC*) encode the polysaccharide intercellular adhesin (PIA) protein, which is attributed to bacteria intercellular adherence and the accumulation of multilayer biofilm (6). Harboring of *ica* genes is more detected among pathogenic CoNS contrary to skin and mucosal flora (19,20). Other virulence factors include the production of toxins, such as hemolysins, Toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins, and enterotoxins (21).

Mutation and biofilm formation which facilitates horizontal gene transfer among bacteria result in antimicrobial resistance genes. Westberg et al.(22) reported antimicrobial resistance genes which were methicillin (*mecA),* macrolides (*mphC*, *ermA*, *msrA*, *vga(A)LC*, trimethoprim-sulfamethoxazole (*dfrG*), β-lactams (*blaZ),* aminoglycosides (*aac(6')-aph(2'')*, and clindamycin (*lnuA*) from neonates with sepsis (22). In this study, MALDI-TOF MS (VITEK MS) was used to identify CoNS to species level, congo red agar, and tube methods were used for screening biofilm former isolates. PCR was performed to detect virulence genes which were *icaA, icaD, icaB,* and *icaD,* and ARG including *blaZ, mecA,* and *aac (6')-Ie-aph(2'')-Ia*.

1.2 Problem statement

CoNS are the most common bacteria isolated from neonate blood samples suffering from sepsis(9,23). In most of our settings, CoNS are considered contaminants although in immunocompromised and individuals with immature immunity like neonates, antibiotics are being prescribed without ruling out if CoNS isolated are a cause of the infection or contaminants. This contributes to the occurrence of antibiotic resistance and increases susceptibility to colonization due to the unnecessary use of antibiotics which cause imbalance of microbiota. Lack of further identification at the species level results in limited information about the commonest species of CoNS associated with neonatal sepsis and their distribution pattern. However, CoNS are considered to be associated with infection due to the existence of several virulence factors. Studies done to assess the role of biofilm formation in the disease process especially BSI have shown that biofilm production was substantially greater among species isolated from blood and the skin of neonates with sepsis (24,25). Most healthcare facilities particularly in developing countries tend to collect a single set of blood cultures contrary to the suggested two to three sets due to expensiveness and hence not affordable to most patients (7). Furthermore, it has been a challenge to collect two sets in neonates due to small circulating blood volumes (4), which leads to difficulty in distinguishing contaminants from true pathogen-causing BSIs. A study done at MRRH where only one set of blood cultures was collected from neonates with sepsis, revealed that the majority of bacteria isolated were CoNS which accounted for 35% (9). These neonates were all treated with antibiotics and this has been a trend in many hospitals in Tanzania. **Furthermore, the Tanzania national guidelines for the treatment of neonatal sepsis stipulate initiating ampicillin and cloxacillin fixed-dose combination plus gentamycin as first-line regimen empirical treatments, with ceftriaxone used as the second-line regimen** (26). **However, with the increased level of resistance to this antimicrobial agents, such a strategy faces stern challenges**(9).

This research provides information on species distribution, **VGs (*icaADBC*) that enhance pathogenicity through biofilm formation which can be used to differentiate pathogenic CoNS and contaminants from neonates with clinical and laboratory indicators of neonatal sepsis. Furthermore, ARGs that confer resistance against β-lactams (*blaZ*), aminoglycosides (*aac(6')-Ie-aph(2'')-Ia*), and methicillin (*mecA*), antibiotics that are empirically prescribed in neonates with sepsis**.

1.3 Rationale of the Study

The study was conducted to provide useful information on species diversity which will help to understand the most common species of CoNS causing neonatal sepsis. The detection of virulence genes (*icaADBC*) can be used as a marker in determining pathogenic CoNS with the aid of clinical and laboratory indicators. Furthermore, findings on antibiotic resistance genes will help to understand different antibiotic resistance mechanisms employed by CoNS.

1.4 Conceptual Framework

For CoNS to be regarded as pathogenic relies on several factors such as clinical indicators, laboratory indicators, species diversities and molecular characteristics such as antimicrobial resistance and virulence genes (*icaADBC*) as indicated in **Figure 1**.

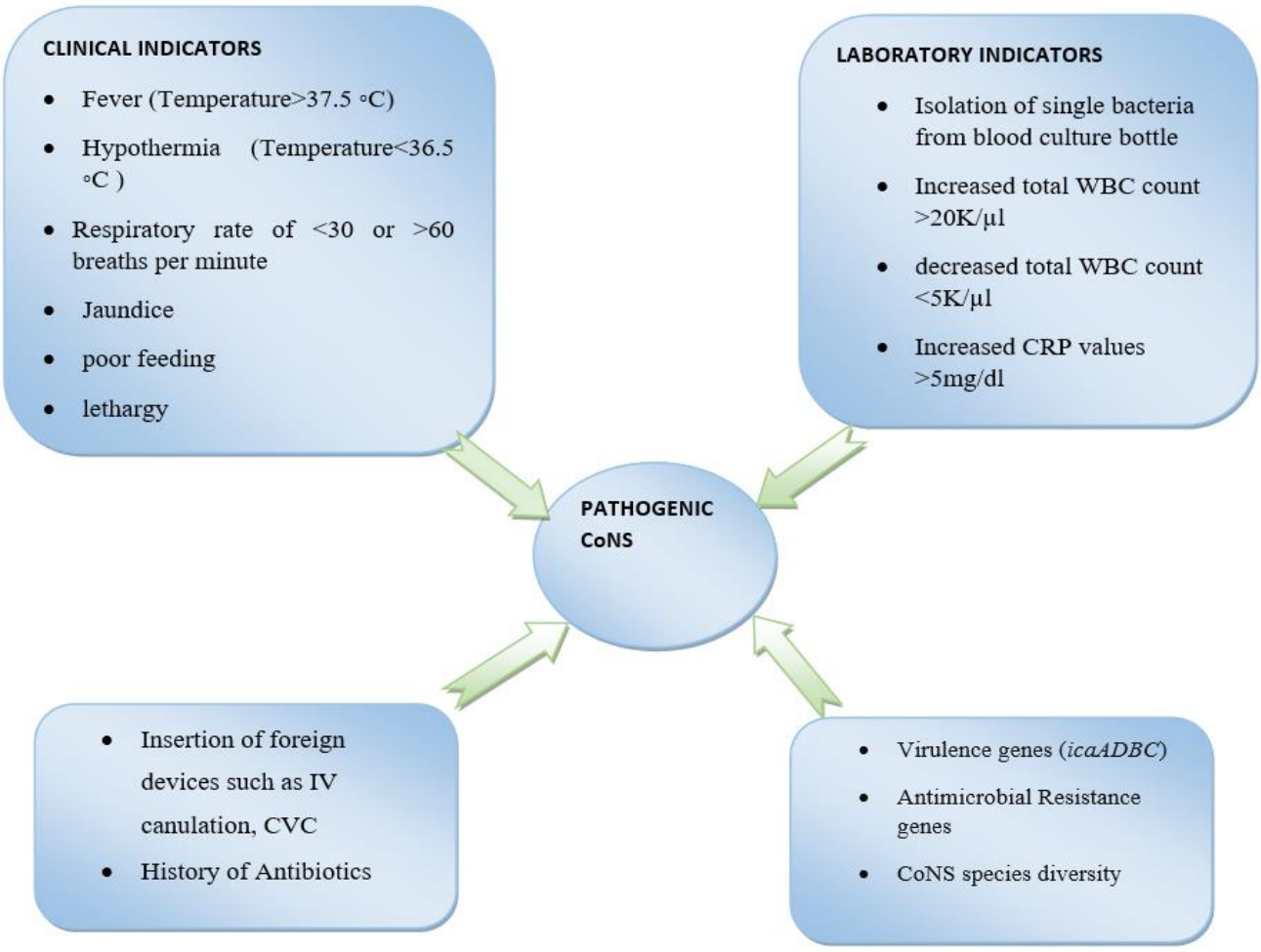


Figure 1: Conceptual Framework showing factors which influence CoNS pathogenicity

1. 5 Research question

Does CoNS isolates from neonates with sepsis express VGs (*icaA, icaB, icaC,* and *icaD*) and ARGs (*blaZ, mecA,* and *aac(6')-Ie-aph(2'')-Ia*) to common prescribed antibiotics in our settings?

1.6 Objective

1.6.1 Broad objective

To determine VGs (*icaA, icaB, icaC,* and *icaD*) and ARGs (*blaZ, mecA,* and *aac(6')-Ie-aph(2'')-Ia*) expressed by CoNS solated from neonates with sepsis at MRRH from January 2021 to July 2022.

1.6.2 Specific objective

1. To determine the diversity of CoNS species isolated from neonates with sepsis at MRRH.
2. To determine the **VGs (*icaA, icaB, icaC,* and *icaD*)** expressed with CoNS isolated from blood culture at MRRH.
3. To determine the **ARGs (*blaZ, mecA,* and *aac(6')-Ie-aph(2'')-Ia*)** expressed by CoNS species isolated from neonates with sepsis at MRRH.
4. To determine the proportion of pathogenic CoNS species isolated from neonates with sepsis at MRRH.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Burden of Coagulase-negative staphylococci in Blood stream infection in Neonates

2.1.1 Burden of CoNS in BSI in developed countries among Neonates

The incidence of neonatal sepsis globally is estimated to be three million cases each year (27), infections is estimated to cause 1.6 million neonatal deaths annually, approximately 40% of all neonatal deaths (28). In study done in Netherlands, CoNS accounted for 66% of nosocomial blood stream infection among preterm neonates admitted between January 2021 and December 2020 (29). Moreover, in a study done in Italy CoNS represented 52% of neonates with sepsis admitted from January 2005 to October 2018 (30). Herbozo et al. (31) reported 49% of neonatal sepsis were due to CoNS among neonates admitted at Peru , neonatal intensive care unit.

2.1.2 Burden of CoNS in BSI among Neonates in Sub-Saharan Africa

In Sub-Saharan Africa neonatal sepsis is estimated to be 57% (32), prevalence of neonatal sepsis due to CoNS in developing countries is 23% (33). In studies done in South Africa reported the prevalence of neonatal sepsis due to CoNS are 19% and 53% respectively (34,35). Also a studies done in Niger reported 19% and 15.8% of neonatal sepsis are due to CoNS respectively (36,37). Furthermore, studies done in Ghana reported 55% and 56% of neonatal sepsis are due to CoNS respectively (38,39).

2.1.3 Burden of CoNS in BSI in East African countries among Neonates

In East African countries estimated prevalence of neonatal sepsis is 30% (40), studies done in Kenya reported 29% and 31% of neonatal sepsis are due to CoNS respectively (41,42). Also, studies done in Ethiopia, Rwanda, and Uganda reported a prevalence of neonatal sepsis due to CoNS of 21%,29.7%, and 21.8%, respectively (43–45).

2.1.4 Burden of CoNS in BSI in Tanzania among Neonates

In studies done in eastern Tanzania CoNS have been reported as the predominant cause of neonatal sepsis, the prevalence of neonatal sepsis due to CoNS is 35%,30%, and 35.6%, respectively (9,23,46). Also, a study done in Northern Tanzania reported CoNS to be accountable for 20% of neonatal sepsis (47). Furthermore, a study done in Lake Zone of Tanzania reported that 23% of neonatal sepsis is due to *Staphylococcus* species (48). **Research conducted in Tanzania showed CoNS are predominant isolates from neonates with sepsis.**

2.2 Distribution of CoNS species from Neonates with sepsis

About 18 species of CoNS have been delineated to be associated with human infections (6), and several studies have identified the distribution of CoNS from neonates with sepsis for example in a study done in Norway distribution of CoNS species from neonates with clinical indication of sepsis were *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *Staphylococcus hominis* (49). Also, Sands et al. (10) reported the mostly commonest CoNS species in developing countries between 2015–2017 associated with neonate sepsis were *S. epidermidis*, *S. capitis*, *Staphylococcus sciuri and S. haemolyticus*. Moreover, in a study done in India by Farheen et al. (50) CoNS accounted for about 13% species distribution were *S. epidermidis*, *Staphylococcus xylosus*, *S. simulans*, *S. capitis*, *S. hominis*, *Staphylococcus cohnii*, *S. warneri*, and *S. haemolyticus*. Furthermore, a systematic review of published articles from 2016-2017 on CoNS in neonate sepsis done in Nigeria reported the predominant species were *S. capitis*, *S. epidermidis*, , *S. hominis* and *S. haemolyticus* (51). **In our settings, there is no published data on CoNS species distribution isolated from neonates with sepsis**.

2.3 Virulence factors associated with Coagulase-negative staphylococci

Biofilm formation is a prime virulence mechanism that facilitates the pathogenicity of CoNS (52–54). Biofilm enhances their capacity to adhere to abiotic surfaces such as invasive devices including pacemakers, catheters, and prosthetic heart valves as well as smooth plastic and tissue surfaces and biotic surfaces such as host tissue and protein (6). Stages involved in the process of biofilm formation are adhesion, accumulation, and

maturation results to complex multicellular structures and finally, biofilm dispersion (4). Polysaccharide intracellular adhesion (PIA) is a main mechanism of biofilm formation in glycocalyx and consists of β-1,6–linked N-acetylglucosamine residues and non-N-acetylated D-glucosaminyl residues of 80–85% and 15–20% respectively (55). Positively charged PIA encoded by *icaB* genes located in the *ica* operon provides intercellular attachment through binding to negatively charged bacteria surfaces (52). The *icaA* gene encodes for the N-acetylglucosamyltransferase enzyme responsible for synthesizing PIA, *icaB* encodes deacetylase accountable for the deacetylation of mature PIA, and the transmembrane protein, *icaC* is involved in externalization and expansion of the growing polysaccharide (56). Ashwalk et al. (52) evaluated CoNS isolated from neonates with sepsis were positive for *ica* operon and biofilm formation than isolates isolated from healthy neonates. CoNS isolated in blood samples, skin swabs from neonates with sepsis, healthy neonates skin swabs (control) and healthcare worker's skin swabs were 13, 40, 8, and 6 CoNS isolates, respectively. The *ica* genes were detected from a blood sample, skin swab from neonates with sepsis and health care worker's skin swabs were 76%, 40% and 33% respectively, *ica* genes were absent among control isolates. Biofilm formation not only relies on *ica* genes which encode PIA once *icaADBC* is deleted, biofilm is formed by PIA-independent which is constructed by the accumulation of Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMMs) such as *SasG*, *SasC*, protein A, fibronectin-binding proteins *FnBPA* and *FnBPB*, cell wall-anchored (*CWA*) proteins including clumping factors A and B, autolysins *AtlA* and AtlE (57). Saghir et al. (55) aimed to assess the role of polysaccharide-dependent and polysaccharide-independent biofilm formation in *S. epidermidis.* Out of 75 isolates, genes encoding fibrinogen binding protein (*be*), extracellular matrix binding protein (*embp*), laminin-binding protein (*eno*), accumulation-associated protein (*aap*), biofilm homologous protein (*bhp*), and biofilm-associated protein (*bap*) 86.6%, 84%, 92%, 46.6%,24%, and 1.3% respectively. This provides evidence that pathogenic CoNS have virulence genes compared to non-pathogenic CoNS. **In our settings a single set of blood culture is collected bringing challenges to discriminate between non-pathogenic and pathogenic CoNS, virulence genes can be used to discriminate between pathogenic and non-pathogenic CoNS with the aid of clinical and laboratory indicators**.

2.4 Antibiotic resistance genes among Coagulase-negative staphylococci

CoNS have several ARGs which are acquired through horizontal gene transfer and mutation (6). In our setting primary antibiotics for the management of neonatal sepsis are ampicillin, cloxacillin and gentamycin, while second-line antibiotics are ceftriaxone and cefotaxime. Studies conducted in Tanzania reported a high resistance rate to the most prescribed antibiotics including; penicillin, ceftriaxone, and gentamycin in most cases, these antibiotics are used empirically in the treatment of neonatal sepsis (9,23,58,59). Sabzehali et al. (60) investigated the distribution of genes encoding aminoglycoside modifying enzymes (AME) which were *aac (6'')-Ie-aph (2'')-Ia*, *ant (4')-Ia* and *aph(3'')-IIIa* among CoNS isolates. About 87% harbor *aac (6')-Ie-aph (2'')-Ia,* 89% of isolates had both *ant(4')-Ia* and 68% had *aph (3')-IIIa* gene. In a study done by Westberg et al. (22) 34 isolates of *S. haemolyticus* from neonates with sepsis were involved in the study, of these 97% harbored multiple ARGs. The most prevalent combination was resistance to aminoglycoside *aac(6')-Ie-aph (2'')-Ia*, β-lactam (*blaZ*), a macrolide (*mphC*), a macrolide (*erm*), and methicillin (*mecA*). Giormezis et al. (61) investigated the presence of ARGs from 226 CoNS isolated from inpatients and prosthetic device-associated infection with BSIs, of which 89.8% of isolates were methicillin and cefoxitin resistant and carried the *mecA* gene. Also, a study done in Iraq discussed the concept of silence genes, where some ARGs can be silenced but can be expressed in some conditions, in their study 15% CoNS phenotypically sensitive to Oxacillin (5 μg) harbored *mecA* gene (62). **In our setting despite high proportion of phenotypic antibiotic resistance among CoNS no any published data on ARGs to common prescribed antibiotics among CoNS isolated from neonates with sepsis.**

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

The study was cross-sectional, laboratory-based study design

3.2 Study area

This study was conducted at MRRH, a government-owned facility under the Ministry of Health, located in Kinondoni Municipal Council. The hospital provides reproductive services for children (antenatal, postnatal and newborn care) and mother health care, with an average of 200 neonate admissions in a month. Currently, the hospital has a bed capacity of 254 with a bed occupancy of 317. It is a referral hospital for more than 200 public and private health facilities such as hospitals, health centers, dispensaries, and clinics located in Kinondoni and Ubungo districts.

3.3 Study population

This study used archived CoNS isolates stored at -80ºC in Tryptic soya broth with 15% glycerol, isolated from neonates aged 0–28 days who were admitted to the neonatal ward with BSIs from a previous study done at MRRH from January 2021 to July 2022 (9). These neonates presented with at least two suggestive clinical hallmarks of sepsis which included an axillary temperature of <36.5 ◦C or >37.5 ◦C, severe respiratory distress (grunting, central cyanosis, hypoxia (SpO2 < 90%), a respiratory rate of <30 or >60 breaths per minute, umbilical cord or skin infection, jaundice, a random blood sugar of <2.5 or >11.0 mmol/L, umbilical cord or skin infection, a pulse rate of <100 or >160 beats per minute, irritability, altered consciousness, lethargy, and altered consciousness.

3.4 Sample size

A total of 160 archived CoNS isolates from neonates with sepsis were stored at -80ºC, of these, 100 CoNS isolates were the targeted sample size. In a descriptive study that describes the characteristics of a certain population Steinberg (63) and Althubaiti (64) recommended a minimum sample size is 30 cases. A convenience sampling technique was used to attain the targeted sample size.

3.4.1 Inclusion criteria

I. Archived CoNS isolates from neonates with laboratory indicators for sepsis (increase or decrease total WBC count and increase in CRP level).

3.4.2 Exclusion criteria

I. Archived CoNS isolated from neonates with missing clinical information such as age, type of neonatal sepsis, and antibiotic susceptibility patterns.

II. Archived CoNS isolates revealed mixed growth after sub-culturing.

3.5 Data collection

Clinical information of neonates from whom CoNS isolates was obtained from the neonatal tool database at MRRH- Neonatal Unit such as the onset of sepsis features, prior antibiotic usage, clinical indicators, laboratory indicators along with patient outcomes.

3.6 Laboratory procedures

Laboratory work including identification to species level and molecular characterization of CoNS isolates was conducted at the National Public Health Laboratory (NPHL) and Microbiology and Molecular Laboratories at the Muhimbili University of Health and Allied Sciences (MUHAS) respectively.

3.6.1 Sub-culture and Re-identification of CoNS isolates

Archived CoNS isolates were **subcultured** on 5% sheep blood supplemented Columbia Blood agar (BA) (Oxoid Ltd., Chester, UK) and incubated aerobically, at 35°C ± 2°C for 18 -24 hours to assess isolates' viability. Viable CoNS isolates were sub-cultured on Nutrient agar (NA) media (Oxoid Ltd., Chester, UK) and incubated aerobically, at 35°C ± 2°C for 18 -24 hours for re-identification.

CoNS were re-identified by conventional methods based on colonial morphology, gram stain, and biochemical tests. Furthermore, biochemical tests such as the catalase test were performed by using 3% of hydrogen peroxide and slide coagulase tests, in both tests pure cultures of 18 hours to 24 hours old were used (11,65). *Staphylococcus aureus* ATCC 25923 was used as a control strain.

3.6.2 Identification of CoNS into Species Level

Speciation of CoNS isolates was performed using VITEK® MS (BioMérieux, France) which works under the principle of mass spectrometry technology – Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF). Briefly, pure culture of 18 hours to 24 hours old colonies were spotted onto the VITEK MS-DS target slide using VITEK PICKME, followed by the addition of 1 µL of VITEK MS- CHCA matrix solution (BioMérieux, Inc.) which extracts proteins from the microorganisms. This was followed by the loading of the metal target into the ionization chamber, crystallized organism proteins were pulsed with a UV laser. An ionized protein entered a flight tube hence, acceleration of cations into a mass analyzer. The mass analyzer separated the ions by their mass-to-charge ratio. Spectra generated were analyzed using VITEK® MS Software Version: 3.2. *S. haemolyticus* ATCC 29970 was used as a positive control strain for quality control. The isolated CoNS species were inoculated into Tryptic soya broth with 15% glycerol for short term stored at -30°C until further analysis (66).

3.6.3 Phenotypic detection of biofilm production

3.6.3.1Congo Red Agar Method.

Congo red agar (CRA) method for qualitative detection of biofilm was performed as described by Freeman et al. (16). Briefly, CRA was prepared by 37g brain heart infusion broth (Oxoid Ltd., Chester, UK), 10g Agar (Oxoid Ltd., Chester, UK), 36 g/mL sucrose and 0.8 g/L congo red stain (VWR International Europe BVBA). Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium components. Once the temperature reached 55°C, congo red stain was added to the mixture of autoclaved brain-heart infusion broth, agar, and sucrose. Test isolates were inoculated on CRA and incubated aerobically, at 35°C ± 2°C for 18-24 hours. Colonial morphology was used to determine biofilm former, black colonies with a dry crystalline consistency indicated a positive result, while a negative result was indicated by pink-red colonies the assays were performed in triplicate.

S. aureus ATCC 25923 was used as the positive control strain and *S. haemolyticus* ATCC 29970 as the negative control for quality control.

3.6.3.2 Tube Method

Tube method (TM) was performed for qualitative biofilm detection as described by Christensen et al. (67). Briefly, test isolates were inoculated on 5% sheep blood supplemented Columbia Blood agar (BA) (Oxoid Ltd., Chester, UK) incubated aerobically at 35°C ± 2°C for 18-24 hours. Then, 18- 24 hours old colonies were inoculated into polystyrene tubes containing 3ml of Tryptic soy broth (Oxoid Ltd., Chester, UK), inoculated tubes were incubated aerobically at 35°C ± 2°C for 18-24 hours. Thereafter, planktonic bacterial cells were discarded, tubes were washed three times with phosphate buffer saline (pH 7.2) and stained with 0.1% Crystal Violet solution for 20 minutes then tubes were washed. Tubes were dried at room temperature in an inverted position. Isolate was considered a biofilm producer if stained bacteria adhered to the tube wall. All assays were performed in triplicate. *S. aureus* ATCC 25923 was used as a positive control strain and *S. haemolyticus* ATCC 29970 as a negative control for quality control.

3.6.4 DNA Extraction and Quantification

Genomic DNA was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit, as per manufacturer instructions (Zymo Research Biotechnology Company, California United States). About 100mg colonies of CoNS isolate were diluted in Phosphate buffered saline (PBS), preceded by the addition of 750 µl Zymo-BashingBead™ buffer Solution to the Zymo-BashingBead™ lysis tube. Rapid and efficiently lysed of bacteria cells by bead beating using vortex Genie (Scientific Industries, USA) without the use of organic denaturants or protease was performed. DNA extracted was then purified using Zymo-Spin™ Technology which contains a unique silica-based matrix that allows purification of up to 25µg of DNA in 100µl of DNA elution buffer. Quantification of DNA was done to determine DNA concentration and purity by measuring absorbance at wavelengths of 260nm and 280nm in a spectrometer (Nanodrop 2000C, ThermoFisher Scientific). Pure DNA had an absorbance ratio (A260/A280) value of 1.8-2.0 (68). Extracted pure DNA was stored in TE buffer containing 10mM of Tris, 1mM of EDTA, and pH 8 at -80^{o}C.

3.6.5 Polymerase Chain Reaction (PCR)

3.6.5.1 Multiplex Polymerase Chain Reaction for virulence genes detection

Multiplex PCR was performed using a C1000 Thermal cycler (Bio Rad Laboratories, USA) for the detection of virulence genes, using multiple sets of primers (forward and reverse primers) for different targeted nucleotide sequences based on targeted genes. Biofilm formation genes that were involved are *icaA, icaD, icaB, icaC,* and *16SrRNA* specific for *staphylococcus* species (17,69).

The volume of the PCR reactions was 25µL, containing 3.75µl of 1µM primers stock for *icaA, icaB, icaC, icaD,* and *16SrRNA* (Inqaba Biotech, South Africa), 2µl of template DNA,5µl of Multiplex PCR 5X Master mix (New England, BioLabs) and 14.25µl of nuclease-free water (Inqaba Biotech, South Africa). PCR conditions involved were initial denaturation at 95°C for 60 seconds, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 53.5°C for 60 seconds, elongation at 62°C for 120 seconds, and final elongation at 68°C for 300 seconds.

Table 1: Multiplex PCR mixture for virulence genes detection

COMPONENTS	REACTION VOLUME	FINAL CONCENTRATION
Multiplex PCR 5X Master mix	5.0µl	1X
1µM Primer Stock	3.75µl	0.15µM
Template DNA	2.0µl	<1000ng
Nuclease free Water	14.25µl	-
Total PCR reaction Volume	25µl	

Table 2: List of primers for detection of Virulence genes and genus *Staphylococcus*

Targeted gene	Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
Intracellular adhesion (icaA)	*icaA*	ACA GTC GCT ACG AAA AGA AA	GGA AAT GCC ATA ATG ACA AC	103
Intracellular adhesion (icaB)	*icaB*	CTG ATC AAG AAT TTA AAT CAC AAA	AAA GTC CCA TAA GCC TGT TT	302
Intracellular adhesion (icaC)	*icaC*	TAA CTT TAG GCG CAT ATG TTT T	TTC CAG TTA GGC TGG TAT TG	400
Intracellular adhesion (icaD)	*icaD*	ATG GTC AAG CCC AGA CAG AG	CGT GTT TTC AAC ATT TAA TGC AA	198
	16S rRNA	CCT ATA AGA CTG GGA TAA CTT CGG G	CTT TGA GTT TCA ACC TTG CGG TCG	791

3.6.5.2 Monoplex Polymerase Chain Reaction for ARGs detection

Monoplex PCR was performed using C1000 Thermal cycler (Bio-Rad Laboratories, USA) for detection of ARGs (*mecA*, *blaZ*, and *aac(6')-Ie-aph(2'')-Ia*) using specific oligonucleotide *mecA* (54,70,71), blaZ (72,73) and *aac(6')-Ie-aph(2'')-Ia*) (74) (Inqaba biotec, South Africa). A total volume of 25µl PCR reactions mixture was used, contained 12.5µl OneTaq Quick Load 2x Master mix with Standard Buffer (New England, Biolabs), 0.5µl of 10 µM of forward primers and reverse primers (Inqaba biotec, South Africa), 2µl of template DNA and 9.5µl of nuclease-free water (Inqaba biotec, South Africa).

PCR conditions were initial denaturation at 94^0C for 30 seconds followed by 40 cycles of denaturation at 94^0C for 30 seconds, annealing at 60.4^0C (*blaZ),* 57.9^0C (*mecA,),* 52.5^0C*(aac(6')-Ie-aph(2'')-Ia)* for 60 seconds, elongation at 68^0C for 60 seconds, and final elongation at 68^0C for 300 seconds.

Table 3: Monoplex PCR mixture for ARGs detection

COMPONENTS	REACTION VOLUME	FINAL CONCENTRATION
One Taq Quick Load 2X Master mix with Standard Buffer	12.5µl	1X
10µM Forward primer	0.5µl	0.2µM
10µM Reverse primer	0.5µl	0.2µM
Template DNA	2.0µl	<1000ng
Nuclease free Water	9.5µl	-
Total PCR reaction Volume	25µl	

Table 4: LIST OF PRIMER FOR DETECTION OF ARGs

Targeted gene	Primer	Forward primer (5'-3')	Reversed primer (5'-3')	Amplicon size (bp)
Penicillin resistance	*BlaZ*	ACTTCAACACCTGCTGCTTTC	TGACCACTTTTATCAGCAACC	173
Methicillin resistance	*MecA*	GTAGAAATGACTGAACGTCCGATAA	CCAATTCCACATTGTTTCGGTCTAA	310
Aminoglycoside Resistance	*aac(6')-Ie-aph(2'')-Ia*	CAGGAATTTATCGAAAATGGTAGAAAAG	CACAATCGACTAAAGAGTACCAATC	369

3.6.6 Agarose gel electrophoresis

PCR amplicons were separated by Agarose gel electrophoresis using 1.3% Tris-acetate-EDTA (TAE) agarose gel. 5µl of PCR amplicons were mixed with 3µl of 6X sample loading buffer bromophenol blue (New England, Biolabs). Agarose gel electrophoresis was run at 100V for 45 minutes. Bands visualization was performed using Molecular Imager Gel Doc XR (Bio-Rad Laboratories, USA) and a DNA ladder (100bp) (New England, Biolabs) was used to determine the size of amplified DNA fragments.

3.7 Result Interpretation

CoNS was considered pathogenic when isolated from neonates with clinical and laboratory indicators of sepsis with the presence of virulence genes (*icaADBC)* in the *ica* operon which is responsible for biofilm formation(19,20).

3.8 Statistical Data Analysis

Analysis of the collected data was performed by importing data into Statistical Package for Social Science (SPSS) software version 23.0, (IBM). Descriptive statistics such as frequency tables were computed to describe categorical variables which were species, virulence genes, and antimicrobial resistance genes distribution among the isolates.

3.9 Ethical consideration

Research ethics approval with reference number DA.282/298/01.C/2010 was sought from the MUHAS Senate Research and Publications Committee.

The authorization to access the gathered patient's clinical information and archived isolates was sought from MRRH management.

3.10 Quality control

Required culture media was prepared following the manufacturer's instructions. For performance check reference strain of *S. aureus* ATCC 25923 was inoculated on prepared media. For sterility check about 5% of the prepared batch of the culture media was incubated aerobically, at 35°C ± 2°C for 18-24 hours to observe for the presence of bacterial growth. Negative control was included during PCR reaction (PCR master mix, primers, nuclease-free water).

3.11 Dissemination plans

The findings of this study were presented to the Department of Microbiology and Immunology. The final report will be available at the MUHAS library. Also, the findings will be presented at scientific conferences and published in a peer-reviewed medical journal.

CHAPTER FOUR

4.0 RESULTS

4.1 Re-identification of CoNS isolates

Out of 160 archived CoNS isolates, 123 met the inclusion criteria. Of these 5 revealed mixed growth, 7 missed clinical history, 21 were non-viable and 20 were not CoNS. Only 70 isolates were re-identified as CoNS and included in the study (**Figure 2**).

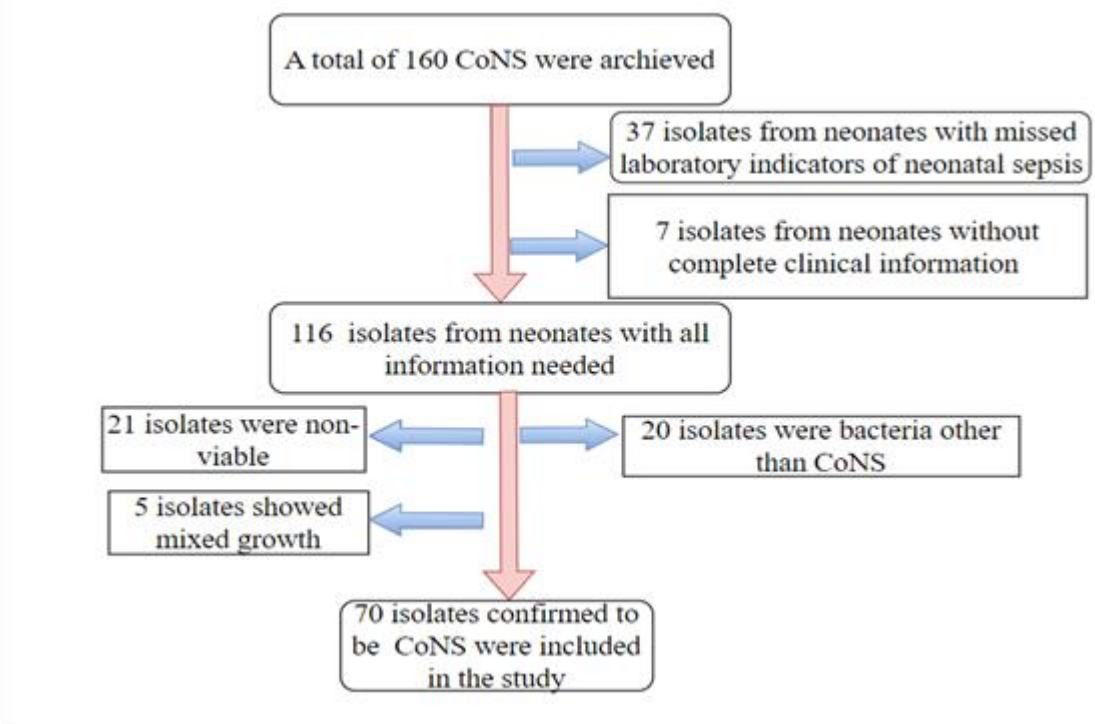


Figure 2: The number and reasons for CoNS isolates to be included and excluded in the study

4.1 Participants' demographic characteristics

The demographic characteristics of 70 neonates from whom CoNS were isolated were obtained from MRRH, neonates were aged 0 to 28 days with median age of 4 [Interquartile range (IQR): 2–10] days. More than half of neonates 39 (55.7%) were aged more than three days, 38(54.3%) of the neonates were males, and majority 64 (91.4%) had 2500g or more birth weight with mean of 3015.6g [Standard deviation (SD) ±562.2]. **More than three quarters** of the neonates 55 (78.6%) were delivered by normal standard vaginal delivery,

of which 10 (14.3%) were preterm delivery and **more than a half** 39 (55.7%) **of neonates** presented with late-onset neonatal sepsis. **All** neonates, 70 (100%) received empirical treatment before laboratory results, 63(90%) of neonates received first-line regimen only, and 7 (10%) of neonates switched from first-line regimen to second-line regimen. The mortality rate was **4** (5.7%)**(Table 5).**

Table 5: Demographic Characteristics of neonates from whom CoNS were isolated

Variables		Frequency (n)	Percent (%)
Sex	Male	38	54.3
	Female	32	45.7
Age (Days)	≤3	31	44.3
	>3	39	55.7
	Median (IQR)	4 (2-10)	
Birth weight (grams)	<2500	6	8.6
	≥2500	64	91.4
	Mean (SD)	3015.6 (±562.2)	
Gestation age (Weeks)	≥37	60	85.7
	<37	10	14.3
Onset of sepsis features	EONS (≤3days)	31	44.3
	LONS (>3days)	39	55.7
Mode of delivery	C/S	15	21.4
	NSVD	55	78.6
Empirical treatment	1st line regimen only	63	90.0
	1st and 2nd line regimens	7	10.0
Outcome	Discharge	66	94.3
	Death	4	5.7

KEY: IQR: interquartile range, SD: Standard deviation, NSVD: Normal standard vaginal delivery, C/S: Cesarean section, EONS: Early-onset neonatal sepsis, LONS: Late-onset neonatal sepsis, 1st line regimen: Ampicillin, Cloxacillin and Gentamycin, 2nd line regimen: Ceftriaxone.

4.2 Speciation and distribution of CoNS species

A total of 70 isolates recovered were confirmed to be CoNS species by VITEK® MS (BioMérieux, France) and six different CoNS species were identified (Figure 3). *S. epidermidis* was the predominant CoNS 27 (38.6%), followed by *S. haemolyticus* 24 (34.3%) and *S. hominis* 12 (17.1%) **(Figure 3).**

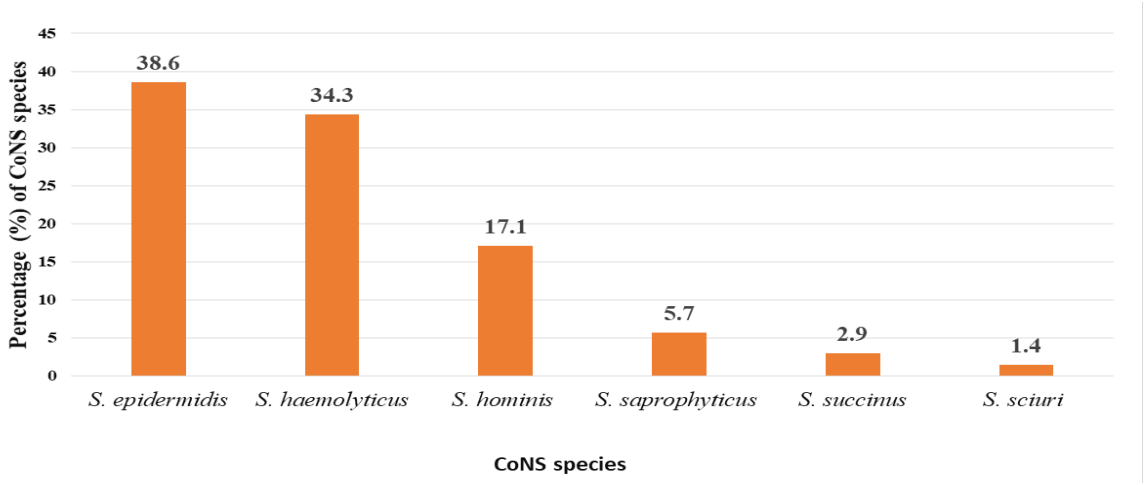


Figure 3: Distribution of CoNS species from neonatal with sepsis

4.3 Distribution of CoNS species according to the onset of sepsis

More than half of neonates 39(55.7%), present with late-onset neonatal sepsis with *S. epidermidis* 15 (21.4%) and *S. haemolyticus* 15(21.4%) being the predominant CoNS species **(Figure 4)**.

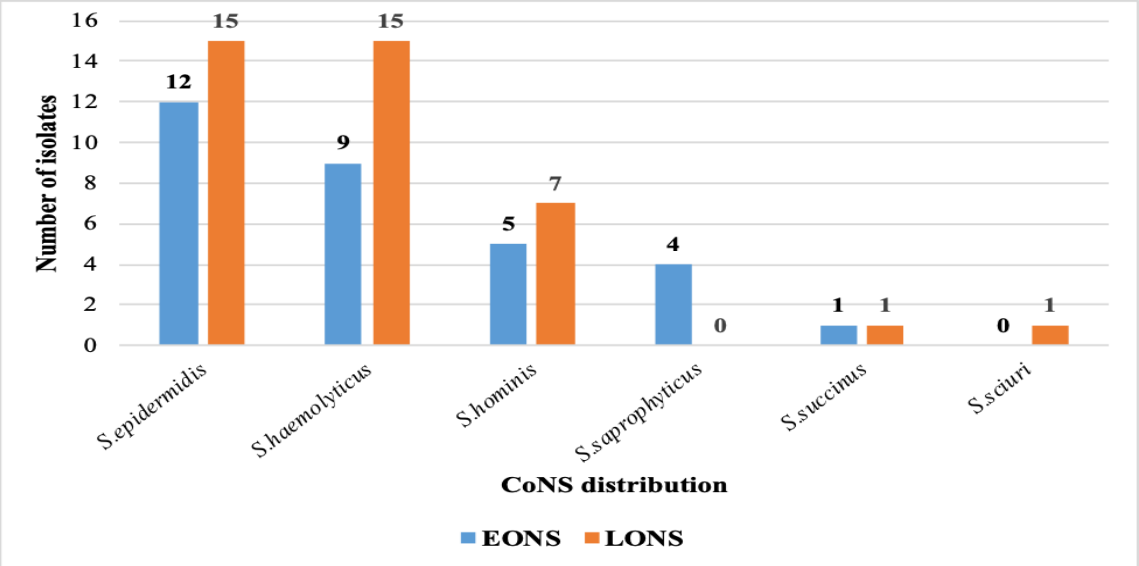


Figure 4: Distribution of CoNS species according to onset of sepsis

4.4 Phenotypically detection of biofilm formation among the CoNS species

The ability of CoNS species to form biofilm was detected phenotypically by congo red agar plate (CRA) and tube method (TM). All 70 (100%) CoNS produced biofilm on CRA and TM **(Figure 5).**

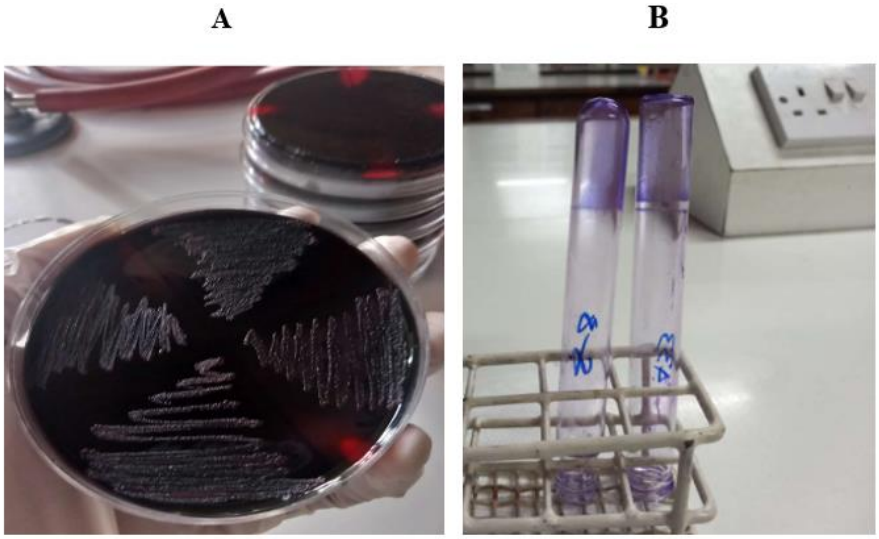


Figure 5: Biofilm formation among the CoNS isolates on CRA (A) and TM (B)

4.5. Detection of Virulence genes (*icaADBC*) among the CoNS isolates

A total of four virulence genes responsible for the formation of polysaccharide intercellular adhesin produced by a gene cluster at the intercellular adhesion (*ica*) locus were detected among 70 isolates. *IcaD* was predominant in all isolates 70(100%), followed by *icaB* 69 (98.6%), *icaC* 69(98.6%), and *icaA* 65(92.9%) **(Figure 6)**.

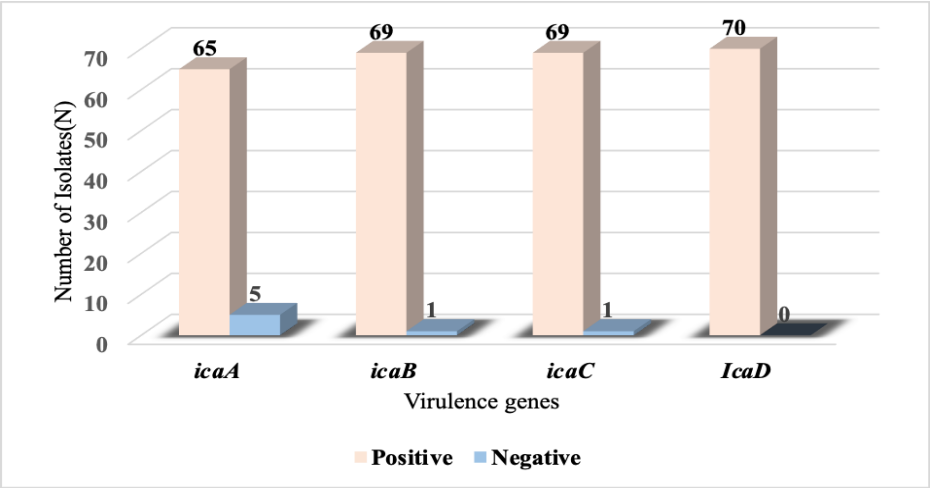


Figure 6: Distribution of *icaA, icaB, icaC,* and *icaD* genes among the CoNS isolates

The distribution of virulence genes per CoNS species was as follows: *icaA* gene was more predominant among *S. epidermidis* 25(92.6%) and *S. haemolyticus* 22 (91.7) while *icaB, icaC,* and *icaD* were predominant in all CoNS species **(Table 6)**.

Table 6: **Frequency and distribution of *icaA, icaB icaC,* and *icaD* genes among the isolated CoNS species**

GENES	**CoNS SPECIES n (%)**					
	S. epidermidis n=27	*S. haemolyticus* n=24	*S. hominis* n=12	*S. saprophyticus* n=4	*S. sciuri* n=1	*S. succins* n=2
icaA	25 (92.6)	22 (91.7)	11(91.7)	4 (100.0)	1(100.0)	2 (100)
icaB	26 (96.3)	24 (100.0)	12 (100.0)	4 (100.0)	1(100.0)	2 (100)
icaC	27 (100.0)	24 (100.0)	11 (91.7)	4 (100.0)	1(100.0)	2 (100)
icaD	27 (100.0)	24 (100.0)	12 (100.0)	4 (100.0)	1(100.0)	2 (100)

All 70 CoNS isolates harbored either *icaA, icaB, icaC,* or *icaD* genes. Four virulence gene combinations were detected among *Staphylococcus* species *icaADBC, icaBCD, icaACD,* and *icaABD*. The most predominant gene combination detected was *icaADBC* 63 (90%), which was more predominant among *S.epidermidis* 24 (38.1%) and *S.haemolyticus* 22 (34.9%) **(Table 7)**.

Table 7: Frequency of virulence gene combinations among the isolated CoNS species

Species N=70	N	**Virulence genes in combination (%)**			
		icaADBC	*icaBCD*	*icaACD*	*icaABD*
S. epidermidis	27	24(38.1)	2(40.0)	1(100.0)	
S. haemolyticus	24	22(34.9)	2(40.0)		
S. hominis	12	10(15.8)	1(20.0)		1(100.0)
S. Saprophyticus	4	4(6.3)			
S. succinus	2	2(3.2)			
S. sciuri	1	1(1.6)			
TOTAL		63 (90.0)	5 (7.14)	1 (1.42)	1(1.42)

Representative pictures of PCR amplicons for detection of *icaADBC* where all isolates harbored *icaADBC* genes (**Figure 7)** while some isolates miss one of *ica* gene in *ica* locus (**figure 8)**.

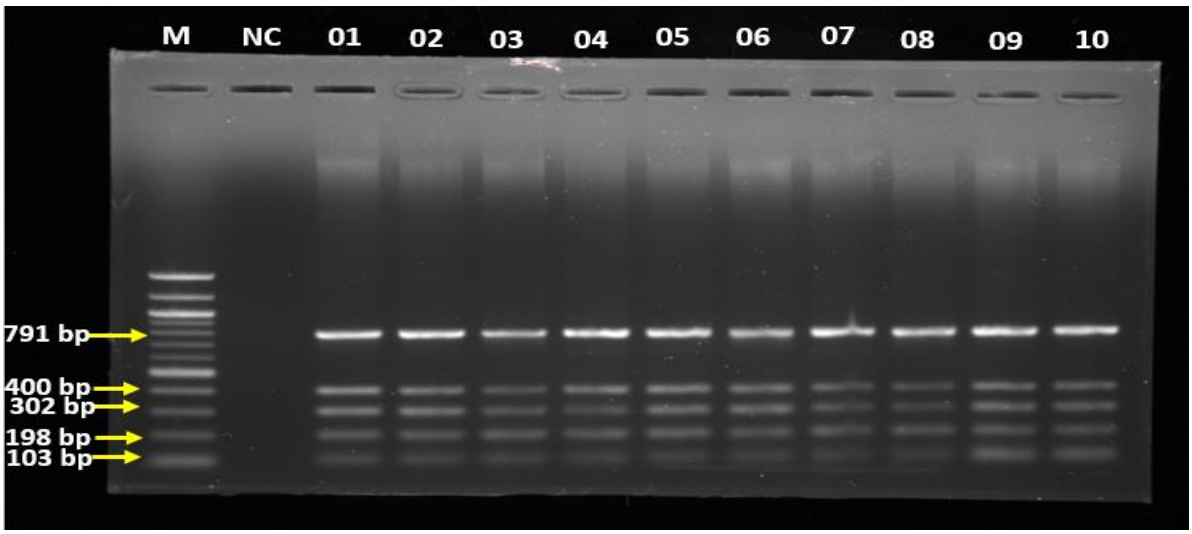


Figure 7: Gel image showing PCR amplicons of 10 CoNS isolates harbor *icaADBC*. M: 100 bp Marker, NC: Negative control, *icaA*-103bp, *icaB*-302bp, i*caC*-400bp, *icaD* -198bp, 16r RNA -791bp.

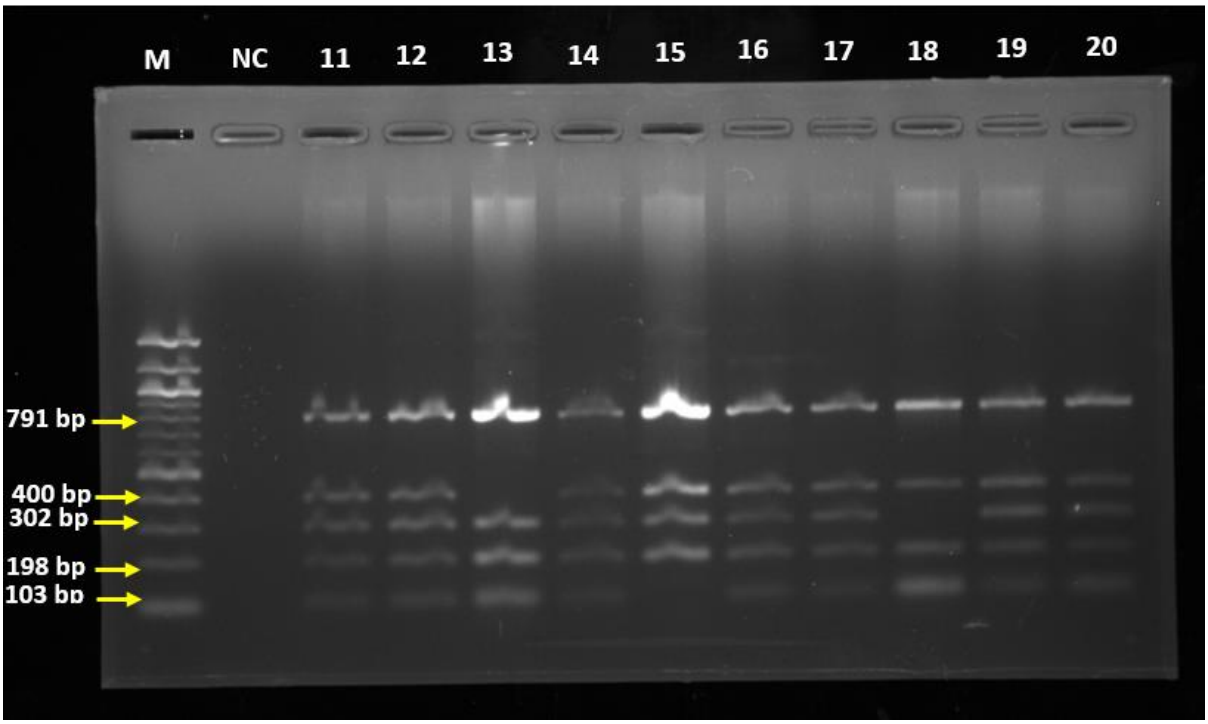


Figure 8: Gel image showing PCR amplicons of 10 CoNS isolates. M: 100 bp Marker, NC: Negative control *icaA*-103bp, *icaB*-302bp, i*caC*-400bp, *icaD* -198bp, 16r RNA -791bp.

4.7 Proportion of pathogenic CoNS

Among the 70 CoNS isolates, 63 (90%) harbored *ica* genes (*icaADBC*) in the *ica* locus and were isolated from neonates with clinical and laboratory indicators of neonatal sepsis were considered pathogenic CoNS, while 7 (10%) CoNS isolates misses *ica* genes were classified as non-pathogenic CoNS (**Figure 9).**

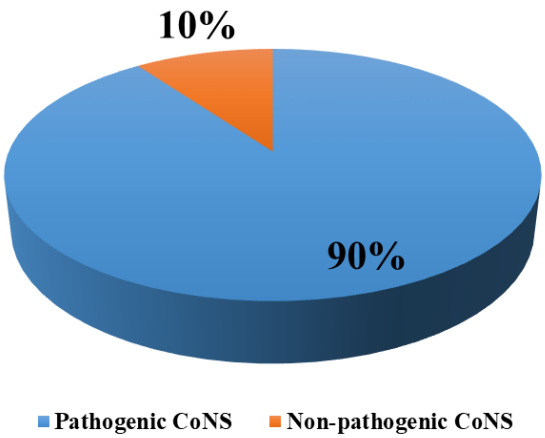


Figure 9: Proportion of Pathogenic CoNS versus non-pathogenic CoNS.

4.6 Detection of ARGs (*mecA, blaZ,* and *aac (6')-Ie-aph (2'')-Ia*) among the CoNS isolates

A total of three clinically relevant ARGs were detected including *mecA, blaZ,* and *aac (6')-Ie-aph (2'')-Ia* in 70 CoNS isolates. The mostly predominant ARGs detected was *mecA* 69 (98.6%), followed by *blaZ* 67 (95.7%) and *aac (6')-Ie-aph (2'')-Ia* 65 *(*92.7%) (**Figure 10).**

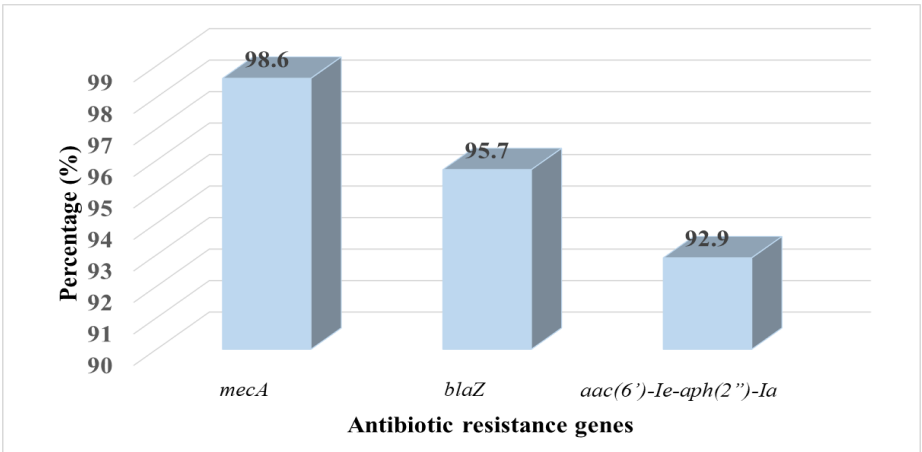


Figure 10: Distribution of ARGs (*mecA, blaZ,* and *aac (6')-Ie-aph (2'')-Ia)* among the CoNS isolates

All ARGs were harbored by 61 (87.1%) isolates were by 24 *S.epidermidis*, 23 *S. haemolyticus*, 9 *S. hominis*, 3 *S. saprophyticus*, and 2 *S. succinus* **(Table 8).**

Table 8: Frequency and distribution of ARGs (*mecA, blaZ,* and *aac (6')-Ie-aph (2'')-Ia*) among the isolated CoNS species

SPECIES	**N**	**ANTIMICROBIAL RESTANCE GENES n (%)**		
		BlaZ	*MecA*	*aac(6')-Ie-aph(2'')-Ia*
S.epidermidis	27	26 (96.3)	26 (96.3)	26 (96.3)
S. haemolyticus	24	24 (100.0)	24 (100.0)	23 (95.8)
S. hominis	12	12 (100.0)	12 (100.0)	9 (75.0)
S. Saprophyticus	4	3 (75.0)	4 (100.0)	4 (100.0)
S. succinus	2	2 (100.0)	2 (100.0)	2 (100.0)
S. sciuri	1	0 (0.0)	1 (100.0)	1 (100.0)

4.7 Comparison between phenotypic and genotypic antibiotic susceptibility test

The comparison between phenotypic antibiotic susceptibility test for penicillin G, oxacillin and gentamicin resistant from previous study (9) and the presence of *blaZ*, *mecA* and *aac(6')-Ie-aph(2'')-Ia* respectively is summarized in **Table 9**. About 59 (84.3%) of isolates were phenotypically resistant to penicillin, gentamycin, and oxacillin. Out of 67 isolates that were phenotypically resistant to penicillin, 65 (97.0%) harbored *blaZ* genes, and all 62 (100.0%) that were resistant to oxacillin harbored *mecA* genes. Sixty-two (93.9%) of 66 CoNS that were phenotypically resistant to gentamycin harbored *aac(6')-Ie-aph (2'')-Ia* gene.

Table 9: Comparison between phenotypic and genotypic antibiotic susceptibility testing among the CoNS isolates.

AST BY DISK DIFFUSION		**ARGS DETECTED BY PCR**	
		Positive	**Negative**
Penicillin		***blaZ***	
Resistant	67	65	2
Susceptible	2	1	1
Intermediate	1	1	
Oxacillin		***mecA***	
Resistant	62	62	0
Susceptible	4	3	1
Intermediate	4	4	0
Gentamycin		***aac(6')-Ie-aph(2'')-Ia***	
Resistant	66	64	2
Susceptible	3	2	1
Intermediate	1	1	

KEY: AST: Antimicrobial susceptability test, ARGs: Antimicrobial resistance genes, PCR:Polymerase chain reaction.

Representative pictures of PCR amplicons for detection of ARGs where all 16 isolates harbored *blaZ* gene (**figure 11**), 12 isolates harbored *mecA* gene (**figure 12**) and 14 isolates harbored *aac(6')-Ie-aph(2'')-Ia* gene, one negative isolate for *aac(6')-Ie-aph(2'')-Ia* gene (**figure 13)**.

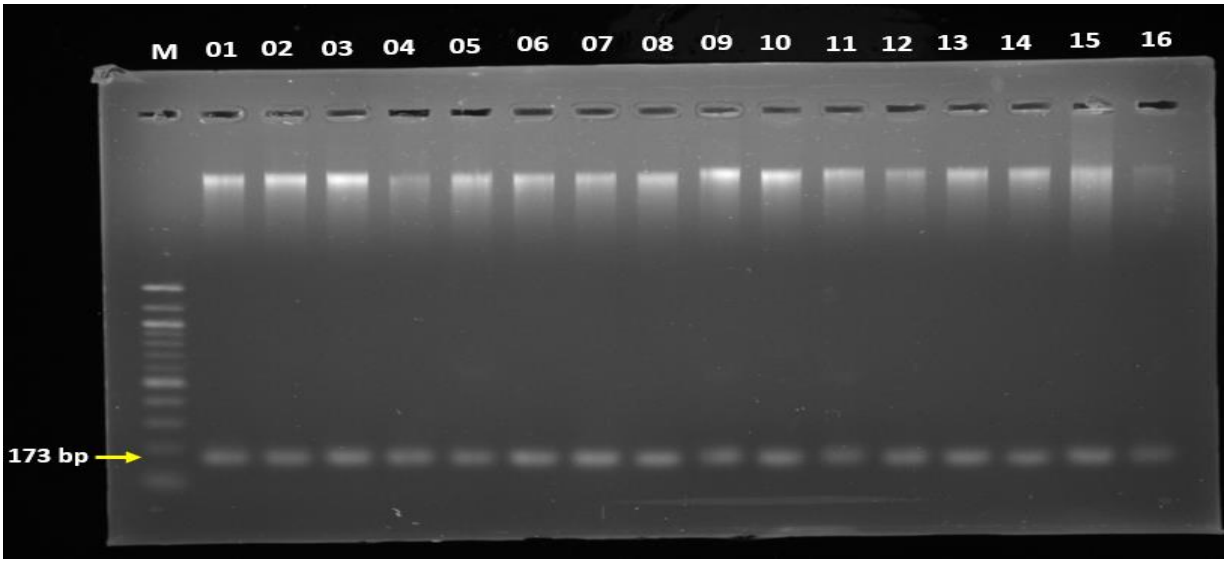


Figure 11: Gel image showing PCR amplicons of 16 CoNS isolates harbor *blaZ* gene. M: 100 bp Marker, *blaZ* -791bp.

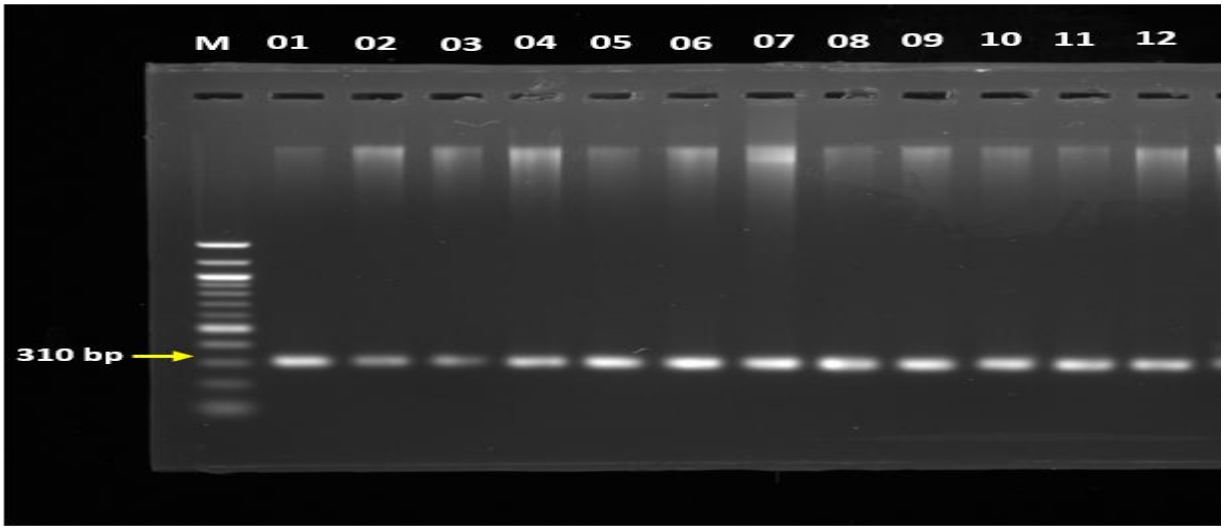


Figure 12: Gel image showing PCR amplicons of 12 CoNS isolates harbor *mecA* gene. M: 100bp Maker, *mecA* - 310bp.

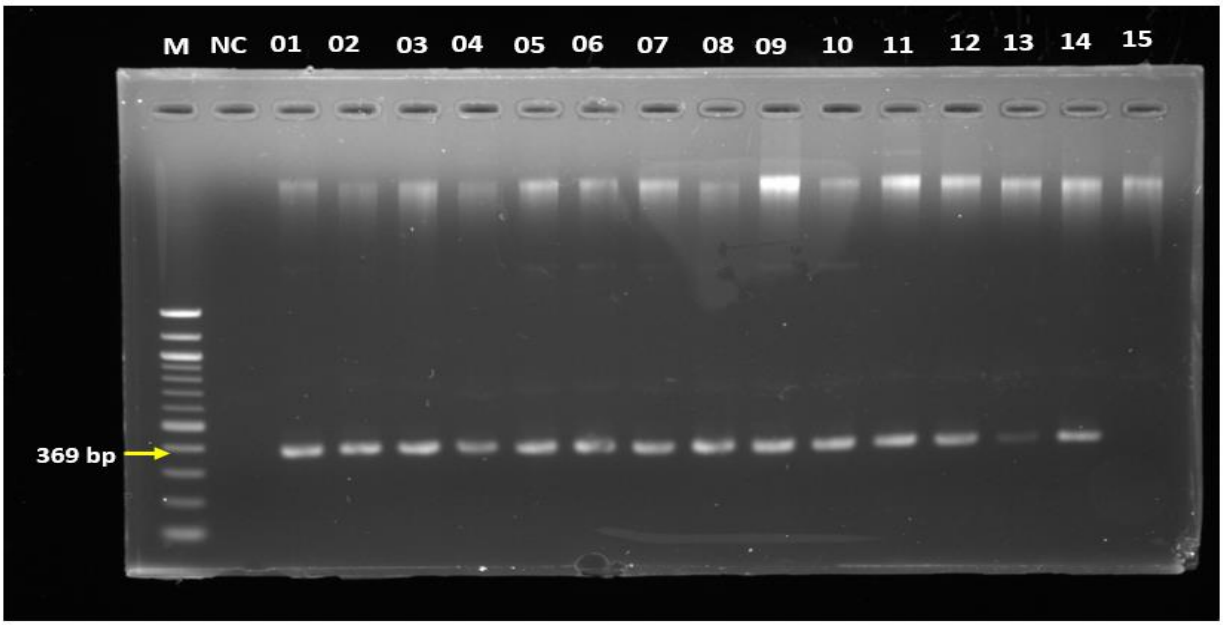


Figure 13: Gel image showing PCR amplicons of 15 CoNS isolates, CoNS isolates (01-14) harbored *aac(6')-Ie-aph (2'')-Ia* gene while 15 were negative. M: 100bp Maker, *aac (6')-Ie-aph (2'')- Ia* - 369bp.

CHAPTER FIVE

5.0 DISCUSSION

The current study screened for *icaADBC* genes that enhance the pathogenicity of CoNS through biofilm formation and ARGs that confer resistance against β-lactams (*blaZ)*, aminoglycosides (*aac(6')-Ie-aph(2'')-Ia*), and methicillin (*mecA)* genes. In overall, 70 CoNS isolates were characterized in this study with *S. epidemidis* and *S. haemolyticus* being the predominant isolate account for about 70% of all isolates. Virulence genes responsible for biofilm formation (*ica* genes) were detected of which 90% of CoNS species harbored all *ica* genes (*icaADBC*) in *ica* locus and were considered as pathogenic species. About 87% of CoNS isolates harbored all the three ARGs against the commonly prescribed antibiotics, where *mecA* around 99% was the most prevalent gene detected followed by *blaZ* 95.7%.

In recent years, CoNS have been reported as the predominant etiology of neonatal sepsis (9,31,36). The current study documents *S. epidemidis* (36.8%) as the predominant CoNS species causing neonatal sepsis followed by *S. haemolyticus* (34.3%). These findings concur with other studies where *S. epidermidis* and *S. haemolyticus* have been reported as the most predominant cause of bloodstream infections in neonates (54,75,76). These species are the among most frequently isolated CoNS species reported to **be** associate**d** with BSIs (3,6,77). Since CoNS especially *S. epidermidis* are present in the skin and mucosal membrane, hence it easily to establish colonization and pose BSI if risk factors such as the use of invasive devices are in place thus, a possible explanation for these findings (78). In this study, **more than half** of neonates had late onset neonatal sepsis caused by CoNS species. Several studies reported CoNS to be more associated with late-onset neonatal sepsis (79,80). The most predominant CoNS species in late-onset neonatal sepsis in this current study were *S. epidermidis* and *S. haemolyticus.* These results **are comparable** with results reported by Kirsty et al. (10) and Ronja et al. (22) where by *S. epidermidis* and *S. haemolyticus* were more associated with late-onset neonatal sepsis.

The ability of CoNS to generate biofilms is a prime virulence in CoNS pathogenesis. Polysaccharide intercellular adhesin (PIA) associated biofilm formation is mediated by the intercellular adhesin (*ica*) locus and represents a major pathomechanism of CoNS (6). This

study found that all CoNS isolates 100% harbored *icaD* gene, 98.6% harbor *icaB* gene, 92.9% harbor *icaA* gene, and 98.6% harbor *icaC* gene. These findings match the report by Rahimi et al. (81) where by all biofilm former CoNS isolates harbored *icaA* gene and *icaD* gene, 93% harbored *icaB* gene, and 83% harbored *icaC* gene. Other studies have reported a similar trend (82,83). In contrast some studies have reported low rates of *icaA, icaB, icaC, icaD* genes carriage among CoNS isolates (17,69,84). Expression of all four genes is required for the synthesis of fully functional PIA, hence *ica* operon deletion results in a loss of the ability to form biofilms (85).The current study detected four gene combinations including *icaADBC, icaBCD, icaACD,* and *icaABD*. About 90% of CoNS isolates were found to harbor *icaADBC* genes in this study **imply that they carry a variety of VGs needed for the synthesis of polysaccharide intercellular adhesin (PIA), which is key in facilitating biofilm formation** (85). Our findings were high compared to the low rates (77.5%) of biofilm producer CoNS harboring *icaADBC* reported in the study done in Iran (84). Similarly, a low proportion of *icaADBC* was also reported in other studies (69,86). The difference could be explained by the patients studied, the current study involved neonates, who are more vulnerable to infection due to CoNS as result of immature or less competent immune systems. While in other studies neonates, infants, children, and adults were involved. Furthermore, our participants had clinical and laboratory indicators for BSI hence, isolated CoNS were more likely to be pathogenic.

The current study observed gene combination with missing either *icaA, icaB,* or *icaC*. For example, 7.14% of CoNS isolates harbored *icaBCD,* 1.42% harbored *icaACD,* and 1.42% of CoNS isolates had *icaABD.* Phase variations in the presence of *icaADBC* genes from studies might be due to frameshift mutations through the Streisinger slippage mechanism since *icaADBC* genes are rich in repetitive -TATTT- motives hence, resulting in gene deletion (87). However, these CoNS produced biofilm phenotypically, biofilm formation might be due to Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMMs) such as (*bap*), *bhp, aap, fbe, embP,* and *atlE* (3,6,82).

About 87.1% of CoNS were resistant to all three antibiotics (penicillin, oxacillin, and gentamycin), recommended in the national guidelines for managing neonatal sepsis. This indicates that only 12.9% of antibiotics used for management of neonatal sepsis

as per to Tanzania guidelines, is effective, suggesting a need to conduct a big or multicenter studies which will provide enough data to guide the review of treatment guidelines. This study found that 95.7% of CoNS harbored *blaZ* gene, which is in keeping with the study done by Sylwia et al. (88) which reported 95.6% of CoNS harbored *blaZ* gene. Similar findings have also been reported in a study done in India (89). The *blaZ* gene encodes for a β-lactamase that is synthesized when staphylococci are exposed to β-lactam antibiotics, thus hydrolyzing the β-lactam ring, hence inactivating penicillin (90). However, two phenotypically penicillin resistance did not harbor *blaZ* gene, phenotypic resistance to penicillin may be due to the presence of *mecA* which they harbored. This finding is supported by reports from other studies where resistance to penicillin was found to be due to the *mecA* gene (91,92).

The current study documents that 98.6% of CoNS harbored the *mecA* gene encoding for an altered penicillin-binding protein (PBP 2a), which confers resistance to methicillin (93). This is high compared to 87% documented by Hira et al. (24). Similarly, studies done by Shrestha et al. (94) and Secchi et al. (95) observed similar trends. The high proportion of the *mecA* gene in our study might be due to the ability of CoNS to form biofilms which facilitate horizontal gene transfer between bacteria. Also, all neonates admitted to hospitals were treated with empirical treatments which might be enhancing gene expression. However, three phenotypically oxacillin-susceptible isolates harbored the *mecA* gene this can be explained by the heterogeneously expressed phenomenon of the *mecA* gene (62). Therefore, all methicillin-resistant staphylococcal strains may not be detectable with phonotypic methods. The detection of the *mecA* gene by PCR techniques is considered the gold standard method (96).

In the current study, 92.9% of CoNS harbor *aac(6')-Ie-aph(2'')-Ia* gene that encodes for aminoglycoside-modifying enzyme AAC(6')-APH(2'') which deactivates a wide range of medically important aminoglycosides such as gentamycin (97,98). Our findings are supported by the study by Mohammad et al. (60) and Fattaneh et al. (99) which reported a proportion of 93.3% and 87.5% respectively. Furthermore, two phenotypically gentamycin-resistant isolates didn't harbor *aac(6')-Ie-aph(2'')-Ia* gene, this may be due to

mutation in *aac(6')-Ie-aph(2'')-Ia* gene which may affect annealing of primer results to a false-negative result (100).

5.1 STUDY LIMITATIONS

1. The study was limited to PCR technology, which interrogates only a fraction of the genome of a bacterium. Whole genome sequencing, which could be more informative on epidemiological bacterial typing and novel antimicrobial resistance and virulence genes among CoNS was not performed due to budgetary constraints.

5.2 CONCLUSION

This study reveals CoNS species causing neonatal sepsis, with *S. epidermidis* followed by *S. haemolyticus* being the predominant species. Nine out of ten CoNS isolated from neonates with clinical and laboratory indicators are more likely to be pathogenic than non-pathogenic. Therefore, CoNS isolation from neonates with clinical and laboratory indicators should be taken into consideration before regard them as non-pathogenic. Furthermore, the study revealed higher carriage of ARGs *blaZ*, *mecA*, and *aac (6')-Ie-aph (2'')-Ia* which confer resistance to most commonly prescribed antibiotics penicillin, methicillin, and gentamycin, respectively. Indicating empirical treatment with the antibiotics recommended in the national guidelines are likely to fail, due to high levels of antimicrobial resistance.

5.2 RECOMMENDATIONS

Given the fact that most CoNS isolated from neonates with sepsis carried virulence genes, and some of them have multiple genes, underscores the need to review the laboratory and clinical guidelines for the management of neonatal sepsis. **CoNS to be included as potential pathogens associated with neonatal sepsis and revise empirical antibiotics recommended in national guidelines.**

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Appendix

DATA COLLECTION TOOL

Sample ID Number ……..

Demographic Information		
Age		
Date of Admission		
Gender	Male	Female
Body weight	≥2500g NBW	<2500g LBW
Gestation age at delivery	≥37weeks	<37week
Clinical Indications		
Axial temperature	<36.5ºC	>37.5 ºC
Respiratory rate per minutes	<30	>60
Jaundice	YES	NO
Fever	YES	NO
Asphyxia	YES	NO
Poor feeding	YES	NO
Laboratory indicators	Total WBCs	
	CRP	
Onset of sepsis features		
Early onset Neonatal sepsis		
Late onset Neonatal sepsis		

Blood culture	
Prior to antibiotics administration	**YES** ☐ **NO** ☐
Clinical Outcome	
Discharged	☐
Death	☐

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES

OFFICE OF THE DIRECTOR – RESEARCH, PUBLICATIONS AND INNOVATION

Ref. No.DA.282/298/01.C/2010

Date: 24/01/2024

MUHAS-REC-01-2024-2010
ANJELA CHARLES
MUHAS

RE: APPROVAL FOR ETHICAL CLEARANCE FOR A STUDY TITLED: MOLECULAR CHARACTERIZATION OF COAGULASE NEGATIVE STAPHYLOCOCCUS ISOLATED FROM NEONATES WITH SEPSIS AT MWANANYAMALA REGIONAL REFERRAL HOSPITAL.

Reference is made to the above heading,

I am pleased to inform you that the Chairman has on behalf of the University Senate, approved ethical clearance of the above-mentioned study, on recommendations of the Senate Research and Publications Committee meeting accordance with MUHAS research policy and Tanzania regulations governing human and animal subjects research.

APPROVAL DATE: 24/01/2024
EXPIRATION DATE OF APPROVAL: 24/01/2025

STUDY DESCRIPTION:
Purpose:

The purpose of this cross-sectional study is To determine the molecular characteristics of Coagulase Negative Staphylococcus (CoNS) isolated from neonates with sepsis from Mwananyamala Regional Referral Hospital from January 2021 to July 2022.

The approved protocol and procedures for this study is attached and stamped with this letter, and can be found in the link provided:

https://irb.muhas.ac.tz/storage/Certificates/Certificate%20-%2014974.pdf and in the MUHAS archives.

UNITED REPUBLIC OF TANZANIA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY

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The PI is required to:

1. Submit bi-annual progress reports and final report upon completion of the study.
2. Report to the IRB any unanticipated problem involving risks to subjects or others including adverse events where applicable.
3. Apply for renewal of approval of ethical clearance one (1) month prior its expiration if the study is not completed at the end of this ethical approval. You may not continue with any research activity beyond the expiration date without the approval of the IRB. Failure to receive approval for continuation before the expiration date will result in automatic termination of the approval for this study on the expiration date.
4. Obtain IRB amendment (s) approval for any changes to any aspect of this study before they can be implemented.
5. Data security is ultimately the responsibility of the investigator.
6. Apply for and obtain Data Transfer Agreement (DTA) from NIMR if data will be transferred to a foreign country.
7. Apply for and obtain Material Transfer Agreement (MTA) from NIMR, if research materials (samples) will be shipped to a foreign country.
8. Apply for and obtain permission to publish the results from National Institute for Medical Research. The application should be before submitting to a publisher and not concurrently through the email publications@nimr.or.tz.
9. Any researcher, who contravenes or fail to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III section 10 (2)
10. The PI is required to ensure that the findings of the study are disseminated to relevant stake holders.
11. PI is required to be versed with necessary laws and regulatory policies that govern research in Tanzania. Some guidance is available on our website https://drp.muhas.ac.tz/.



Dr. Kasusu Nyamuryekung'e
Ag. Chairperson, MUHAS Research and Ethics Committee

UNITED REPUBLIC OF TANZANIA
MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY
MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES
OFFICE OF THE DIRECTOR – POSTGRADUATE STUDIES

In reply quote;
Ref. No. HD/MUH/T.374/2022

29th January, 2023

The Head,
Department of Microbiology/Immunology,
MUHAS

Re: INTRODUCTION LETTER

The bearer of this letter is Anjela Charles (HD/MUH/T.374/2022), a student at Muhimbili University of Health and Allied Sciences (MUHAS) pursuing MSc. Microbiology/Immunology

As part of her studies she intends to do a study titled: **Molecular Characterization of Coagulate Negative Staphylococcus Isolated from Neonates with Sepsis at Mwananyamala Regional Referral Hospital."**

The research has been approved by the Chairman of University Senate.

Kindly provide her the necessary assistance to facilitate the conduct of her research.

We thank you for your cooperation.

Ms. Victoria Mwanilwa
For: DIRECTOR, POSTGRADUATE STUDIES

c.c. Anjela Charles

9 United Nations Road; Upanga West; P.O. Box 65001, Dar Es Salaam: Tel. G/Line: +255-22-2150302/6; Ext. 1015; Direct Line:+255-22-2151378;Telefax:+255-22-2150465;E-mail:dpgs@muhas.ac.tz;Web:https://www.muhas.ac.tz

THE UN THE UNITED REPUBLIC OF TANZANIA
MINISTRY OF HEALTH

Telephone Address:

Telephone: 022-2760500

Mwananyamala Regional
Referral Hospital,
P.O.Box 61665
Dar es Salaam.

RE: NO.: MA. 240/341/01/8

DATE: 31TH JANUARY,2024

Director,
Muhimbili University of Health and,
Allied Sciences,
P.O.BOX 65001,
DAR ES SALAAM.

RE: TO CONDUCTI HIS RESEARCH IN MWANANYAMALA REGIONAL REFERRAL HOSPITAL

The captioned subject refers

2. May you be informed that your prescribed request for your bonafide student **Anjela Charles,** your request to collect data as part of Research Titled ***"Modecular Characterizations of Coagulate Negative Staphylococcus Isolated from Neonates with Sepsis at Mwananyamala Regional Referral Hospital"*** **to 2th – 17th February,2024**, is asserted.

3. The Institution charges **50,000/=**, as Data Collection fee as per student spent. The payments are to be made upon reporting.

4. May she report to the Administration and HR department head for further instruction.

4. May she report to the Administration and HR department head for further instruction.

Thanks.

Dr. Mkiwa Akida
RESEARCH COORDINATOR
FOR: MEDICAL OFFICER INCHARGE
MWANANYAMALA REGIONAL REFERRAL HOSPITAL

COPY:
Heads of Paediatric Department - **MWANANYAMALA REGIONAL REFERRAL HOSPITA**

Report to the head of Paediatric Department