ASSOCIATION OF METHICILLIN RESISTANCE AND VIRULENCE GENES IN STAPHYLOCOCCI ISOLATED FROM PAPER CURRENCY AND NASAL CAVITY OF BUS CONDUCTOR

A Research Report

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DECLARATION

The study entitled “Association of methicillin resistance and virulence gene in staphylococci isolated from paper currency and nasal cavity of bus conductor” has been submitted to the Research section, Institute of Science & Technology (IoST), Dean’s Office. Tribhuvan University. This study is conducted under the supervision of Lecturer Dr. Sarita Manandhar. To the best of my understanding, no other university has received this submission.

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ABSTRACT

Because of their resistance to a variety of medications, Methicillin-Resistant Staphylococci (MRS) poses a serious threat to public health. These organisms also harbor various virulent factors that make them significant concerns in public health. They survive on various environmental surfaces including paper currencies. The purpose of this research was to assess the possible presence of MRS on bus conductor's nasal swabs and the paper money they carried. A total of 100 samples (50 nasal swab and 50 paper currency) were collected in peptone water and saline water respectively. The samples were processed in Microbiological laboratory of Tri-Chandra Multiple Campus, Ghantaghar and Institute for Research in Science and Technology, Thamel from July 2023 - December 2023. The samples were exposed to standard bacteriological techniques for the purpose of isolation and identification of *S. aureus* and coagulase negative Staphylococci (CNS). Cefoxitin disc was used to confirm MRS. Modified Kirby-Bauer disc diffusion technique, an antibiotic susceptibility test was carried out and, *tsst*, *sea* and *pvl* gene were amplified by conventional PCR- Polymerase Chain Reaction. Of 50 nasal swab samples, 25 (50%) were identified as CNS and 8 (16%) as *S. aureus*. Among them, 14 (56%) and 4 (50%) were identified as MR-CNS and MRSA respectively. Similarly, from 50 paper currency, 11(22%) as CNS and 21 (42%) were identified as *S. aureus*. All CNS were methicillin resistant while only 7 (33.3%) were MRSA. Higher prevalence of MRSA and MRCNS were identified from age group 15-30. All MRSA and MRCNS were resistant to penicillin but sensitive to amikacin, meropenem and chloramphenicol. All MRSA and 9 (81,1%) MRCNS from nasal swab and all MRCNS and 4 (57.1%) MRSA from paper currency were MDR. None of the isolate harbor *pvl* gene whereas 52.2% MRS isolate carried *tsst* gene and 50% MRS isolate carried *sea* gene. High occurrence of MDR staphylococci & virulence gene containing MRS in community shows the need of regular surveillance & implementation of awareness programmed that help to reduce its hazards.

Keywords- Staphylococci, MRSA, MR-CNS, MDR, *tsst*, *sea*, *pvl*

शोधसार

Staphylocci जीवाणुको औषधि प्रतिरोध गर्ने, विषालु तत्व उत्पादन गर्न सक्ने क्षमताले गर्दा विश्वभर धेरै मानिसहरुलाई संक्रमण गर्ने र मृत्यु भैराखेको छ । सबै जसो औपधिहरु जुन संक्रमण विरुद्ध प्रयोग गरीन्छ, जस्तो की Methicillin, Penicillin सँग प्रतिरोध गर्ने क्षमताले गर्दा समाजमा MRS फैलिरहेको छ । यो अध्ययनको उद्देश्य बस कन्डक्टरहरुमा MRSA पहिचान गर्ने, औषधिहरुको activity थाहा पाउने र विषालु तत्वहरु (*TSST, sea, pvl*) जीनहरु छ कि छैन भनि पत्ता लगाउनु हो । १०० वटा नमूना (५० वटा नाकको र ५० वटा पैसा) हरु जम्मा गरी त्रि-चन्द्र कलेजको माइक्रोबायोलोजी प्रयोगशालामा परिक्षण गरियो । Standard Bacteriological Method र Kirby-Bauer diffusion method ले MRSA र MRCNS पहिचान गरियो र जीनहरु पोलिमरेज चैन रियाक्सन (PCR) बाट थाहा पाइयो ।

५० वटा नाकको नमूनाबाट २५ (५०%) CNS र ८(१६%) *S. aureus* जसमध्ये १४(५६) MRCNS र ४(५०%) पाईयो । त्यस्तै ५० वटा पैसा नमूनाबाट ११(२२%) CNS र २१ (४२%) *S.aureus* जसमध्ये ११(१००%) MRCNS र ७(३३.३%) MRSA पाईयो । त्यस्तै सबैभन्दा बढी MRSA र MRCNS १५-३० वर्षका मानिसहरुमा पाइयो । सबै जीवाणुहरु औषधिसँग प्रतिरोधात्मक नै देखियो । Chloramphenicol, gentamycin, amikacin, meropenem औषधिहरु यस जीवाणुको संक्रमण विरुद्ध उपयुक्त भएको पाइयो । ५०% MR staphylococci मा *sea* जीन देखियो । त्यस्तै ५२.८% MR staphylococci मा *tsst* जीन देखियो । *pvl* जीन कुनै पनि जीवाणुमा भेटिएन । हाम्रो समाजमा घातक जीनहरु भएका MRSA र : MRCNS फैलिरहेकाले यसबाट जोगिन यस्तै धेरै अध्ययनहरु गर्नु पर्ने र समयमै मानिसहरुमा सचेतनामूलक कार्यक्रमहरु संचालन गर्नु पर्ने जरुरी भएको देखियो ।

मुख्य शब्दहरु : staphylococci, MRSA, MRCNS, *tsst, pvl, sea*

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ABBREVIATIONS

AST- Antibiotic Susceptibility Test

CA-MRSA- Community Acquired Methicillin Resistant *Staphylococcus aureus*

CDC- Central for Disease Control and Prevention

CLSI- Clinical Laboratory Standard Institute

CNS- Coagulase Negative staphylococci

CPS- coagulase positive staphylococci

HA-MRSA- Hospital acquired methicillin resistant *Staphylococcus aureus*

HCW- Health Care Worker

MDR- Multi Drug Resistance

MRCNS- Methicillin resistant coagulase-negative staphylococci

MRSA- Methicillin resistance *Staphylococcus aureus*

MRS- Methicillin resistance staphylococci

MSA- Mannitol Salt Agar

MS-CNS- Methicillin sensitive coagulase positive staphylococci

MSSA- Methicillin sensitive *staphylococcus aureus*

NA- Nutrient Agar

NB- Nutrient broth

O/F- Oxidative / fermentative

PCR- Polymerase Chain Reaction

Pvl- Panton Valentine Leucocidin

SA- *Staphylococcus aureus*

sea- Staphylococcal enterotoxin A

TSB-Tryptone Soya Broth

tsst- toxic shock syndrome

WHO- World Health Organization

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background of study

Staphylococci are Gram positive cocci, occur in pairs, tetrads and clusters (Wilkinson, 1997). They do not form spores & are facultative anaerobes and grow in an aerobic environment with temperatures ranging from 15 -45°C and concentration of sodium chloride up to 15%. Staphylococci are significant pathogenic bacteria accountable for various diseases in humans (Beck et al., 2012).

Robert Koch discovered staphylococci within human pus for the first time in 1878. The uneven grouping of these cells of bacteria led Alexander Ogston to suggest the name "Staphylococcus" in 1884. The word is taken after the Homeric Greek word "staphyle," which means grape. In the same year, Rosenbach separated two colonies into *Staphylococcus aureus* and *Staphylococcus albus* based on their coloration, which was white and yellow. Later (Ucuncu et al., 2009), the latter was called *Staphylococcus epidermidis.*

As an opportunistic pathogen, staphylococci are human commensals that live as normal microflora in many regions of the human body and can cause a variety of illnesses (Gotz, 2002). According to Morgenmeier et al. (2014), *S. aureus* may cause a variety of ailments, from mild skin infections like pyoderma to more serious ailments like bacteremia, osteomyelitis, and endocarditis, which can lead to toxic shock syndrome.

Because they may use the coagulase enzyme to coagulate blood plasma, they are divided into two groups: coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS). According to Becker et al., (2014*), S. aureus* and *S. epidermidis* are among the more common CPS in human infections. *S. aureus* can settle in and cause infections in various animals used for food production.

Additionally, CNS are also becoming increasingly recognized as a significant group of pathogenic staphylococci. They are often discovered in human and other animal mucous and skin membranes. Generally, CNS are relatively less virulent and expresses fewer virulent factors (Von Eiff et al., 2002).

Due to *S. aureus's* great antibiotic tolerance, it may readily colonize healthy persons, which increases the risk of infections and spread among people (Chambers et al., 2009).

S aureus can cause different types of infections (Chakraborty, 2011)-

- Skin infection- the most common types of infection caused by *staphylococcus* species
- Bacteremia- infection of bloodstream that led sepsis
- Bone infection- infection of bones and joints
- Endocarditis- infection of inner layer of heart and valve that lead heart fail
- Toxic shock syndrome- life threatening infection caused by toxin
- Pneumonia- infection of lungs
- Food poisoning – due to staphylococcal enterotoxin

The bacteria's ability to withstand antibiotics, either bacteriostatic or bactericidal, is known as antibiotic resistance. Resistance to the majority of antibiotics is mostly caused by the overuse and abuse of antibiotics as well as by horizontal and vertical gene transfer. The increasing level of resistance to numerous ranges of drugs by staphylococci represent a significant threat to treatment efficacy. The commonly used drugs to treat staphylococcal infections have been ineffective due to developed resistivity particularly beta-lactams (Goldstein et al., 2007).

Multi-drug resistance in pathogens is induced by the rise in the number of genes on resistance plasmids, each of which codes for resistance to a specific agent as well as the activity of multi-drug efflux pumps. Most strains are capable of developing resistance to at least one medication across at least three antimicrobial

groups. There are two possible processes by which bacteria develop multidrug resistance.

First, the bacteria can collect many genes from a single cell, all that stands for resistance to a different medication. Usually, R (resistance) plasmids the site of this buildup. Second, extensive use of medications may be extruded by genes that code for multidrug efflux pumps, hence increasing the possibility of multidrug resistance (Godebo et al., 2013). MRSA is a class of multi drug-resistant bacteria that increases morbidity and mortality. MDR organisms are pathogens that are no longer susceptible to the effects of antibiotics, making them difficult to control or eradicate (Gedebo et al., 2013).

In the course of the 1950s and 1960s, penicillin-resistant *S. aureus* became extremely widespread and eventually became a worldwide pandemic. Penicillin-resistant *S. aureus* was treated with semi-synthetic penicillinase stable methicillin in 1959 as a therapeutic agent. CNS then developed resistance to methicillin following its introduction to medical practice in the UK in 1961 (Kejela & Bacha, 2013). The rise of MRSA and increasing treatment resistance among *S. aureus* pose a global threat. It is a leading cause of nosocomial infections (Bhatta et al., 2016). A mutation in the penicillin binding protein, a chromosome-encoded protein, results in methicillin-resistant *S. aureus*. This kind of resistance is dispersed across *S. aureus* organisms via bacteriophages. The *mec*A gene in MRSA makes it resistant to beta-lactam drugs. According to Siddiqui and Koirala (2024), this gene reduces reactivity to beta lactam antibiotics and creates transpeptidase PB2a.

MRSA infections are classified as either community-acquired (CA-MRSA) or hospital-acquired (HA-MRSA) (Lowy, 1998). HA-MRSA infections are common in hospitals and healthcare settings, particularly among patients receiving medical care and those with weakened immune systems. It is an emerging health problem, increasing from 2% to 63% (Chang et al., 2004).

CA-MRSA is prevalent in the community, particularly among individuals who have recently used antibiotics and those living in crowded environments such as

schools, markets, public vehicles, etc. (Mendes et al., 2015, Marasini et al., 2021). MRSA is a usual inhabitant in a healthy population. There is evidence of increasing community infection with the transfer of methicillin resistant strains from hospitals to the public (Turner et al., 2019; Kumar et al., 2011; Tolba et al., 2007). CA-MRSA may cause a variety of infections, including simple skin infections to more serious manifestations like necrotizing pneumonia, bone and joint infections as well as endocarditis (Sousa & Lancastre, 2004).

S aureus is leading normal flora of human colonized on the skin and nasal cavity. The anterior nares are primary habitat due to its moist squamous epithelium and leads to the dissemination of other sites of body (Aquino et al., 2012). *S aureus* colonizing in the nasal cavity, especially among health care workers and healthy individuals results in the spread of resistant CA-MRSA infections (Bharati & Padmaja, 2012, Von Eliff, 2001).

The organism has the presence of numerous virulent factors associated with the organism's capability to induce various infections. These components, which include α hemolysin (Hla), Panton valentine leucocidin *(pvl)* toxin, and toxic shock syndrome toxin-1 (*tsst-1),* are highly expressed, which facilitates bacterial attachment to tissues and immune system penetration, leading to toxicity and pathogenesis (Turner et al., 2019).

One strain of *S. aureus* produces an exotoxin known as *pvl.* It enhances transmission of infections and treatment resistance. It can lead to the lysis of human neutrophils and potentially result in recurrent skin infections. According to Bhatt (2016), *pvl* is one of the major staphylococci virulence factors that cause WBC cell necrosis and death.

tsst is superantigen, causing toxic shocked syndrome by stimulating the releasing large amount of interleukin 1 &2 and tumor necrosis factor. It is produced at the site of infection and enters the blood stream. Another superantigen staphylococcal enterotoxin, emetic toxin, responsible to cause food poisoning in human. It is pyogenic toxin, divided into 5 serological types *sea*A *to sea*E on basis of their antigenicity (Chakravorty, 2011).

Efflux pumps encoded on chromosomes, help to acquire resistivity to the antibiotics in *S aureus*. The main function of efflux pumps is to divert toxins & harmful compounds out of the cytoplasm or cell (Costa et al., 2013).

The self-secreted extracellular polysaccharide matrix (EPS) i.e. biofilm, performs as an effective barrier against antimicrobial agents. Biofilm helps to adhere to host cell and buries the bacterial cell from host immune cells. *S aureus* can produce biofilm on both abiotic and biotic environments, is its primary pathogenicity characteristic. Biofilm has significant consequences in medicine and their importance in human illness (Manandhar et al., 2018).

Different forms of currency, including banknotes and metal coins, are widely utilized for purchasing goods and services, acting as a common channel for the spread of microorganisms in the surroundings and between individuals (Tolba et al., 2007; Demirci et al., 2010). Paper currency often becomes contaminated by microorganisms due to factors such as handling by money counting machines, exposure to the atmosphere, accumulation of dust and soil, storage procedures, and regular usage. The unsanitary habits of individuals while handling currency, such as sneezing and coughing onto their palms, using saliva for counting notes, and inadequate hand washing after using the restroom, contribute to making currency a reservoir for various microorganisms found on human hands, in the mouth, and the gastrointestinal tract (Demirci et al., 2010; Marasini et al., 2021; Tolba et al., 2007).

Many studies reported currency as a reservoir of Gram-positive bacteria including staphylococci that arises from normal skin flora (Tolba et al., 2007; Kramer et al., 2006). The exchange of these contaminated notes through shopkeepers, vendors as well as bus conductors acts as a transmission vehicle of infections including antibiotic resistance strains and virulent genes in the community (Marasini et al.,2021, Demirci et al., 2010).

In developing countries like Nepal, people prefer to use paper money for commercial transactions rather than digital alternatives. The transfer of MRS strains from contaminated banknotes to individuals, and subsequently to

healthcare environments, may compromise infection control practices and aid in the spread of strains resistant to drugs leading to serious public health issues.

In Nepal, there are no strict regulations regarding the antibiotics use. This has also led to strains developing of resistance (Ucuncu et al., 2009). Lack of MRSA carriage screening among health care workers and healthy carriers contributed to profound transmission occurring not only in healthcare facilities but also within community settings. Effective management of MRSA necessitates a blend of interventions alongside robust organizational backing. Documented infection control practices proven to diminish MRSA transmission include proper hand hygiene, implementation of precautions for MRSA-positive patients, conducting active surveillance cultures, educational initiatives, thorough environmental cleaning, as well as proper management of patients with MRSA infections. Multiple strategies should be run either concurrently or subsequently (Siegel et al., 2007).

1.2 Objectives

1.2.1 General objective

To identify methicillin resistance and virulence genes in staphylococci isolated from nasal swab and paper currency carried by bus conductors of Kathmandu

1.2.2 Specific objectives

i. Isolation and identification of the *Staphylococcus aureus* and coagulase negative staphylococci from nasal swab and paper currency carried by bus conductors
ii. To determine methicillin resistivity among the isolates
iii. To detect *pvl, TSST-1* and *sea* genes

CHAPTER II

LITERATURE REVIEW

2.1 Staphylococci

Staphylococci are Gram positive cocci, which are non-motile, and non-spore measuring 0.5-1 μm in diameter. The cocci are typically grouped in grape-like clusters, occur individually, in tetrad pairs or in short chains. These facultative anaerobes grow under aerobic conditions at temperatures of 15-40℃. Skin, skin glands, mucus membrane and anterior nares are its primary colonization sites (Ucuncu, et al., 2009).

2.2. *Staphylococcus aureus*

According to Jawetz et al. (2010), the yellow pigment acts as a kind of protection against the antibacterial properties of sunlight. These bacteria thrive in nasal secretions and the skin because they prefer environments with high osmotic pressure and low moisture (Ryan and Ray, 2004). Their capacity for adaptation also makes it easier for them to develop in low and high-osmotic-pressure diets. (Ryan and Ray, 2004). *Staphylococcus aureus* is a common microbe that can be found in a variety of settings, such as environmental surfaces, domesticated animal nasal passages, and human body as a typical element of the microorganism. (Cimolai, 2008; Arjyal, 2015; Maina et al., 2012). Three types of *S. aureus* carriers can be identified: persistent, intermittent, and non-carriers (Dora, 2011).

S. aureus was initially identified in 1880 by surgeon Alexander Ogston, who elucidated its role in staphylococcal disease, sepsis, and abscesses (Ogston, 1984). Over a century later, *S. aureus* still poses a major threat, being a leading cause of hospital-acquired infections globally (Ogston, 1984). Infections were often severe in the early 1940s, with the mortality rate of around 80%. Despite its initial susceptibility to antimicrobials agents, the emergence of penicillin-resistant

strains in the mid-1940s marked a turning point. In the 1950s, a highly virulent penicillin-resistant clone known as the 80/81 strain emerged in Australia, leading to hospital outbreaks worldwide (Isbister et al., 1954). The incidence of *S. aureus* resistant to penicillin had increased by the middle of the 1950s to the point that penicillin was not a viable therapeutic choice anymore (Oliveira et al., 2002). This resistance spread globally for years to come. The development of methicillin in the 1960s represented a significant advancement in the fight against *S. aureus* infections. Methicillin, the first semi-synthetic penicillin derivative, was chemically altered to resist the effects of penicillinase, an enzyme that breaks down penicillin (Oliveira et al., 2002). However, methicillin-resistant *S. aureus* (MRSA) was first identified in 1961, the same year methicillin was introduced (Lowy, 2003).

2.3. Coagulase negative staphylococci (CNS)

CNS are Gram-positive bacteria that test positive for catalase and typically form irregular grape-like clusters. CNS being human normal flora inhabiting in skin and mucous membrane, causes infection. Most of the CNS are associated with clinical diseases. *S. epidermidis* is the most found species, constituting around 60-70% of CNS present on the skin. *S. saprophyticus* is responsible for up to 10% of uncomplicated UTI in young women.

2.4. *S. aureus* and Virulent factors

S. aureus possesses a variety of virulent factors like surface proteins, enzymes and toxins. These features allow the microorganism to be effective as a pathogen, that causes various diseases. Virulence factors help to adhere in host cell, immune system breakdown, sepsis, tissue invasion and cause toxin mediated syndromes.

This is the reason for staphylococcal infections that are classified according to their pathogenicity and mode of action and that last in a strong host (Greenwood et al., 2012). (Hennessy et al., 2015; Arvidson & Tegmark, 2001)

Staphylococci are clinically significant because of their high pathogenicity, which is mainly caused by a variety of surface proteins, toxins, and enzymes, including their capacity to quickly acquire resistance to drugs. These virulence genes enhance the pathogenicity and survival of MRS strains, making them a significant concern in clinical settings (Arvidson & Tegmark, 2001; Hennessy et al., 2015)

S. aureus has various virulence factors with different mechanisms

- Various surface proteins found in Staphylococci facilitate adhesion to the extracellular matrix.
- Toxic shock syndrome (*TSST*-1) Seb, sec with super antigenic activity
- Hemolysin- alpha cause cellolysis
- Lipoteichoic acid and peptidoglycan with interaction to Toll Like Receptor
- Protein A with interaction to Tumor Necrosis Factor- RI (INF-RL)
- Panton Valentin leucocidin with polymorph neutrophils lysis
- *TSST-1, pvl* collagen binding protein causes necrotizing pneumonia, *S. aureus* secrets several cytolytic toxins among then α, β and δ hemolysin and *pvl* are considered as important (Krishnamurthy et al., 2014)
- Exfoliatin B causes scalded skin syndrome

Panton Valentine Leukocidin (*pvl*), a common marker gene for CA-MRSA, causes minor to major skin infections (Havaei et al., 2010). The majority of these infections (75.5%) are brought on by microbes that produce *pvl.* This is because the leukocidin action gives the bacteria a survival advantage (Bhatta et al., 2016). *pvl* related diseases in human ranges from acute to mild and even resulting in a serious life-threatening infection (Miller et al., 2005). By the combinational mechanism of clonal expansion and horizontal gene transfer, the *pvl* gene spreads among Staphylococcal strains (O'Hara et al., 2008).

According to research, MSSA strains with *pvl* can transfer virulent elements to other MRSA strains often, acting as a reservoir for *pvl* and raising the risk to the public's health (Rasigade et al., 2010). *pvl* was more common among MRSA isolates in Nepal, particularly in pus samples. According to Bhatta et al., (2016),

this shows that *pvl* could be a major factor in pyogenic infections in community settings.

Toxic shock syndrome toxin (*TSST)*-1 and staphylococcal enterotoxin (SE) are considered superantigens out of more than 20 toxins found in Staphylococci. Common *seb* and *sea* enterotoxins are responsible for staphylococcus-related food poisoning (Regenthal et al.,2017). The Staphylococcal Enterotoxins (SEs) exhibit high toxicity in humans, requiring only minute quantities to induce toxic effects. Additionally, they exhibit exceptional resilience to extreme conditions, including low pH, heat and proteolytic digestion which allow them to remain active in the digestive system after ingestion. Food handlers infected with enterotoxigenic staphylococci are thought to be the main cause of contaminated food (Fisher et al., 2018).

Toxic shock syndrome (TSS) is thought to be caused by Staphylococci's Super Antigen (SAg), *TSST*-1. It functions by forming a cross-link between T cell receptors (TCRs) and Major Histocompatibility Complex (MHC) Class II molecules. Severe intoxication caused by SAgs then activates T cells, which causes TSS. TSS symptoms include fever, nausea, skin rash, diarrhea and complications such as multiple organ failure (Andrey et al., 2010; Tuffs et al., 2019).

2.5. Staphylococcal infections

S. aureus is an organism that inhabits the skin, the upper respiratory tract and gut mucosa. The organisms in carrier condition are present in the anterior nares of certain hosts under certain environments. It is one of the most dangerous pathogens, spreading and colonizing asymptomatically among 30% of healthy individuals due to its diverse biological properties.

It can lead to various skin and soft tissue infections, especially when the skin or mucosal barrier has been compromised. These organisms can be deadly pathogens

if they enter the bloodstream, lungs or heart causing bacteremia and infective endocarditis (Kwiecinski and Horswill, 2020).

An individual can get an infection through several different channels, such as touching an infected wound, coming into close contact with an infected person, or coming into contact with items that an infected person has used, including clothes, towels, etc. (Bash, 2023).

S. aureus can cause three main categories of infections: superficial lesions, toxicosis and systemic infections.

The most prevalent form of infection are Superficial lesions which may be characterized as vesicular pustules and crusting of the skin (impetigo) or sometimes cellulitis or focal with nodular abscesses (furuncles and carbuncles). In rare cases, Scalded Skin Syndrome can develop which is a serious complication, e.g. mastitis (Chakraborty, 2011).
S. aureus can cause toxicosis infection as well caused by the production of toxin like enterotoxin, a super antigen, that affects the intestine by inducing the secretion of large amounts of fluids and electrolytes resulting in muscular contractions, diarrhea and vomiting. (Boucher, et al., 2010).

A further kind of toxicosis infection is represented by TSS. The symptoms of this severe illness include vomiting, a high temperature, and muscular pains. Hypotension may ensue, which has the potential to be fatal (Chakraborty, 2011). As a community-acquired illness, toxic shock syndrome typically affects women, while it can also affect men (Boucher et al., 2010).

When *S. aureus* enters the bloodstream and spreads to other organs, it can cause systemic infections. Numerous severe infections, including bacteremia or sepsis, endocarditis, osteomyelitis, staphylococcal pneumonia, septic arteritis, and thrombophlebitis, may arise from this (Sousa and Lencastre, 2004).

2.6. Resistance to antimicrobial agents

Antibiotic resistance refers to the bacteria’s ability to sustain the work of antibiotics, either bacteriostatic or bactericidal. Excessive misuse of antibiotics,

and horizontal & vertical gene transfer are significant on the rise of resistance on most antibiotics. The rising resistance to a wide range of drugs by staphylococci represent a significant threat to treatment efficacy. The drugs available to treat staphylococcal infections have been reduced due to developed resistance to the most active antimicrobials, particularly beta-lactams (Goldstein, et al, 2007). The resistivity of organism is caused by the gene. A few genes have continued to evolve and mutate due to modern medicine therapy. The gene of bacteria can be transferred through plasmid, a mobile genetic material.

The organism becomes resistant to the antimicrobial agents because of bacterial cell wall permeability, active efflux of antibiotic through the bacterial cell, enzymes that degrade antibiotic becomes ineffective, the formation of metabolic pathway that inhibit medication etc. (Goldstein et al., 2007).

2.6.1. Penicillin resistance

Penicillin was first introduced for medical treatment purposes in 1940. Since then, the resistivity against antibiotics *S. aureus* has been developing. Penicillin is rendered ineffective by penicillin-binding proteins (PBPs) present within the bacterial cell wall. (Topley, et al., 1990).

The discovery of penicillin and its ability to cure disease was accepted worldwide. Soon after its use as antibiotic, penicillin resistance strains emerged, mediated by the *blaZ* gene, codes for β-lactamase enzyme. (Lowry, 2003).

2.6.2. Methicillin resistance and its mechanism

Methicillin is a semi synthetic derivative of penicillin. To acquired resistant to methicillin is another important β lactam drug resistance mechanism in staphylococci. Methicillin resistant staphylococci infections are significantly increasing and acquiring resistance for many other widely used antibiotics. The MRSA strains which had an average resistance to Vancomycin have been reported recently, which is a concern for treatment options for serious MRSA infections. (Tiemersma, et al., 2004).

The penicillin-binding protein PBP2a is responsible for methicillin resistance (Yadav et al., 2012). It is coded by the *mec*A gene. It is a housed of staphylococcal cassette chromosome *SCC*mec elements.

2.7. Methicillin resistant *S. aureus*

MRSA has certain factors or genetic traits that make them more potent and virulent. (Lowy et al., 2008). Treating complicated diseases can be challenging because of multiple antibiotics resistance, and currently, the vaccine is not available (Cheng et al., 2004). Beckham launched methicillin in 1959. In 1960, after less than a year of methicillin use, Professor Patricia Jevons first observed methicillin-resistant *S. aureus*. He found that methicillin is no longer effective against *S. aureus.* A year later in 1961 the British Medical Journal recognized Professor Jevons as the founder of MRSA (British Medical Journal, January 14, 1961). Then, strains began to show elevated (MIC) values for methicillin in UK hospitals. Within 2 years, invasive infections were identified in Denmark which were the first 1960s epidemic clones. Then after, many countries like Japan, Australia, U.S have reported MRSA. In 1974, there were only 2 % of MRSA present in the US, but in 1995 it rose to 22% and 63% in 2004. In the first two decades, it increased by 20% and then by 40% in just a decade later (Leslie et al., 2008). MRSA infections start with HA-MRSA and continue with CA-MRSA in the US which then occurred worldwide (Boucher et al., 2008).

2.7.1. Hospital acquired MRSA

In hospitals and health care center HA-MRSA infections frequently occur where patients are under invasive medical treatment and have weak immune responses. These types of infections are rapidly growing as life threating infections and increased from 2% to 6% of all Staphylococcal infections from 1974 to 2004 (Chang et al., 2004). Many researchers demonstrated that MRSA from patients and health care workers could transmit to the community or the environment (Siddiqui & Koirala, 2023).

2.7.2. Community acquired MRSA

In 1996 CA-MRSA was first reported at the Minnesota Department of Health in drug users. It was the first case without a traditional healthcare association. Four deaths were reported from 1997 to 1999. People in other communities like prisoners, soldiers and athletes, were also infected (CDC 1999).

CA-MRSA is prevalent in non-hospital community settings as well (Ellis et al., 2004). It is linked with individuals who have recently used antibiotics and those in dense environments like schools. It is also transmitted among passengers who travel in local buses, micro-buses (Angbuhang et al., 2018) and trains (Mendes, 2015). As the organisms are inhabiting in the skin, MRSA are known to survive for long periods on dry surfaces touched by humans. MRSA and CNS present in paper currencies pose a high risk to people handling them as well as to those in the community (Marasini et al., 2021).

CA-MRSA is generally more treatable than healthcare-associated MRSA (HA-MRSA), yet it exhibits higher virulence (Ellis et al., 2004). CA-MRSA harbors the Pantone Valentine Leukocidin (*pvl*) virulence genes, which can lead to necrotizing infections in the skin and lungs. Individuals of any health status, including those without recent hospitalizations, can contract CA-MRSA infections, with young people being particularly susceptible (Richard, 2010). Unlike HA-MRSA, CA-MRSA lacks the pvl gene however it carries other Staphylococcal cassette chromosomes. CA-MRSA can cause a range of community-acquired infections, including boils, folliculitis, cellulitis including more severe infections to major organs which can result in death. Most MRSA infections are now community-associated, representing a shift from previous associations solely with healthcare settings like hospitals (CDC, 2005).

Recent studies have reported that spread of MRCNS in community have increased because of MRCNS acting as a source of *SCC*mec for CA-MRSA and associated diseases. In Brazil 38% of MRCNS isolates were reported and 86% were multi drug resistance (Aquino et al., 2012*). Staphylococcus epidermidis* and other CNS

species are prominent agents of the human skin and mucosa (Lebeaux et al., 2012). (Abimanyu et al., 2013). This indicates a significant proportion of *S. epidermidis* strains are methicillin resistant.

2.8. *S. aureus* and MRSA carriage in Nasal Cavity

S aureus colonizing in the nasal cavity of health care workers plays a crucial role in the development of resistant CA-MRSA. The rising prevalence of *S. aureus,* including MRSA, in the nasal passages of patients and hospital staff leads to the rapid spread of these bacteria throughout various hospital environments. This, in turn, contributes to the quick colonization of the nasal passages and skin of other individuals visiting hospitals. The anterior nares of humans are the primary site for *S. aureus (*Peacock, et al., 2001; Giri et al., 2021).

The prevalence of nasal *S. aureus* among healthcare workers ranges widely (Ahmed et al., 2013, Bharati and Padmaja, 2012). From a clinical sample in Nepal, presence of *S. aureus* isolated is 34.5%, multi-drugs resistance is 57.1% and MRSA accounts for 41.7%. Whereas inducible clindamycin resistance is 35%. (Shrestha et al., 2021). Similar study conducted by Giri et al., (2021) reported that 5.2% of healthcare workers were carrying nasal staphylococci asymptomatically, where *S aureus* among healthcare workers 14,7% and MRAS is 35.3%. Thus, the nasal bacteria being a risk factor for infections as well as transmission. The number of MRSA infections is high and remains a major risk to patients, especially when they are hospitalized (Shanmugam and Gopal, 2008).

The Canadian Nosocomial Infection Surveillance program reports that in 2003, the rate of MRSA infection rose from less than 1% to 10%. Since 20% of infections result in death, HA-MRSA infections are a major source of medical as well as public concern worldwide. An estimated 126,000 people are hospitalized annually in the United States alone as a result of MRSA-related infections (Dey et al., 2013).

Historically, hospital environments have been the only places where epidemic MRSA isolates have spread (Thomson et al., 1982). Globally, the prevalence of nasal carriers among adult community has been reported at between 0.8% to 3.0 % MRSA carriage rate (Giri et al., 2021; Khanal et al., 2015; Jernigan et al., 2003).

The nasal carriage rate of MRSA among healthcare professionals in Nepal has not been extensively studied. However, in medical centers in Kathmandu, previous research has found considerable prevalence of MRSA among patients and personnel. In conducted research, for example, patients, staff, and the hospital environment had a 29.1% MRSA prevalence (Rai et al., 1990) in a teaching hospital in Kathmandu. This emphasizes how hospital atmosphere contributes to the spread of MRSA within healthcare facilities.

In a different study, Rai and Pant found that among medical staff at a medical college teaching hospital in Kathmandu, nasal carriage rates were 43.8% (Pant et al., 2007).

More recently, a study conducted in a hospital in Birgunj, found an MRSA carriage rate of 8% among patients, visitors/patients' attendants, and health personnel. This study also revealed the highest nasal colonization rate (25%) and MRSA prevalence rate (10%) among healthcare workers, followed by visitors/patient attendants (8.2%) (Dimitrov et al., 2003).

2.9. Multi drug resistance

Bacteria that are resistant to at least one antimicrobial agent in three or more classes of antibiotic, based on sensitivity patterns of the isolates are identified as Multi drug Resistant (MDR) (Magiorakos et al., 2012). MDR staphylococci have been increasingly reported worldwide, including methicillin-resistant staphylococci (Godebo et al., 2013).

The recent rise in resistant organisms can be attributed to several factors, including improper dosing, prescription of drugs without proper susceptibility testing, self-medication practices, and prolonged hospitalizations. The MDR in bacteria can be induced by the activity of multidrug efflux pump, which is encoded on chromosomes, helping to acquire resistivity to the antibiotics in *S aureus*. The main function of efflux pumps is to divert or remove toxins & harmful compounds, out of the cytoplasm or the cell (Costa et al., 2013).

2.10 Paper currency and transmission of staphylococci

Amid concerns over the potential transmission of pathogens through fomites, paper currency has garnered attention as a potential vehicle for the dissemination of MRSA (Demirci et al., 2020). Paper money, like any other surface, has the potential to harbor various microorganisms. However, the transmission of MRSA through paper money is likely to be less common relative to direct transmission through personal contact.

Several studies have also shown the presence of diverse bacterial species on paper money including both potentially harmful and harmless bacteria. *S. aureus* as well as other Gram-positive cocci were also frequently encountered, along with other fungi (Demirci et al., 2020; Marasini et al., 2021; Girma, 2014).

It was also reported in 2021 that *S aureus* presence in paper currency was 24.2% followed by CNS 22.09%, *Diphtheroid* 16.3%, *Bacillus spp*. 15.11%. Another study conducted by Girma et al., (2014) reported predominate isolates of bacterial groups are *S. aureus* 34.6% followed by *Bacillus* spp. 31.38% Enterobacteriaceae 13.39% and *Micrococcus* 9.55%. Similarly, Allan (2018) reported 45% of *S. aureus* are isolated from Ugandan paper currency.

Several studies have investigated the prevalence of MRS on paper currency, with varying results depending on the geographical location and sampling techniques employed. These investigations have detected the presence of MRSA and

MRCNS on banknotes, highlighting the potential for contamination. The prevalence rates reported in these studies range from a few percentage points to over 90%, indicating the need for further research and standardization of sampling protocols (Maritz et al., 2017).

The presence of organic material, temperature, and humidity significantly influence the survival of MRS on banknotes. Studies have reported viable MRS on banknotes for extended periods, with survival times ranging from hours to several weeks. Such findings raise concerns about the persistence of MRS on paper currency and subsequent transmission potentially (Tolba et al., 2007). MRSA transmission from paper currency to individuals is a complex process influenced by multiple factors. Direct contact with contaminated banknotes, particularly when hands are not properly sanitized, has been identified as a primary route of transmission. Additionally, another source of spread is hand-to-mouth contact following the exchange of contaminated currency during commercial transactions (Tolba et al., 2007; Demirci et al., 2020).

Widespread contamination poses a significant risk of serious public health disasters. Transmission of MRS strains from contaminated banknotes to individuals, and subsequently to healthcare environments, may compromise infection control practices and lead to more drug-resistant strains. However, only limited research has been done on antibiotic resistant strains and virulence genes of staphylococci as *pvl*, *tsst-1* and *sea* in paper money.

Genotypic detection of *pvl*, *sea* and *tsst*-1 genes are carried out by polymerase chain reaction (PCR) amplification method, which is regarded as ‘gold standard’ method for identifying these special genes. This method can be performed by conventional PCR either Uniplex or multiplex (Abimanyu et al., 2013). Various other methods can be used for gene detection such as Sanger’s sequencing, pyrosequencing, iron conductor sequencing etc. (Aquino et al., 2012).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

The equipment, reagents, culture media, antibiotic and biochemical required for the study were listed on appendix.

3.2 Methods

3.2.1 Study design

A cross-sectional study was performed in the Department of Microbiology, Tri-Chandra Multiple Campus and the Institute of Research in Science and Technology Kathmandu, Nepal from July 2023 to December 2023. A total 100 samples (50 nasal swab and 50 paper currency) were collected from bus conductor from different area of Kathmandu. Standardized questionnaire was used to interview all participants to gather detailed information about their clinical histories and demographics.

3.2.2. Ethical consideration

The research has got approval from the Institute for Science and Technology (Registration number: IRCIOST- 23- 0065.

3.3. Sample collection

3.3.1. Collection of nasal swabs

The sterile cotton swab moistened in normal saline was used to collect nasal swab by inserting 2-3 cm into nasal cavity. Then the swab was rotated clockwise and anticlockwise direction before withdrawal. The same cotton swab was used for sampling the both nostrils. The swab was then immediately placed inside a labeled screw-capped tube containing 5ml peptone water and brought to the laboratory.

3.3.2. Collection of paper currency

Similarly, Nepali Rs. 5 and Rs 10 were selected for the study and the participants were instructed to dip the currency into labelled screw capped tube containing normal saline and transported to the laboratory as same.

3.3.3. Sample processing

Nasal swab samples were directly inoculated on Mannitol Salt Agar and aerobically incubated at 37℃ for 24 hrs.

Similarly, the money samples were vortexed for 5 minutes and spin 5 mins at 2000rpm. Then 50ul pellet was enriched with TSB containing 7% sodium chloride and incubated aerobically for 24 hrs. at 37℃. After 24hrs a loopful of sample was aseptically inoculated on MSA and aerobically incubated at 37℃ for 24 hrs.

3.3.4. Identification of *S aureus* and Coagulase- negative staphylococci

The standard biochemical techniques and conventional methods were followed for identification of *S. aureus* and CNS.

The colony showing yellow and pink colored colonies on MSA were preliminarily chosen as *S. aureus* and CNS respectively. Then subculture on nutrient agar and incubated at 37℃ for 24hrs. The golden yellow color colonies on nutrient agar having round, smooth, convex with a diameter 2-3mm were indicative as staphylococci. Further phenotypic identification of *S. aureus* and coagulase negative staphylococci (CNS) were done by Gram's staining, suspected organism was observed as Gram positive cocci in cluster and different biochemical tests were performed to confirmed *S. aureus* and CNS as catalase, oxidase, O/F test, DNase test. A coagulase test (tube and slide) was performed to differentiate coagulase positive and negative staphylococci. The procedure of biochemical tests are given in appendix III

3.3.5. Antibiotic susceptibility test (AST)

The modified Kirby- Bauer disc diffusion method and CLSI guidelines 2015 were followed to perform AST. The antibiotic disc such as gentamycin, erythromycin, clindamycin, ciprofloxacin, tetracycline, penicillin, co-trimoxazole, amikacin, meropenem, and ofloxacin were used. The concentration of antibiotic disc is given in appendix v.

3.3.6. MDR Analysis

Multi-drug resistant was determined as the isolate resistance to one antibiotic in three or more classes of antibiotics based on zone of inhibition patterns of isolates (Gedebo et al., 2013).

3.3.7. Detection of MRSA and MRCNS

All isolates were performed for MRSA screening using 30ug cefoxitin on Mueller Hinton Agar. The zone size was interpreted according CLSI guideline. The inhibition zone ≤ 21mm was identified as MRSA and MRCNS (Raut et al., 2017).

The identified isolates were preserved for further testing.

3.3.8. Extraction of DNA

DNA from all identified isolates were extracted by the boiling techniques. Overnight culture bacterial suspension was centrifuged at 14000rpm for 5min and the pellet was collected. The pellet was suspended in 100ul 0.1M TE buffer and gently vortexed. Then 20ul 25mg/mi lysozyme was added and incubate at 37 ℃ for 10min. After incubation it was boiled at 100℃ for 10 min and centrifuged at 12000rpm for 5min. The supernatant was collected in new sterile Eppendorf tube (1.5ml) and purified by adding equal volume of chilled ethanol. After purification the pellet was resuspended in 50ul TE buffer and stored at -20℃ until use. (Hoveida et al., 2020).

3.3.9. PCR Amplification

Specific primers used to amplify the *tsst*, *sea*, *pvl* genes were given in (Table 1). The PCR amplification was conducted out in a total volume of 15ul, which contained 2.5ul DNA, 7.5ul Master Mix PCR (Taq2x master mix, New England Bio-labs ins. Company, England), 0.45ul each primer (10pmol) (the forward and reverse). Finally, 4.1ul nuclease free water was added to a tube total volume 15ul. The thermal cycling conditions were performed according to type of gene amplified with the protocol (table 2). The PCR products were electrophoresed in a 1.75% agarose gel and stained with ethidium bromide (0.5ug/MM).

Table 1. Primer sequence used for amplification of *tsst, sea* and *pvl* gene

Target genes	**Primer Sequence**	**Primer Size (bp)**	**References**
Pvl	F: 5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3' R: 5'-GCATCAAGTGTATTGGATAGCAAAAGC-3'	433	Karmakar et al, 2018
tsst1	F: 5'-CTGGTATAGTAGTGGGTCTG-3' R: 5'-AGGTAGTTCTATTGGAGTAGG-3'	271	Koosha et al, 2016
Sea	F: 5'-TTGGAAACGGTTAAAACGAA-3' R: 5'-GAACCTTCCCATCAAAAACA-3'	120	Johnson W.M et al, 1991

Table 2. The specific genes were amplified by following standard protocol

PCR Conditions					
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Final Extension**	**Number of Cycles**
94°C, 3 minutes	94°C, 30 seconds	55°C, 45 seconds	72°C, 45 seconds	94°C, 3 minutes	36
94°C, 5 minutes	94°C, 1 minutes	54°C, 2 minutes	72°C, 1 minutes	72°C, 7 minutes	40
94°C, 5 minutes	94°C, 1 minutes	54°C, 2 minutes	72°C, 1 minutes	72°C, 7 minutes	40

3.4. Quality control

3.4.1 Routine inspection of laboratory equipment, reagents and media

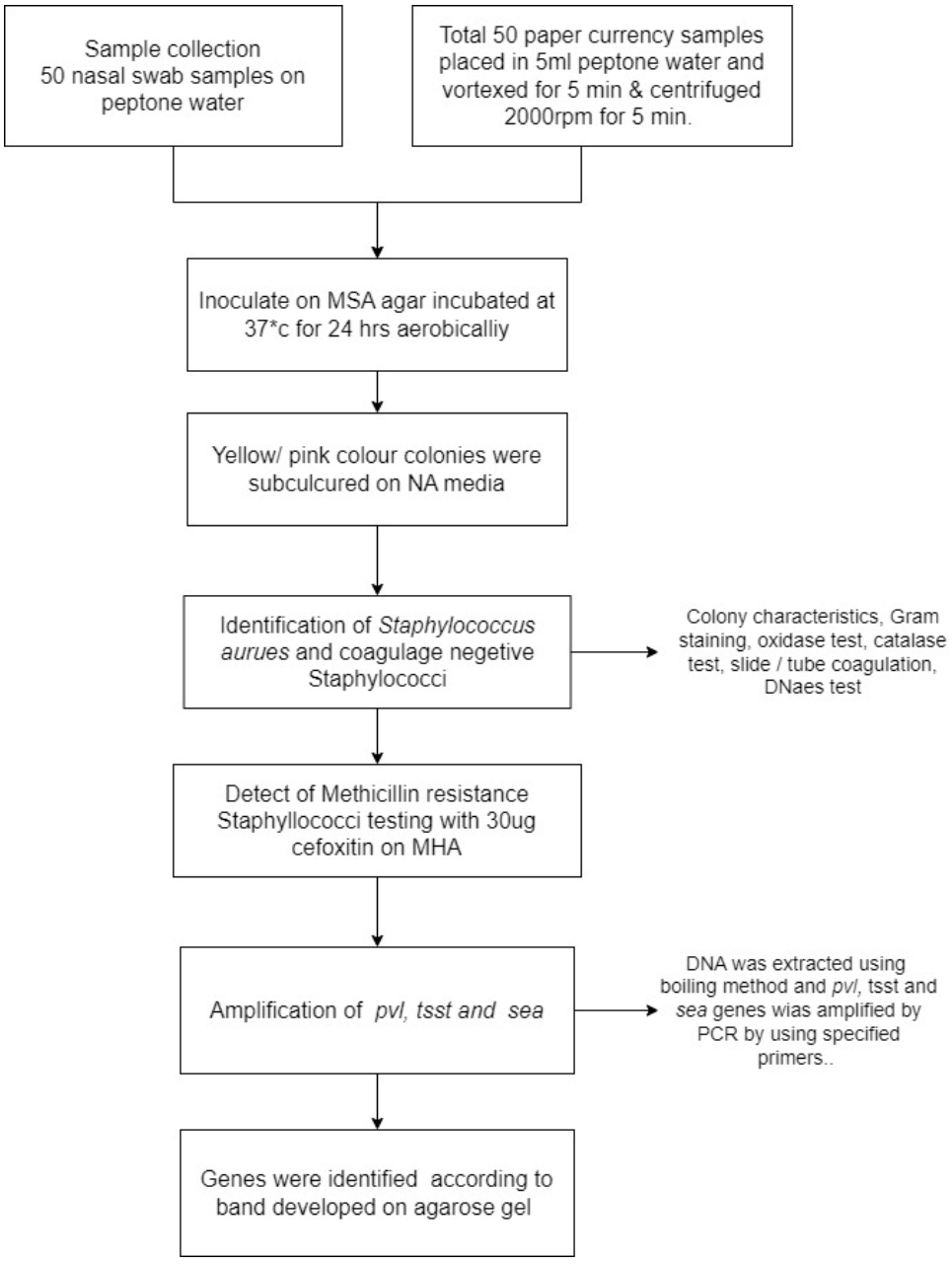
All equipment used in the laboratory underwent routine inspections to ensure all tests were following standard procedures. Periodically, expiration dates as well as storage needs of reagents and media were confirmed.

3.4.2 Quality control during isolation and identification

During the identification the pure culture of isolates were used. Freshly prepared reagents and plasma were used to performed biochemical tests. The expiry dates of antibiotic disc were also checked.

3.5 Data analysis

All the data is presented on percentage, bar graphs and pie charts and the data obtained were statically analyzed by using GraphPad Prism. P-value of 0.05 was regarded as significant result.



Flow chart showing for processing and detection *tsst, sea* and *pvl* genes of isolates.

CHAPTER- IV

RESULTS

4.1 Distribution of Staphylococci isolate from nasal swab

A total of 100 samples were collected from the bus conductors from different places in Kathmandu. Out of the 50 nasal swab samples, a significant number were identified as CNS that counts 25 (50%) and 8 (16%) as *S. aureus* and 17 (34%) were others.

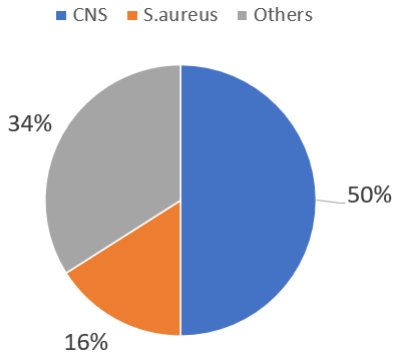


Fig 1: Pie chart showing Staphylococci in Nasal Swabs

4.2 Distribution of Staphylococci isolate from paper currency

Similarly, out of 50 paper currency samples, the higher number identified as *Staphylococcus aureus* was 21 (42%) and 11(22%) as CNS and 18 (36%) isolates were others.

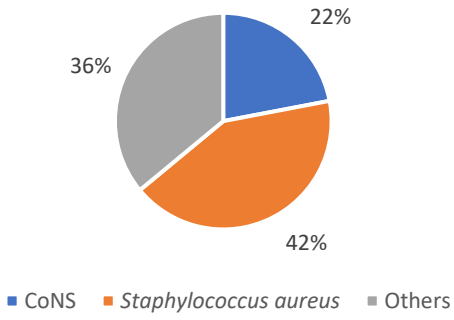


Fig 2: Pie chart showing Staphylococci in Paper currency

4.3. Percentage of MRSA and MRCNS in nasal swab

Out of 25 CNS, 14 (56%) were identified as MRCNS and 11 (44%) as MSCNS. Similarly, among 8 isolates of *S. aureus*, 4 (50%) identified as MRSA and 4 (50%) as MSSA.

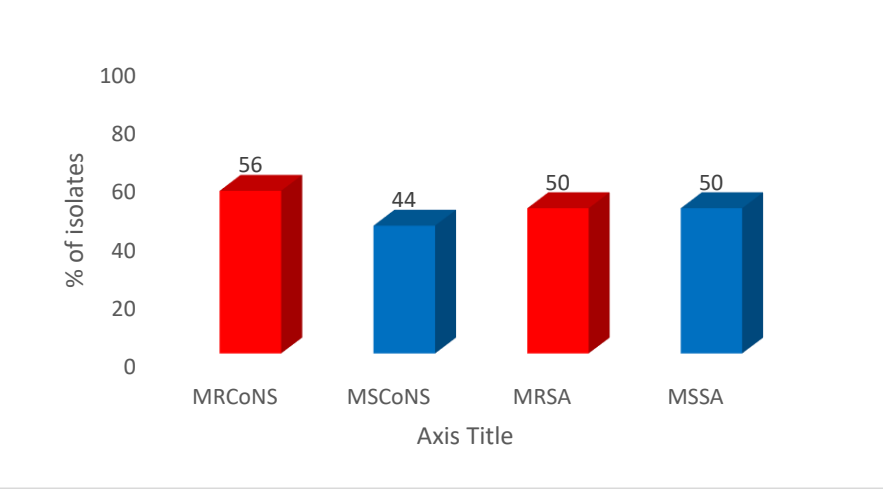


Fig 3: Bar Graph showing MRSA and MRCNS in Nasal swab

4.4. Percentage of MRSA and MRCNS in Paper currency

Out of 21 *S. aureus* isolates, 7 (33.3%) were identified as MRSA and 14 (66.6%) as MSSA and all 11 CNS were identified as MRCNS.

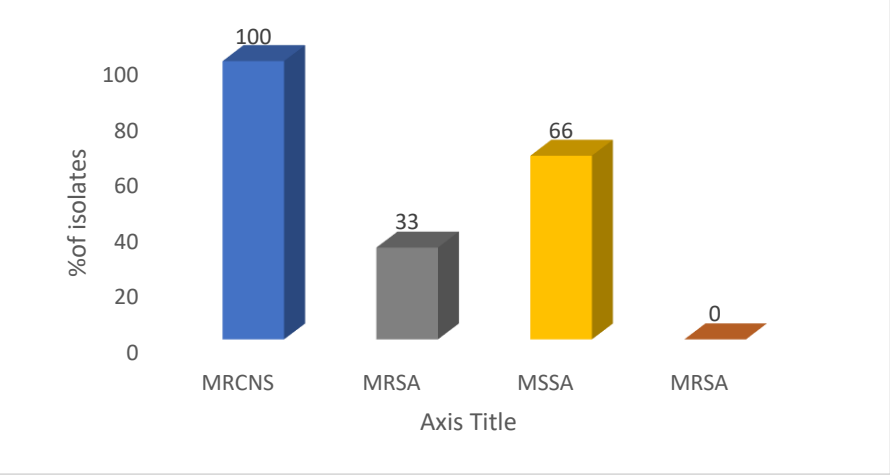


Fig 4: Bar Graph showing MRSA and MRCNS in Paper currency

4.5. Age-wise distribution of MRSA and MRCNS in nasal swab and paper currency carried by bus conductor

Higher prevalence of MRSA and MRCNS isolates from nasal swab were identified from age group 15-30. Out of 4 MRSA isolates 3 (75%) and out of 14 MRCNS isolates, 11 (78.6%) were identified from age group 15-30.

Similarly higher prevalence of MRSA and MRCNS isolates were identified from paper currency carried by bus conductor of age group 15-30. Out of 7 MRSA isolate, 5 (71.4%) and out of 11 MRCNS isolate 8 (72.7%) were identified from age group 15-30.

Table 3: Age-wise distribution of MRSA and MRCNS in nasal swab and paper currency carried by bus conductor

	Nasal Swab			Paper currency		
Age	MRSA (n=4)	MRCNS (n-14)	p-value	MRSA(n=7)	MRCNS(n=11)	p-value
15-30	3(75%)	11(78,6%)	>0.05	5(71.4%)	8(72.7%)	>0.05
31-45	1(25%0	3(21.4)		2(28.6%)	3(27.2%)	

4.6. Antibiotic susceptibility patterns of MRSA &MRCNS of nasal swab

All MRSA and MRCNS isolated from swab nasal samples were 100% resistance to penicillin but sensitive to chloramphenicol and meropenem. The MRSA were also sensitive to amikacin and ofloxacin.

Table 4. AST patterns of MRSA & MRCNS of nasal swab

	MRSA (n=4)		MRCNS (n=14)	
Antibiotics	Resistance n(%)	Sensitive n(%)	Resistance n(%)	Sensitive n (%)
Gentamycin	1 (25%)	3 (75%)	1 (7%)	13 (92.8%)
Penicillin	4 (100%)	-	14 (100%)	-
Chloramphenicol	-	4 (100%)	-	14 (100%)
Erythromycin	1 (25%)	3 (75%)	10 (71.4%)	4 (28.6%)
Clindamycin	2(50%)	2(50%)	2 (14.3%)	12 (85.7%)
Tetracycline	1 (25%)	3 (75%)	3 (21.4%)	11 (78.6%)
Co-trimoxazole	2 (50%)	2 (50%)	3 (21.4%)	11 (78.6%)
Amikacin	-	4(100%)	1 (7%)	13 (92.8%)
Meropenem	-	4(100%)	-	14(100%)
Ofloxacin	-	4(100%)	2 (14.3%)	12 (95.7%)
Ciprofloxacin	1 (25%)	3 (75%)	3 (21.4%)	11 (78.6%)

4.7. Antibiotic susceptibility patterns of MRSA & MRCNS of paper currency

MRSA & MRCNS isolated from paper currency showed 100% resistance to penicillin but 100% sensitive to amikacin, chloramphenicol, ciprofloxacin, gentamycin, meropenem and ofloxacin.

Table 5: AST patterns of MRSA & MRCNS of paper currency

	MRSA (n=7)		MRCNS (n=11)	
Antibiotics	Resistance (n%))	Sensitive (n%)	Resistance (n%)	Sensitive (n%)
Gentamycin	-	7 (100%)	-	11 (100%)
Penicillin	7 (100%)		11 (100%)	-
Chloramphenicol	-	7 (100%)	-	11 (100%)
Erythromycin	4 (57%)	3 (42.9%)	9 (81.8%)	2 (18.2%)
Clindamycin	1 (14.3%)	6 (85.7%)	1 (9.9%)	10 (90.9%)
Tetracycline	1 (14.3%)	6 (85.7%)	1 (9.9%)	10 (90.0%)
Co-trimoxazole	3 (42.9%)	4 (57%)	6 (54.5%)	5 (45.5%)
Amikacin	-	7(100%)	-	11 (100%)
Meropenem	-	7(100%)	-	11(100%)
Ofloxacin	-	7(100%)	-	11(100%)
Ciprofloxacin	-	7 (100%)	-	11 (100%)

4.8. MDR pattern among MRSA and MRCNS

All MRSA isolates from nasal swab were MDR but out of 11 MRCNS, 9 (81.8%) isolates were MDR. Similarly, all MRCNS and 4(57.1%) MRSA from paper currency, were MDR.

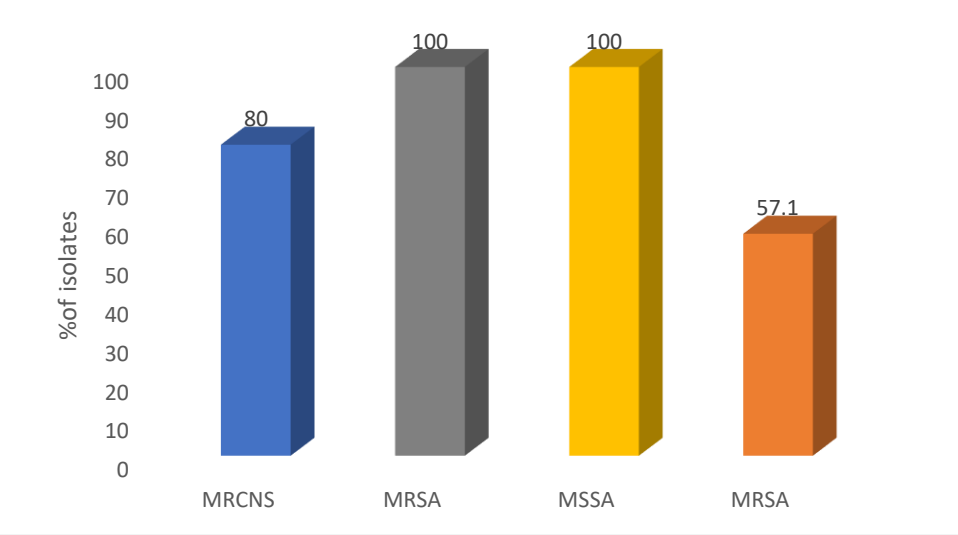


Fig 5: Bar Graph Showing MDR on MRSA and MRCNS isolates

4.9. Detection of *tsst* gene among MRS isolates

The *tsst* gene was amplified in all isolates. Among them,19 MRS isolates harbor *tsst* gene. Out of 19 isolates, 15 isolates of paper currency sample and 4 isolates of nasal swab sample showed *tsst* gene. The highest *tsst* gene was identified from paper currency sample.

Table 6: Prevalence of *tsst* gene among MRS isolates

Sample	*tsst* positive	*tsst* negative	total	p-value
Paper Currency	15 (41.7%)	3 (8.3%)	18	<0.05
Nasal Swab	4 (11.1%)	14 (38.9%)	18	
Total	19 (52.8%)	17(47.2%)	36	

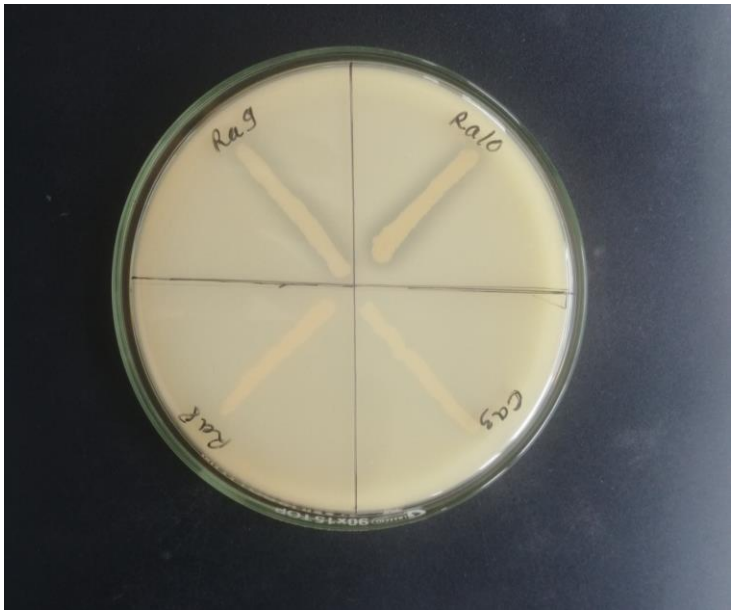
4.10. Detection of *sea* gene among MRS isolates

The *sea* gene was amplified in all isolates. Among them 18 isolates showed *sea* gene. Out of 18 isolates, 12 isolate of paper currency sample and 6 isolate of nasal swab sample showed *sea* gene. The highest *sea* gene was found in MRS isolated from paper currency.

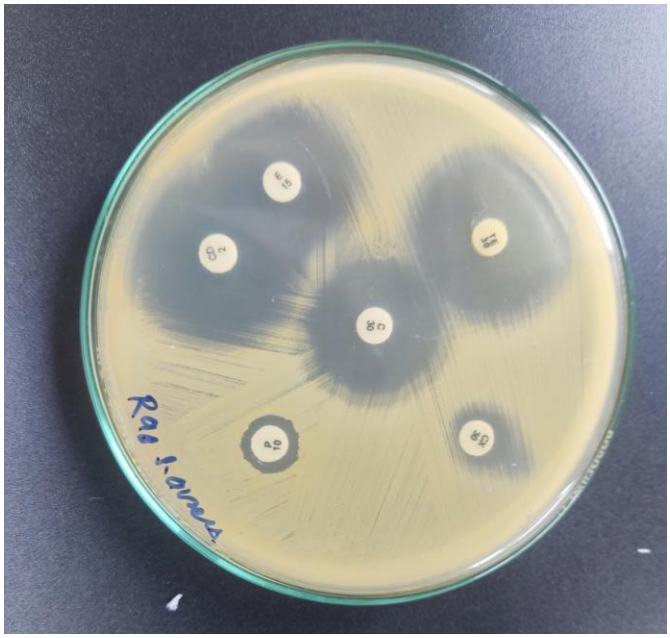
Table 7: Prevalence of *sea* gene among MRS isolates

Sample	*sea* positive	*sea* negative	total	p-value
Paper Currency	12 (33.3%)	6 (16.7%)	18	<0.05
Nasal Swab	6 (16.7%)	12 (33.3%)	18	
Total	18 (50%)	18 (50%)	36	

Furthermore, all MRS isolates, isolated from paper currency and nasal swab were *pvl* gene negative.



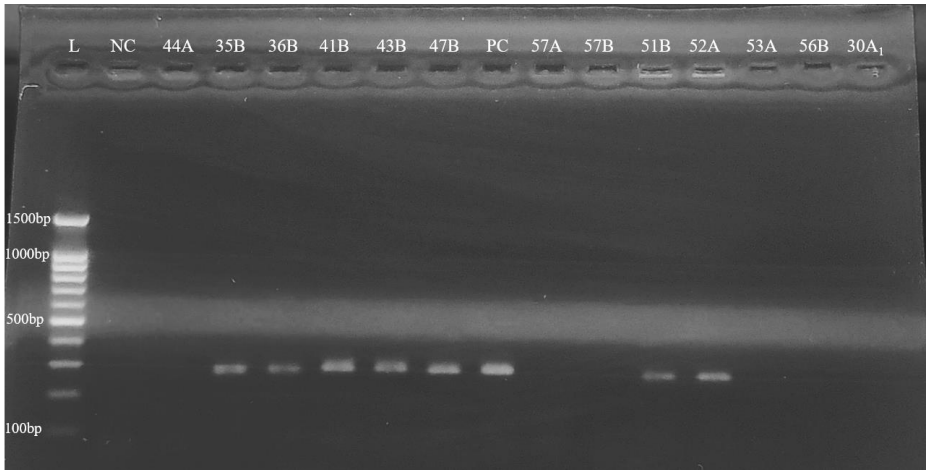
Photograph 1: The clear zone around the growth shows DNase positive



Photograph 2: Antibiotic susceptibility of MR Staphylococci

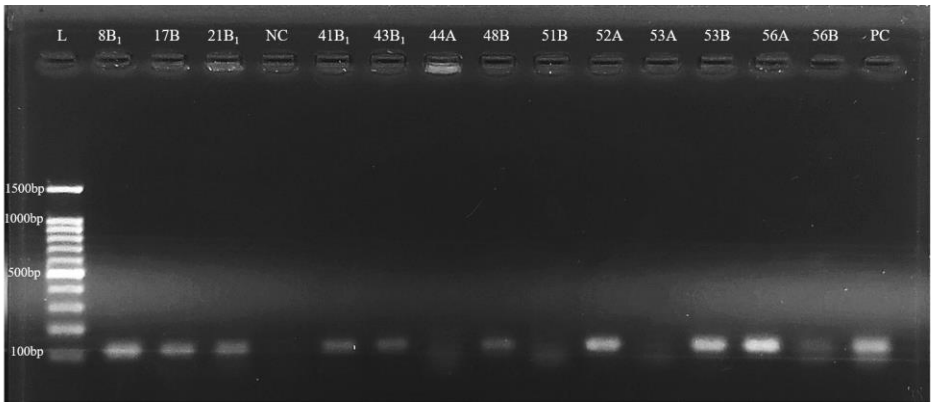
Resistance: Cefocitin CX (30 µg), Penicillin P (10 Unit)

Sensitive: Clindamycin CD (2 µg), Erythromycin E (15 µg), Chloramphenicol C (30 µg), Tetracycline TE (30 µg)



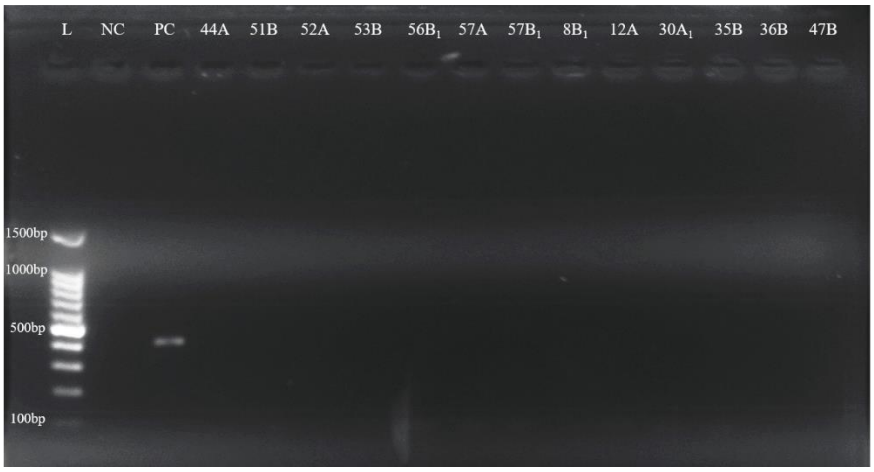
Photograph 3: Visualization of amplified *tsst* gene under UV illuminator

L-100 bp DNA ladder, lane NC-negative control, lane 44A-negative, lane 35B,36B,41B,43B,47B-positive, lane PC-positive control, lane 57A,57B-negative, lane 51A,52B-positive and lane 53A,56B,30A1-negative.



Photograph 4: Visualisation of amplified *sea* gene under uv illuminator

L-100 bp DNA ladder, lane 8B1,17B,21B1-positive, lane NC- negative control, lane 41B1,43B1-positive, lane 44A51B,55A56B-negative, lane 52A,53B,56A,-positive and lane PC-positive control.



Photograph 5: Visualisation of amplified *sea* gene under uv illuminator

L-100 bp ladder, lane NC- negative control, lane PC- positive control and rest of other lane shows negative for *pvl* gene.

CHAPTER- V

DISCUSSION

Staphylococci are a highly infectious agents involved in community as well as hospital illness. A total of 100 samples (50 nasal swabs and 50 paper currency) were processed. Significant growth of CNS was isolated from nasal swab which is in concordance with Shiff et al., (2013), carried out in blood sample, but lower percentage was reported by Khabri & Alzohozt, (2010) than the current study. Similar research outlined by Marasini et al., (2021) in Pokhara and Angbuhang et al., (2021) in Kathmandu reported high prevalence rate of CNS from human touched surfaces. Colonization of various parts of the body and mucous membranes of host is a primary source of endogenous infection increases.

In paper currency, a higher number *of S. aureus* (42%) were isolated. Comparable studies were outlined by Shrestha et al., (2021) and Raut et al., (2021) Kandel et al., (2020). Tyagi et al., (2021) Sunil et al., (2020) had also reported higher prevalence of *S. aureus* from money circulating in market. Currencies, especially paper money and coins, is a common element of daily life and is routinely handled by several people. The frequent interchange of paper currency among people facilitates the spread of organisms.

The higher number of MRCNS were identified from nasal swab than MRSA. which was acceptable with Acquino et al., (2002), Mir (2013) Giri et al., (2021). Bharati et al., (2012) also announced high prevalence of MRCNS in healthy individuals. MRCNS have emerged as important causative agents, being main colonizer of anterior nares & skin, have acquired and integrated into their genome *SSC*mec.

The highest percentage (100%) of MRCNS was present in paper currency. Same type of study was outlined by Angbuhang et al., (2021), Marasini et al., (2021) in Pokhara from money circulating in market, Kumar, (2009) and Sunil et al., (2021) reported higher prevalence of MRCNS from paper currency. High frequency

associated with the methicillin resistance CNS might be its ubiquitous nature and the unhygienic practice of human. The composition of paper notes (cotton-based paper notes) also plays a significant conducive environment for colonization numerous resistant bacteria.

In this study, higher prevalence of MRSA and MRCNS was identified from nasal swab and paper currency from bus conductors from age group 15-30. From this distribution, the presence of MRSA and MRCNS was notably high among the individuals aged 15-30 compared to 31-45 age group. This study suggests that younger individuals in this age group might be more susceptible to colonization by MRS. As the age increased a shift in the distribution of staphylococci was observed. Similar study was carried out by Kluytmans et al., (2006) and reported that prevalence of CA-MRSA was higher in younger individuals compered to older age groups. The potential age group difference in the distribution of MRS might be influenced by factors like immune response, environmental exposure and other individual specific characters.

Antibiotics sensitivity pattern of MRSA isolated from nasal swab and paper currency samples were found 100% resistance to penicillin. Ansari et al., (2014), Manandhar et al., (2021), Giri et al., (2021), Khatri et al., (2007) announced that MRSA were resistant to penicillin which was like the current study. MRSA strain carries mobile genetic element *mec*A, encoded in penicillin binding protein (PBP2a) that helps to avoid the inhibitory effects of the β- lactam antibiotics. Most effective antibiotic was found to be gentamycin, chloramphenicol, amikacin. Pandey et al., (2020) the MRSA isolates were 100 % sensitive towards chloramphenicol and 96% of isolates were sensitive to gentamycin. The research published by Giri et al., (2021), Manandhar et al., (2021), Khatri et al., (2007) and Khanal et al., (2010) were reported chloramphenicol, Gentamycin were sensitive towards MRSA which was acceptable to present study. These antibiotics were used as the first lines of treatment for MRSA infection. The low cost of these antibiotics will also be advantage to underdeveloped country like Nepal.

In this study MRCNS isolated from nasal swab and paper currency were resistant to penicillin. Many studies carried out on different samples showed all MRCNS were resistant towards penicillin which was similar to current study. The MRCNS acquires resistivity towards the β- lactam antibiotics, encoded by *mec*A gene. MRCNS were susceptible to gentamycin, chloramphenicol, amikacin, meropenem. Khanal et al., (2010), Manandhar et al., (2018) reported the isolates were sensitive to gentamycin and chloramphenicol which was agreeable to present study. The study performed by Marasini et al., (2021), Dhungel et al., (2021) reported Meropenem, amikacin were sensitive antibiotics to the isolates which aligns to the current study. Meropenem, broad spectrum antibiotics, have excellent activity against penicillinase producing organism associated with complicated infection.

All MRSA isolates from nasal swab was showed MDR. The same studies were published by Neupane et al., (2023), Kandel et al., (2020), Shrestha, (2021) and observed high prevalence of MDR-MRSA from nasal carrier. Higher prevalence of MDR could be the abuse of antibiotics for treatment, overuse and self-medication, which is practice in Nepal. Similarly, all MRCNS isolated from paper currency was MDR in present study**.** Similar high resistant- patterns have been observed against multiple antibiotics in other studies and reported that MRCNS carry various new variant *SCC*mec A gene that predict MDR (Singh et al., 2016, Asanta et al., 2020, Shrestha et al.,2018, Shrestha, 2013). High ability to produce biofilm and presence of biofilm associated gene among MRCNS also helps to resistance against multiple antibiotics.

In this study, 19 (52.8%) isolates of MRS were detected with *tsst* gene. A same finding was published by Bergdoll et al., (1981) in a clinical sample, which showed 93.8% MRSA isolates harbor *tsst* gene which was similar to current study. Alm et al., (2018) reported 45% MRSA isolates, isolated from health worker and patient harbor *tsst* gene. A similar investigation was carried out by Sharma et al., (2018) reported 39% community associated MRSA isolates harbor *tsst* gene in UK that was same to current finding. Similar study observed by Laham et al.,

(2015) on community-based sample reported 27.4% *tsst* gene. The distribution of *tsst* gene amid MRSA has been increasing. The β- lactam antibiotics resistance also helps to harbor pyogenic toxins such as *tsst* gene.

In the present study 78.9 % (15/19) MRS isolate of paper currency sample carried *tsst* gene. A similar study was studied by Wierzchowsha, (2020) and reported 31.4% MRCNS isolate harbor *tsst* gene from ready to eat food samples which was acceptable to present study. Unhygienic practices during food processing and handling also contaminate virulent gene carrying organisms in foods. In current study, 22.2% (4/19) MRS isolate of nasal swab bear tsst gene. A similar study was published by Mohonadoss, (2021) reported 7.5% isolates from clinical sample harbor *tsst* gene, Nasaj, (2020) reported 25.3% were positive for *tsst* gene, Bertelloni, (2015) showed 4.1% isolate carrying *tsst* gene in community. This result gives strong evidence that normal human flora carries virulent genes which are circulating in the community. The antibiotic resistance and ability to produce biofilm adds to speed up organism in community.

The current study showed 18 (50%) MRS were positive for *sea* gene. This finding was acceptable to Bertelloni, (2015), Nasaj et al., (2020) reported 41.9 % and 27.5% isolates harbor *sea* gene respectively. The *sea* gene is highly transformable, also called pyogenic. It is linked with many human diseases carried by MR staphylococci in community. In this study, 66.6% (12/18) MRS isolated from paper currency harbor *sea* gene. Aung et al., (2017) reported 12% of isolates were carrying *sea* gene from food poisoning patients. Wierzchowsha, (2020) reported 14% MRSA from ready to eat food sample harbor *sea* gene which was similar to this study.

None of the isolates harbored *pvl* gene. Most of the researchers reported that the MR staphylococci isolated from clinical sample harbors *pvl* gene. The *pvl* gene is an important marker of virulence factor of staphylococci and it is less common in community samples.

CHAPTER -VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

A significant number of CNS were isolated from the nasal swabs, but a higher number *S aureus* was identified from paper currency. The higher number of MRCNS were identified from nasal swab and paper currency. The isolates show significant resistance to penicillin. Chloramphenicol and gentamycin were found to be effective antibiotics to care the infection caused by MRSA & MRCNS. All MRSA isolated from nasal swab was identified as MDR whereas all MRCNS from paper currency was MDR. One third of methicillin resistance staphylococci carried *tsst* gene and half of the methicillin resistance staphylococci carried *sea* gene. None of isolates bear *pvl* gene.

6.2 Recommendation

- Good personal hygiene should be practiced by individuals to reduce staphylococci associated infections.
- Continuous surveillance should be carried out covering larger groups of people of all ages and backgrounds to determine the circulating virulent gene.
- Paper currencies used in the community should be monitored to reduce the spread of resistant genes.

6.3. Limitations

Due to limited time frame and budget the virulence gene profile will not be prepared and only selected genes will be studied.

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APPENDICES

APPENDIX- I

List of equipment

A. 0.5 McFarland standard

Antibiotic discs

Catalase reagents

Oxidase reagent

Gram staining reagents

Human plasma

Sodium chloride solution

B. Glass slides, test tubes

Conical flask, Beaker

C. Nutrient Agar and broth

Mannitol Salt Agar

Muller Hinton agar

O/F media

D. others

inoculating Loop/ Wire

Cotton Swab

Screw Capped Test tubes

Ice Box

APPENDIX- II

Composition and Preparation of different Culture and biochemical media

i. Culture media

1. Nutrient agar

components	Gram/LIt
Peptone	5.0
Beef extract	1.5
Yeast extract	1.5
NaCl	5.0
Agar	15.0
pH (at 25℃)	7.4 ± 0.2

- 28 gm of NA powder was weighted and dissolved by heating in 1000ml of the distilled water. Then it was autoclaved for15 minutes at 15lb pressure at 121℃. Then the media was solidified by aseptically pouring in petri-disc

2. NB (nutrient broth)

components	Gram/lit
peptone	5.0
NaCl	5.0
yeast extract	1.5
beef extract	1,5
pH at 25℃	7.4 ± 0.2

- 6.5 grams of NB media was taken and dissolved by heating in 500ml distilled water. The broth media was dispensed in clean test tube in amount 3ml in each. Sterilized by autoclaving for 15 minutes at 15lb pressure at 121℃ and cool it.

3. Mannitol Salt Agar

components	Gram/lit
peptone B	1.0
Proteose peptone	10.0
NaCL	75.0
D mannitol	10.0
Phenol Red	0.025
Agar	15.0
pH	7.4 ± 0.2

- 111,02 gms of MSA media was taken and dissolved by heating in 1000ml distilled water and sterilized by autoclaving for 15 min at 15lb at 121℃. Then aseptically poured in petri-disc.

4. Muller Hinton Agar

components	Gram/lit
in form of Beef infusion	300.0
acid hydrolysate of casein	17.50
Starch	1.5
Agar	17.0
pH at 25℃	7.3 ± 0.2

- 38gms of MHA media was taken and dissolved by heating in 1000ml water. The MHA was sterilized by autoclaving at 121℃ for 15 min at 15 lbs. Aseptically poured in petri disc.

5. Trypticase Soya Broth

components	Gram/lit
Tripton (pancreatic digest)	17.0
Soyatone (peptic digest)	3.0
Glucose	2.5
Sodium Chloride	5.0
Dipotassium phosphate	2.5
pH	7.3

- 30 g of TSB was dissolved by heating in 1000ml distilled water and dispensed in screwed capped test tube and autoclaved at 15 lbs at 121℃ for 15 min.

6. DNase medium

components	Gram/lit
Pancreatic digest of casein	10.0
Yeast extract	10.0
Deoxyribonucleic acid	2.0
NaCl	5.0
Agar	15.
PH	7.5

- 39 g of medium was dissolved by heating in 1000ml D/w and autoclaved at 15 lbs at 121℃ for 15 min. Aseptically poured in petri-disc

ii. Preparation of reagents

A. Reagent for Gram's staining

i) Stock solution of crystal violet

For preparation of crystal violet, 40 g of crystal violet was weighted and dissolved in 400 ml of 95% ethyl alcohol. Then the solution was leached

and stored. The working solution was prepared by mixing 40ml of stock solution and 160 ml 1% ammonium oxalate solution

ii) Stock solution of Gram's iodine

- The concentrated solution was prepared by dissolving 50gm of potassium iodide & 25gm of iodine crystal in conical flask containing 500ml of D/w and store in dark glass container. The practical solution was prepared by mixing 60 ml stock solution and 60ml of 5% NaHCo3 solution to 220ml of distilled water

iii) Safranin

- 5gm of safranin was dissolved in 200ml of 95% ethyl alcohol. From this, practical solution was prepared by mixing 20ml of concentrate solution to 180ml of distilled water.

iv) 95% ethanol

- with help of alcohol meter 95% ethyl alcohol was prepared by adding D/W

B. Biochemical Test Reagents

i. 3% Hydrogen peroxide

Hydrogen peroxide	1ml
Distilled water	9ml

- 9ml distilled water and I ml of hydrogen peroxide were vortexed

ii. Oxidase Reagent

Oxidase reagent	1.0 g
Distilled water	100ml

- 1gm of oxidase reagent was mixed in 100ml of distilled water. and allowed to sock by pieces of Whatman filter No.1 for 30 sec. Then it was dried and store in air tied container.

iii. McFarland 0.5 solution

- 1.175gm of BaCl2 was mixed in 100ml distilled water and 1ml conc. sulphuric acid was mixed in 100ml distilled water.
- Then previously prepared, 0.05ml of BaCL2 solution and 9.95ml of sulphuric acid were mixed and autoclaved in test tube.
- Then the sterilized & stored at 4℃

APPENDIX – III

i. Procedure for Gram's staining

- Aseptically a uniform smear was made on glass slide.
- Then it was air dried and heat fixation.
- Primary stain crystal violet was floated on smear for 1 min and washed by distilled water (D/W).
- Then iodine solution was added for 1 min and washed by D/W.
- Then washed by absolute ethyl alcohol for few sec. and again washed by D/W.
- Lastly, safranin was floated to the smear for 30 sec & washed with D/W
- Then air dried and observed under oil immersion objective.
- Violet colored cocci in cluster were indicative of *S. aureus*.

ii. Procedure for Catalase Test

- On clean slide, a drop of 3% H2O2 was taken.
- Then with help of glass rod aseptically transferred a pure colony from NA and mixed it.
- Gas bubbling was seen immediately showed positive for catalase test.

iii. Procedure for Oxidase Test.

Aseptically isolated colony form NA was transferred on oxidase paper using glass rod.

- Development of purple color within 10 sec showed positive for oxidase test.

iv. Procedure for slide Coagulase Test

- One drop of normal saline solution was put on either side of slide.
- Then, with help of glass the suspension of test sample was prepared in both sides.
- Then mixed the plasma on test sample and for next not to add plasma.
- Then clumping was observed with plasma showed positive for coagulase test which was compared with another side of suspension.

- If the clumping was not observed with plasma, then tube coagulase test was performed.

v. Procedure for tube coagulase test

- Labelled the test tube as A (test), B (positive test) and C (negative test) and dispense 0.5ml of plasma in each test tube.
- Then in A tube, put 0.5ml test organism. In B tube 0.5ml S. *aureus* and in C tube sterile broth was added.
- Then Shaked gently and kept at 37℃ in incubator.
- The gel formation was observed in every 30 min up to 6 hrs by tilting the test tube that showed positive test for tube coagulation test.

vi. Procedure for Oxidative/ fermentative (O/F) test

- Aseptically the test organism was inoculated in both O/F media.
- Then sterilized paraffin oil was added in one media to form 1cm depth and both were kept in incubator at 37℃ for 24 hrs.
- Yellow in both tubes showed fermentative test.

vii. Procedure for Deoxyribonuclease test

- Aseptically streaked culture on DNase agar and incubated at 37 ℃ for 24hrs.
- Then IM hydrochloric acid was flooded onto the DNase plate and excess of hydrochloric acid was discarded.
- After about 5 mins, the halo zone was observed around the inoculums showed positive for DNase test.

viii. Procedure for AST By Disc Diffusion Method

- The 4 hrs. cultured test organism on nutrient broth was compared with 0,5 McFarland turbidity.
- Then aseptically entire MHA media was swabbed and dried for 5 min.
- After that antibiotic were applied & gently pressed to stick on media.
- Then the plate was kept in incubator at 37℃ for 18 hrs.
- Measured the inhibition zone around the disc.
- The result was interpretated using CLSI antibiotic interpretative chart.

APPENDIX IV

Questionnaires:

क्र.स. -.

१. तपाईको नाम के हो?

२. तपाईं कति बर्ष हुनु भयो?

३. कति बर्ष देखि यो पेशामा हुनहुन्छ ?

४. तपाई दिनमा कति समय यो काम गर्नु हुन्छ

५.१ महिना एता तपाईलाई कुनै रोग लागेको छ ?

६. यदि लागको छ भने, कुन रोग लागेको र सो को औषधी लिनु भएको छ, औषधीको नाम थाहा छ भने कुन औषधी हो ?

७. विरामी हुदाँ धेरै जस्तो के हुने गर्छ ?

APPENDIX V

			Diameter of zone of inhibition	
Antibiotics	**Symbol**	**Strength**	**Resistance**	**Sensitive**
Chloramphenicol	C	30 µg	18	18
Ciprofloxacin	CIP	5 µg	15	21
Cotrimoxazole	COT	25 µg	14	17
Erythromycin	E	15 µg	18	21
Tetracycline	TE	30 µg	14	19
Penicillin	P	10 unit	28	29
Clindamycin	CD	2 µg	19	22
Cefoxitin	CX	30 µg	21	22
Amikacin	AK	30 µg	18	18
Ofloxacin	OF	5 µg	14	18
Gentamycin	GEN	10 µg	22	22
Meropenem	MRP	10 µg	13	16

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