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Highly Resistant *Staphylococcus aureus* Isolated from Patients Attending a Tertiary Hospital, South Western Uganda

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: *S. aureus* is a frequent cause of human infections and is one of the most important nosocomial pathogens. Widespread antimicrobial resistance has limited therapeutic choices to treat *S. aureus* infections. Methicillin-resistant *S. aureus* has continued to cause significant infections today challenging public health initiatives to a better healthcare.

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Materials and methods: This was a cross-sectional study carried out between February 2016 and January 2017 among inpatients at Kabale Regional Referral Hospital, Various clinical specimens were collected basing on nature of infection, and analysed using standard phenotypic methods to characterise *S. aureus*. Presence of MecA gene was detected by PCR to confirm methicillin resistance strains. Antibiotic susceptibility tests were done to determine resistance patterns using standard methods.
Results: The study revealed the prevalence of *S. aureus* at 86.7% (n = 390) among inpatients, where; 223(57.2%) were Methicillin-resistant *S. aureus.* Methicillin-resistant *S. aureus* was predominantly isolated from surgical sites; 56.9 %(n = 166), Road traffic accident wounds 67.9% (n = 19) and Burn wounds; 56.3% (n = 09).
Conclusion: There was high prevalence of *S. aureus* among inpatients and majority of the isolates were methicillin-resistant. Methicillin resistant strains were highly resistant to multiple antibiotics that are commonly used.

Keywords: MRSA; inpatients; antibiotic resistance.

1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is the major pathogen responsible for various infections, including bacteraemia, skin and soft tissue (SST) and surgical site infections [1]. The presence of methicillin-resistant *S. aureus* (MRSA) imposes a significant burden on the public health care system due to emergence of antimicrobial resistance (AMR) [2]. S*. aureus* is widespread bacteria freely living in different environmental niches, and to human host, it colonises the axilla, perineum and the anterior nares of the nostrils [3,4]. Skin-to-skin contact from an infected host or colonised individuals forms the basis of common mode of transmission [5]. In the immunocompromised hosts, *S. aureus* infections can be serious and severe leading to increased mortality and morbidity [6,7].

The distribution of MRSA in any given healthcare setting varies depending on hospital wards, locations and hygiene [8]. MRSA remains a public health challenge due to limited availability of therapeutic options to manage infections [9,10], and it is increasingly reported in Uganda [11-14]. Therefore, this study aim to determine the prevalence and antibiotic susceptibility patterns of *S. aureus* isolated from clinical specimens of inpatients admitted at Kabale Regional Referral Hospital (KRRH), a tertiary health care settings located in Kabale District, South Western Uganda.

2. MATERIALS AND METHODS

2.1 Study Design

This was a cross sectional study carried out between February 2016 to January 2017 onto non repetitive clinical specimens (n = 450) collected from consented inpatients who were admitted in various wards at KRRH.

2.2 Sampling Procedure and Specimen Collection

The inpatients were selected randomly from various wards at KRRH. The study specimens were collected and transported to the laboratory for bacteriological analysis. Specimens were collected from surgical sites, sputum, blood, wound abscess, trauma wounds, burns and Cerebral spinal fluids (CSF) and Urine. Sterile cotton swabs (Fisherbrand ^{TM}) were used to collect pus from bed sores other types of wounds. CSF and Knee joint aspirates were appropriately collected in a clean and sterile plain vacutainer universal tubes (BD vacutainer®- yellow top) by clinicians and sent to the laboratory. Sputum samples were collected and received in clean plastic sputum container (Biosigma). Commercially prepared blood culture bottles (BACTEC^{TM} plus Aerobic/F culture vials and BACTEC^{TM} plus anaerobic/F) were used to collect and transport blood for culture from patients suspected with bacteraemia. Urine samples were collected into sterile wide mouth urine containers (SARSTEDT).

2.3 Isolation and Identification of the *S. aureus*

All specimens were processed at KRRH microbiology laboratory. Blood culture bottles were accessioned and incubated in BACTEC ^{TM} FX 40 blood culture system for a maximum incubation period of 168 hours [15]. Pus swabs, urine and sputum samples were separately inoculated into the Mannitol salt broth (MSB)

(HiMedia- India) and incubated aerobically at 37°C for a maximum period of 48 hours. The broth with presumptive *S. aureus* growth was determined by the presence of yellow colour of the culture broth media [16]. Positive broth cultures, as well as positive blood culture vials, were subcultured onto Mannitol salt agar (MSA) media (Oxoid™) and incubated at 37°C± 2 for 24 - 72 hours until appropriate growth was observed [16]. Every new batch of MSB, MSA and blood culture media bottles (Bactec TM plus aerobic/F medium) were quality controlled using *S. aureus* ATCC 6538 as positive control and *Escherichia coli* ATCC8739 as negative control prior to use [17].

S. aureus was phenotypically identified as yellow colonies which were phenotypically identified using biochemical tests that included catalase, coagulase and Staphaurex™ Plus Latex Agglutination Test (Remel™) test [18]. Every isolate that was both catalase, coagulase and Staphaurex™ Plus Latex Agglutination test positive was presumed to be *S. aureus* and subcultured on to the Nutrient agar as a purity plate and incubated aerobically at 37°C± 2 for 24 hours.

2.4 Antimicrobial Drug Susceptibility Testing

All the phenotypically confirmed *S. aureus* isolates were screened for methicillin resistance using cefoxitin (30 µg) disk diffusion method, where a suspension of organism equivalent to 0.5 McFarland turbidity standard were prepared and inoculated onto Mueller-Hinton agar (Oxoid™) by swabbing over agar surface. Cefoxitin 30 µg disk was applied to the plates which were later incubated at 35°C± 2 for 18-24 hours. The zone of inhibition diameter around Cefoxitin disc were measured using a digital Vanier calliper (Fisherbrand™) and results recorded. The results were compared with the Clinical and Laboratory Institute Standard (CLSI, 2015) where any isolate with zone diameter of ≤ 21 mm was labelled as MRSA while those with ≥ 22 mm were labelled as MSSA (CLSI, 2015) [19]. All the phenotypic MRSA and MSSA were inoculated into 30% glycerol in brain heart infusion (BHI) broth (Difco Laboratories) in cryovial tube and stored at -80°C until retrieved for further testing.

All the isolates were tested for susceptibility to selected antibiotics which included; Amoxycilin and clavunate (20/10 µg), Ciprofloxacin (5 µg), Gentamycin (10 µg), Imipenem(10 µg), Trimethoprim and sulfamethoxazole (1.25/23.75 µg), Tetracycline (30 µg), Vancomycin (30 µg), Erythromycin (15 µg), Clindamycin (2 µg), Rifampicin (5 µg), Linezolid (30 µg), Teicoplanin (30 µg) using Kirby Bauer disk diffusion technique according to CLSI, 2015 guidelines [19]. The *S. aureus* colonies were emulsified in approximately 5mL of sterile normal saline to 0.5 McFarland turbidity standard equivalent of the bacterial suspension. After 15 minutes, a sterile cotton swab was used to inoculate dried Mueller Hinton agar plate by streaking the swab over the entire sterile agar surface repeatedly, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. After 5 minutes, the drug-impregnated disks were applied in no closer than 24 mm from center to center. The plate was incubated at 35± 2°C in ambient air for 18 to 24 hours.

The zones of inhibitions were determined by measuring the size of clear zones using a digital Vanier calliper (Fisherbrand™), comparing the zone sizes within the CLSI, 2015. The results were reported as Resistant (R), Intermediate (I) or Sensitive(S). The zone of clearance with flattening characteristics (D-shaped) around clindamycin in the area between the former and Erythromycin was observed to detect inducible clindamycin resistance. All the phenotypic Vancomycin resistance strains were further tested using Biofire FilmArray for presence of vanA/B gene [20].

Mueller Hinton agar for this study was quality controlled (QC) using S. *aureus* ATCC 25923, *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). In addition, weekly Antibiotic QC was constantly carried out to ensure the quality and validity of the antibiotic susceptibility study results. All the isolates were preserved in 30% glycerol in sterile tryptic soy broth and stored in the biorepository at the Uganda Central Public Health Laboratories (CPHL).

2.5 DNA Extraction and PCR detection of mecA gene

DNA was extracted from all the phenotypic MRSA and MSSA colonies previously stored in 30% glycerol in BHI broth (Difco Laboratories). The isolates were subcultured onto sheep blood agar (SBA) and incubated at 35±2°C for 24 hours to obtain pure colonies. About 1-2 pure colonies of *S. aureus* were suspended in 25 µl of

sterile distilled water and boiled at 100°C in a digital heat block (Thermo scientific™) for 15 minutes [21]. About 05 µl volume was directly used as a template for PCR amplification. The mecA gene was amplified using the primers ‘’MecA F; 5'TCC AAT TAC AAC TTC ACC AGG3' and “MecA R; 5'CCA CTT CAT ATC TTG TAA CG3'”as described by Wang et al. [22] with the Amplicon size approximately 180 bp which were consistent with *mecA* gene amplification. A 50 µl PCR reaction mixture was used which included; 45 µl of master mix containing PCR buffer (x1), dNTP mix (0.2 mM of each), primer (0.5 µM), Taq DNA polymerase (0.25U), and MgCl 2 (1.5 mM) with 5 µL of template DNA. Cycling conditions were set at 94°C as hot start for 4 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute and final extension step at 72°C for 3 minutes. PCR products were loaded onto 2% agarose gel (Invitrogen, Carlsbad, CA, USA) previously mixed with ethidium bromide gel dye (0.5 µg/mL) and run electrophoresis followed by gel visualisation using UV transilluminator (Fig. 2).

2.6 Data Analysis

The data was entered in EpiData version 3.1.2701.2008, and statistical analysis done by descriptive statistics using SPSS version 23.

2.7 Ethical Considerations

The study was approved by Institutional Review Board of Mbarara University of science and technology (MUST) and Uganda National Council of Science and Technology (UNCST) study Number 13/08–15. The procedures followed were in accordance with the ethical standards of these committees on human experimentation, and with the Helsinki Declaration of 1975 as revised in 2000.

3. RESULTS

A total of four hundred and fifty non-repetitive specimens were collected from 450 inpatients and those from females (n= 241) were slightly higher than those from males (n = 209). The mean age of patients hospitalised was 35.5years and the overall age range was from 5days old to 88years. Specimen were collected from Children; 57(12.7%), teenagers; 71(15.8%), youth; 151(33.6%), middle-aged; 56(12.4%) and the old;113(25.1%). The mean length of hospitalisation was higher in elderly patients compared to other patients age groups (Table 1).

Among all the clinical specimens analysed (n = 450), *S. aureus;* 390(86.7%) was the common bacterial pathogen isolated among which 223(49.6%) were MRSA and 167(37.1%) were MSSA. *S. aureus* was high among surgical site specimens; 74.9%(n =292), which also yielded the highest number of MRSA organisms 166 (74.4%). In general, the MRSA prevalence among the clinical specimens was 49.6% and among the *S. aureus* isolates was 57.2% (Table 2).

MRSA isolates highly resistant to resistant to Amoxicillin - clavunate;100%(n=223), TMP–SMX; 100% (n = 223), Ceftriaxone; 100%(n =220) and Imipenem;100%(n = 49). While MSSA were highly resistant to TMP-SMX; 77.8%(n = 130) and Erythromycin 59.9%(n = 100). Among the 223 of MRSA isolates, 03(1.3%) were vancomycin-resistant (VRSA) confirmed by BioFire FilmArray (Fig. 1).

Table 1. Age characteristic of patients (in years) and mean length of hospitalisation

Age group	Age range (years)	MLH (Hours)	Male (n = 209)	Female (n =241)	Total (n=450)
Child	≤ 12	96	25	32	57(12.7%)
Teenagers	13 – 19	120	33	38	71(15.8%)
Youth	22 – 35	120	73	78	151(33.6%)
Middle age	36 – 45	168	27	31	56(12.4%)
Old	> 45	196	51	62	113(25.1%)

Footnote: n = sample size; MLH =Mean length of hospitalisation; P>.005

Table 2. *S. aureus* profile isolated from various clinical specimens

Type of specimen	Specimen	*S. aureus*	MRSA	MSSA
Surgical site swabs	308	292(74.9%)	166(74.4%)	126(75.4%)
Bed sore swabs	10	07(1.8%)	05(2.2%)	02(1.2%)
Abscess swabs	7	06(1.5%)	02(0.9%)	04(2.4%)
Burn wound swabs	20	16(4.1%)	09(4.0%)	07(4.2%)
Blood culture	12	09(2.3%)	04(1.8%)	05(3.0%)
Sputum	30	11(2.8%)	08(3.6%)	03(1.8%)
Knee joint aspirate.	5	03(0.8%)	02(0.9%)	01(0.6%)
Urine	25	18(0.8%)	08(3.6%)	10(6.0%)
RTA wounds swabs	33	28(7.2%)	19(8.5%)	09(5.4%)
Total specimen and isolates	450	390(86.7%)	223(49.6%)	167(37.1%)

Footnote: n= sample size; RTA = Road traffic accident; MRSA = Methicillin resistant S. aureus; MSSA = Methicillin sensitive S. aureus. P>.005

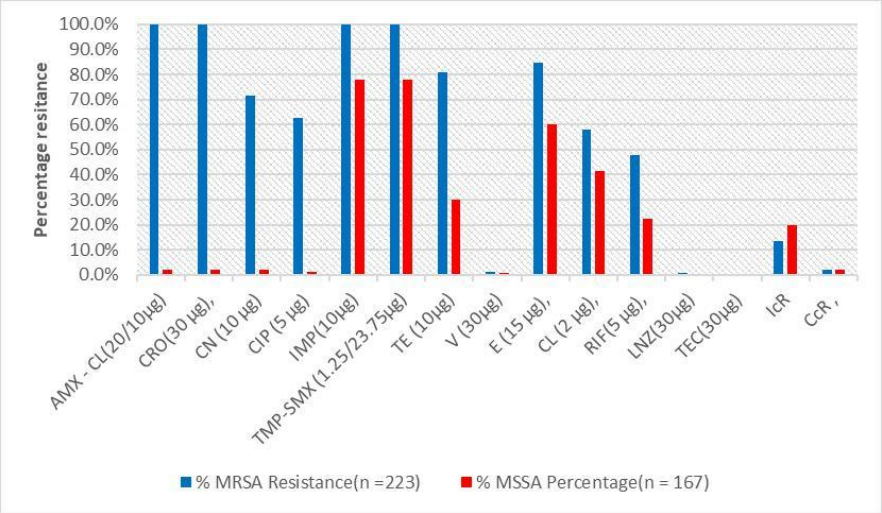

Fig. 1. Antibiotic resistance pattern of MRSA and MSSA isolates

Footnote: AMX –CL = Amoxycilin and clavunate; CRO = Ciprofloxacin; CN = Gentamycin; CIP = Ciprofloxacin; IMP = Imipenem; TMP – SMX = Trimethoprim and sulfamethoxazole TE= Tetracycline; V = Vancomycin; E= Erythromycin; CL = Clindamycin; RIF = Rifampicin; LNZ = Linezolid; TEC = Teicoplanin; MRSA= Methicillin resistance S. aureus; MSSA = Methicillin sensitive S. aureus.

4. DISCUSSION

The prevalence of *S. aureus* was 86.7% among inpatients admitted at KRRH. This is higher than previously described in other Ugandan hospitals; 46% in Mulago National hospital [14], 27% in Mbarara Regional Referral Hospital [13] and 31.6% in neonatal ward at Mulago national hospital [23]. Also, the findings confirm reports of earlier studies implicating *S. aureus* as the common bacterial infections affecting hospitalised patients [24]. Bacterial infection due to *S. aureus* is always associated with increased mortality, morbidity, and length of hospital stay [25]. Potential predisposing risk factors like previous surgeries, severe burns, road traffic accident trauma, the presence of indwelling devices such as catheters, ward overcrowding and prolonged lengthy hospital stays are probable reason for high prevalence at KRRH. Enhanced infection prevention and control strategies is required to reduce the prevalence.

S. aureus infections of the surgical sites was 74.9% which is higher than previously reported in other Ugandan Hospitals [13,29]. Invasive surgical procedures are the common mode of treatment at Surgical and obstetric wards. However, bacterial colonisation of the surgical sites may reveal a glaring lack of adequate postoperative care and failure to maintain sterility during surgical procedure. Postoperative surgical site infections with *S. aureus* remains a significant cause of morbidity in surgically

treated patients that requires additional cost of care [27].

We report frequency of *S. aureus* in urine samples was 0.8% among inpatients especially those with urinary catheter devices. Isolation of *S. aureus* from urine samples probably indicates colonisation. The presence of a urinary catheter is the most important risk factor for bacteriuria which adversely affect patient outcomes [30]. Blood borne *S. aureus* isolates was 2.3% than previously reported in Jimma University Specialized Hospital, Ethiopia and Mulago National hospital, Uganda [31,23]. Previous antibiotic therapy before blood culture is taken could probably suggest presence of low prevalence [30]. Physical trauma due to burns, bed sores (pressure sores) or road traffic accident predisposes *S. aureus* infection. The overall prevalence of *S. aureus* infection of burn wounds was 80% with majority of the isolates being MRSA. This is much higher than previously reported in tertiary care health care facilities of Ethiopia [31] and Ghana [32]. Bed sores are more strongly associated with prolonged hospital stays [33]. The incidence of pressure ulcers in hospitalised patient is often a direct measure of the quality of medical care provided, particularly in the meticulous attention paid to careful positioning and frequent turning of the bedridden patient. Wounds due to road traffic accident (RTA), had *S. aureus* prevalence of 85%. This is consistent with Wong et al who reported similar prevalence in Malaysia [34,35]. Wounds are a risk factor for colonisation by pathogenic bacteria, including multidrug-resistant microorganisms like *S. aureus* particularly MRSA which interferes with wound healing with consequent increase in the severity of the lesions.

The emergence and spread of antibiotic resistance remains a global public health concern. Continuous update about the prevalence and distribution of antibiotic resistance mong MRSA isolates is valuable for clinical and epidemiological intervention. We also report the prevalence of MRSA as 49.6% among hospitalised patients. This is consistent with Chakrakodi et al. in the Tertiary Care Indian Hospital [26]. However, this is higher than is previously reported in S. Korea [28], Australia [8] and India [36]. Probable attributes are suggested to be the inappropriate use of antibiotic or inadequate drug dosage during treatment. It has been argued that, previous hospitalisation is among the contributing factor that results in the selection of multidrug resistance MRSA due selective antibiotic pressure [36]. Presence of MRSA in hospitalised patients indicates a need for greater infection control measures, proper use of antibiotics, and careful inpatient management. Among the MRSA; there was 100% resistance to TMP - SMX, and Amoxicillin – clavunate. The findings are consistent with a previous report from Democratic Republic of Congo (DRC) in which MRSA collected from different clinical specimens showed TMP - SMX resistance prevalence of 100% and Amoxicillin – clavunate resistance prevalence of 89% [37]. The long term use of these antibiotics and concurrent colonisation with other resistant bacteria [36,37] explains emergence of high resistance rates.

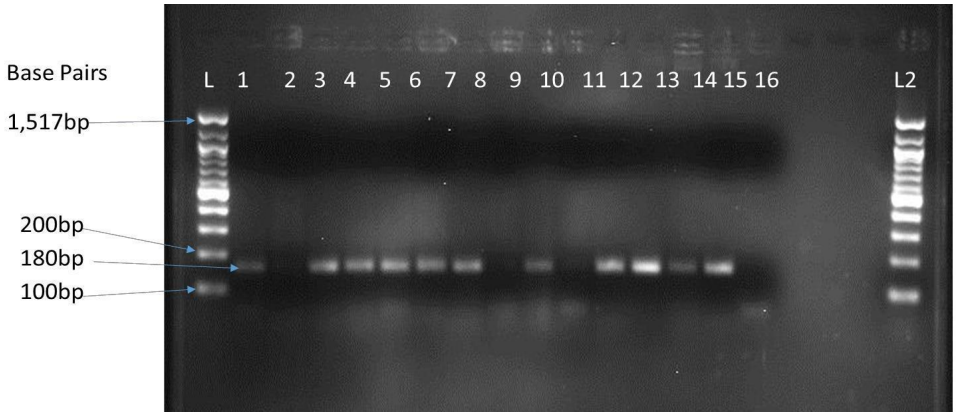

Fig. 2. Results of mecA gene PCR where an Amplicon of approximately 180 bp was expected
L is 100 bp ladder,1- Positive control (MRSA ATCC 43300), 2 – negative control (MSSA contains a Methicillin Susceptible S. aureus strain ATCC 25923) Lanes 3, 4,5,6,7,9,11,12,1314 and15 mecA positive (MRSA) from clinical samples of inpatients. Lane 8, 10 and 16 mecA negative (MSSA) isolates from clinical samples. L2 is the Negative amplification control.

The widespread and uncontrolled use of antibiotics undoubtedly accelerates the evolution of *S. aureus*, acquiring multiple resistance genes and therefore capable of surviving in almost all antibiotic families, i.e., beta – lactams, Tetracyclines, fluoroquinolones [38], Macrolides [39] and aminoglycosides [40]. In addition, MRSA isolates resistance to gentamycin was 71.7%. this is higher than 47.8% previously reported in Iran [40]. A resistance rate of 84.5% reported in the Sub Himalayan Region of India [41], reflects and increasing resistance nature of MRSA towards aminoglycosides. Similarly, the report indicates Tetracycline resistance rate of 80.7%, which is much higher than previous reports from Tehran (61%) [42]. Tetracycline is a broad-spectrum antibiotic and have been widely used indiscriminately which probably explain the high resistance rate. Generally, there is significantly higher antibiotic resistance among MRSA strains observed towards the set commonly used antibiotics with exception of Vancomycin, Teicoplanin and Linezolid. MRSA resistance to various antibiotics has increased dramatically at an alarming rate and could probably due to unrestricted use this antibiotic [43,38].

Clindamycin has been an exceptional antibiotic *Staphylococcal* infections and an alternative in penicillin-allergic patients. Its desirable oral bioavailability has been credited as an excellent option for outpatient treatment and changeover after intravenous antibiotics. However, the rate of inducible clindamycin resistance (iCR) (16.2%) requires an urgent response, and has been previously reported elsewhere [44,45]. The consequence of excessive use of macrolides is responsible for raising resistance in this class of antibiotics. However, differences in the resistance rate among regions emphasise the need for generating a local resistance data to guide empirical therapy. Detection of iCR is important for the use of clindamycin in Staphylococcal infections including MRSA.

Treatment of VRSA infections is challenging today often associated with persistent infections, treatment failure, and poor clinical outcomes. However, there is limited literatures regarding VRSA from clinical isolates in Uganda. The current study report of 1.3% resistance rate of MRSA to Vancomycin, is probably the first of its kind to be reported in this region. Disk-diffusion sensitivity systems and automated methods are not very reliable in detecting VRSA [46] and therefore, we confirmed these isolates for the presence of vanA/B gene using Biofire FilmArray. Vancomycin has had perhaps the slowest rate of development of resistance among antibiotics as a glycopeptide antibiotic, inhibits cell wall biosynthesis and is among the drugs of choice for treatment of severe MRSA infections. The molecular basis of resistance in vancomycin resistance *S. aureus* is polygenic and involves stepwise mutations in genes encoding molecules primarily those in cell envelope biosynthesis. The *vanA* gene and operon, which is present on a plasmid is responsible for reduced susceptibility of *S. aureus* toward vancomycin, believed to be transferred from *Enterococcus faecalis* and *E. faecium* [47]. Environmental factors contributing to vancomycin resistance include irrational use of antibiotics; over-the-counter availability without prescriptions; injudicious use in hospitals, agriculture, fisheries and animal husbandry, which could result in increased selective pressure of vancomycin. Finding VRSA isolates in a rural setting represents a serious public health concern. We suggest stringent infection control policies to prevent transmission of such life-threatening isolates in the hospital setting.

There was no MRSA isolates resistance against teicoplanin (Glycopeptides) and Linezolid observed. Walkey et al recommended empirical use of these antibiotics because of their presumed efficacy against MRSA [48]. However, we suggest that such decision should be based routine and regular antimicrobial surveillance, local availability, and local cost. The limitation of this study did not differentiate community acquired and hospital acquired *S. aureus* and MRSA.

5. CONCLUSION

There was high rate of MRSA with multi drug resistance infections among inpatients. This justifies the need to develop a sustainable policy on screening and decolonisation of MRSA from patients as they are admitted to wards and prompt infection control and prevention at the hospital. The high incidence of MRSA and multidrug resistance permits for the judicial use of antibiotics to avoid therapeutic calamity. Vancomycin is no longer taken as excellent antibiotic with activity against clinical isolates of MRSA. We recommend strengthening on -site laboratory capacity to routinely perform antibiotic susceptibilities on all isolates from clinical specimens to detect emerging resistance and spread. This will reduce empirical antibiotic

prescription, facilitates the positive clinical outcome.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included within the article and excel file attached.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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