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Research Article

ANTIBIOTIC RESISTANCE PATTERNS OF *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES OBTAINED FROM PATIENTS VISITING GONDAR UNIVERSITY HOSPITAL.

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ABSTRACT

***S. aureus* is opportunistic bacterial pathogens and characterized by rapidly developing antimicrobial resistance. The purpose of this study was to assess the antibiotic resistance pattern of clinical isolates of *S. aureus* from patients visiting Gondar University Hospital. Sixty *S. aureus* confirmed clinical isolates were collected. Inoculum was prepared from three to four well-isolated colonies. Disc diffusion method susceptibility test was done for amoxicillin (20µg), ampicillin (10µg), chloramphenicol (30µg), erythromycin (15µg), tetracycline (30µg) and vancomycin (30µg). After 24 hrs of time incubation time at 37 °C inhibition zones were measured, recorded and evaluated. MIC was determined using an agar dilution method and broths that don't show any bacterial growth at MIC were taken as MBC. Mean inhibition zones, amoxicillin 5.33±0.58 mm and 6.33±1.53 mm against isolate 15 and 36; p=0.015; ampicillin 9.00 ±1.00 mm against isolate 56; p=0.033; chloramphenicol 5.33±0.58 mm against isolate 60; erythromycin, 4.67±0.58 mm, 6.00±1.00 mm and 6.35±1.53 against isolate 22, 15 and 48; p=0.031; tetracycline 8.00±1.00 mm and 10.00±1.00 mm against isolate 36 and 45 and vancomycin 12.00±1.00 mm against isolate 31; p=0.00 had been found. Amoxicillin (MIC 24 µg/ml, MBC 32 µg/ml); ampicillin (4 µg/ml MIC and MBC); chloramphenicol (40 µg/ml MIC and MBC); erythromycin (0.5 µg/ml MIC; 24µg/ml MBC); tetracycline (2µg/ml MIC and 24 µg/ml MBC) and vancomycin (3 µg/ml MIC and MBC) have shown. Half of isolates 31(51.7%) were found to be resistance to amoxicillin. Chloramphenicol and tetracycline had shown equal resistance 9(15%). Blood specimen 18 (72%) has shown the highest resistance. Incidence of resistant isolates in neonates 10 (83.33%) were higher than children 18 (75%) and adults 13 (54.17%). In this study multiple antibiotic resistance increment was seen.**

Keywords: S. aureus, Antibiotics, MIC, MBC, Antibiotic Resistance

INTRODUCTION

*Staphylococcus aureus*remains the most important opportunistic bacterial pathogens of humans. It is characterized by the existence of many virulence factors and rapidly develops antimicrobial resistance (Bryant *et al*, 1998; Hall *et al.*, 2001). Antibiotic resistance is unhidden and a frequent phenomenon. Not all bacteria are affected similarly by antibiotics, some bacteria are genetically able to protect against the killing effects of an antibiotic (He*et al*., 2010). These resistant bacteria replicate and others have gained ‘‘resistance’’ through transfer that antibiotic has lost its effectiveness (Phongpaichit *et al.*, 2008). As time passes, resistance builds up for various antibiotics and these are referred to as "multiple-drug resistant." Multi-drug resistant bacteria are now serious health concern because they may cause diseases that are complicated or impossible to cure (Bryant *et al.*, 1998).

More and more significant contributor to the emergence of microbial threats to health is a drug [antibiotic] resistance (He*et al*., 2010; Raza *et al*; 2013). Microorganisms that on one occasion were easily controlled by antimicrobial drugs are more and more causing infections that no longer respond to treatment with these drugs. Accustomed bacterial infection now becomes cause for more mortality due to no or very less treatment choices left (Hall *et al.*, 2001).

Acquisition and transfer of antibiotic resistance and virulence factor genes by the bacteria via horizontal transfer of the integrons, resistance (R) plasmids and transposons are increasing problems in infectious diseases (Hall *et al.*, 2001).In recent times, virulence genes have been reported in *Enterobacteriacea* (Hall *et al.*, 2001) bacteria found from food (Sunde, 2005)and environment (Roe *et al.*, 2003). The intention of this study was to assess the antibiotic resistance pattern of clinical isolates of *S. aureus* from patients visiting Gondar University Hospital.

MATERIALS AND METHODS

Study area,sample collection, sampling techniques and type of study design

This study was conducted in North Western Ethiopia, at Gondar University, Department of Biotechnology (Molecular Biology laboratory).In thisstudy, sixty (60)*Staphylococcus aureus* confirmed clinical isolates were collected from 25 blood specimens, 17 drainage fluid specimens, 6 urine specimens and 12 pus and wound specimens. *S. aureus* confirmed samples were collected Purposive non-random sampling was used using till the sample size reaches indicated sample size. The study design was experimental/interventional using appropriate methods to determine antibiotic resistance patterns in these isolates. The purified strains were stored at −80°C.

Antibacterial agents

Amoxicillin,ampicillin, chloramphenicol, erythromycin, tetracycline and vancomycin, were used. The sensitivity of specimen to 6 types of antibiotics was examined using the K-B method. The susceptibility test results were compared to the Clinical Laboratory Standard Institute (CLSI, 2013) antibacterial susceptibility testing standard.

Inoculum Preparation

S. aureus isolates were isolated and collected separately from samples by culturing on Mannitol salt agar at 37°C for 24 hrs by using streak plate method. Then

three to four well-isolated colonies of identical morphological character were taken from Mannitol salt agar culture and colonies of each specimen was transferredand mixed into sterile normal saline solution. The suspension turbidity was comparable to 0.5 McFarland standards which is equivalent to a bacterial suspension of 1.5×10^8 Colony Forming Unit CFU/ml (CLSI, 2013).

Susceptibility Test

Bacterial suspensions of 60 µl were cultured on Muller-Hinton agar. Inoculums were uniformly distributed and streaked on agar surface through sterile cotton swab. This was achieved by rotating the Petri-plate to have equal distributions of cultures. Antibiotic discs were dispensed using antibiotic disc dispenser. Disc diffusion method for antibiotic susceptibility testing was implemented as described by(Vivekanandhan *et al.*, 2006) against antibiotic discs, amoxicillin (20µg), ampicillin (10µg), chloramphenicol (30µg), erythromycin (15µg), tetracycline (30µg) and vancomycin (30µg). After 30 min of prediffusion time, the plates were incubated at 37 °C for 24 hrs and the inhibition zones were measured and recorded. The inhibition zones were compared with the susceptible control strainof *S. aureusATCC 25923*, whose susceptibility pattern is known and used as positive controls (Bauer *et al.*, 1996). Moreover, the recorded inhibition zones were evaluated according to the Clinical and Laboratory Standards Institute (CLSI, 2013) to determine the susceptibility, intermediate and resistance pattern of isolates for the aforementioned antibiotics. Testswere performed in triplicates and discrepancy results were repeated. Analysis of Variance (ANOVA) was usedto determine number of resistance, intermediate resistance and susceptible isolates in the group.

Determination of Minimum Inhibitory Concentrations (MICs)

The MIC of antibiotics was determined using an agar dilution method (Islam *et al.*, 2008). Tryptic Soya broth was used to determine the MIC of isolates. Stock solutions of antimicrobial agent at a concentration of amoxicillin 5mg/ml, ampicillin 5 mg/ml, chloramphenicol 10mg/ml, erythromycin 1mg/ml, tetracycline 5mg/ml and vancomycin 2.5mg/ml were prepared using sterile distilled water. From these stock solutions antimicrobial agent of different volume (amoxicillin, 3.2µl, 4.8µl,6.4µl and 8µl; ampicillin, 3.2µl, 6.4µl, 8µl and 16µl; chloramphenicol, 3.2µl, 6.4µl, 9.6µl, and 12.8µl; erythromycin, 2.4µl, 4.8µl, 9.6µl and 19.2µl; tetracycline, 1.6µl, 3.4µl, 4.8µl and 9.6µl and vancomycin, 8µl, 16µl, 24µl and 32µl were added in to the test tube containing 4ml broth using filter sterilization method to prepare four different concentration of each antimicrobial agent (amoxicillin; 4µg/ml, 6µg/ml, 8µg/ml and 16µg/ml; ampicillin; 4µg/ml, 8µg/ml, 16µg/ml and 32µg/ml; Chloramphenicol; 8µg/ml, 16µg/ml, 24µg/ml and 32µg/ml; erythromycin; 1µg/ml, 2µg/ml, 4µg/ml and 8µg/ml; tetracycline; 2µg/ml, 4µg/ml, 8µg/ml and 16µg/ml and vancomycin; 1µg/ml, 2µg/ml, 3µg/ml and 4µg/ml) respectively. From the bacterial suspension 80µl volume were taken and added in to broth antibiotics mixture and incubated at 37°C for 24 hrs. After 24 hrs, the growth turbidity was compared with that of negative control broth and antibiotic concentration that inhibit microbial growth

was taken as minimum inhibitory concentration.

Determination of the Minimum Bactericidal Concentrations (MBCs)

Broth containing test tubes that don't show any bacterial growth at MIC were used to determine MBC. Small volume of these broths were transferred and streaked on to the surface of nutrient agar medium by sterile wire loop. The medium was incubated at 37°C for 24 hrs. The least concentration of antimicrobial agent that effectively inhibits any bacterial colony growth on the agar plate was recorded as MBC of antimicrobial agent.

Data Analysis

Individuals who were positive to *S. aureus* and the parameters obtained from them like sex, age, occupation, educational status and employment and correlation between Antibiotic susceptibility patterns were analysed using SPSS software package version 20.0.Analysis of variance (ANOVA) was used to determine number of antibiotic resistance, intermediate resistance strains and susceptible isolates in the group.

RESULT

Antibiotics Susceptibility (Growth Inhibition Zone) of Isolates

In this study, growth inhibition zones of amoxicillin, ampicillin, chloramphenicol erythromycin, tetracycline and vancomycin were evaluated against sixty *S.aureus* isolates. The mean inhibition zones of amoxicillin 5.33±0.58mm and 6.33±1.53 mm against isolate 15 and 36 respectively, were the least (*p=0.015.* ampicillin has shown considerably lowest inhibition 9.00 ±1.00 mm against isolate 56(*p=0.033*).Chloramphenicol, 5.33±0.58 mm least inhibition had also shown against isolate 60.Erythromycin, 4.67±0.58 mm, 6.00±1.00 mm and 6.35±1.53 mm killing efficacy has recorded against isolates 22, 15 and 48 correspondingly, were less than all other isolates (p=0.031). Similarly tetracycline showed 8.00±1.00mm and 10.00±1.00mm inhibition against isolate 36 and 45, respectively were theleast (p=0.09)between them. Finally,meaninhibition value of Vancomycin 12.00±1.00 mm against isolate 31 were least (p=0.00) compared to all other isolates.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

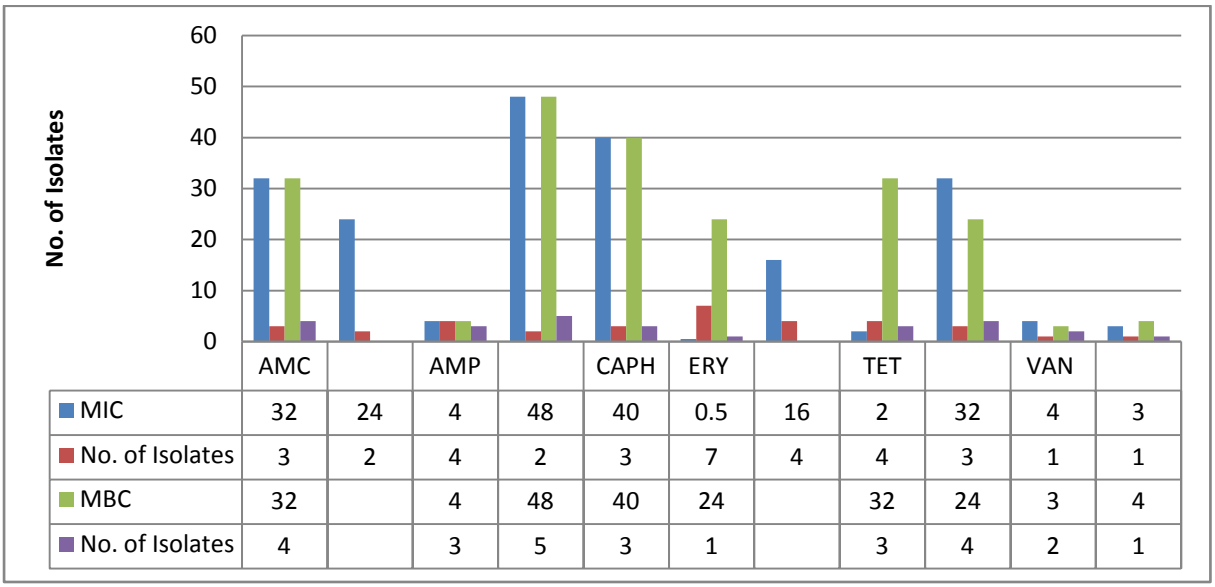
Minimum Inhibitory Concentration of each Antimicrobial Agent

The MICsof the six aforementioned antibiotics were assessed as shown in **figure 1.**The highest amoxicillin MIC 32 µg/ml against 5, 27 and 37 isolates and 24 µg/mlinhibitions against 4 and 45 isolates were observed. Leastconcentration of ampicillin 4µg/mlhad shown against 1, 2, 25 and 35 isolates however, highest 48 µg/ml concentration isolate 17 and 56. MIC of chloramphenicol was found to be 40 µg/ml against isolate 11, 41 and 60.MICof 0.5 µg/mlhad shown against 2, 16, 35, 39, 50, 56 and 58 isolates by erythromycin, but highest MIC 16µg/ml was also shown by isolates of 4, 15, 52 and 59. Likewise, tetracycline 2µg/ml MIC againstisolate 2, 14, 20, 25 and 40 was the least concentration and highest 32 µg/ml against isolate 12, 45 and 54 was also recorded. In this study, the MIC of vancomycin 4 µg/ml against isolate 31 and 3 µg/ml against isolate 32 was seen.

Minimum Bactericidal Concentration of each Antimicrobial Agent

As shown in figure 1, from different concentrations isolate 5, 15, 27,

37 and 45 were shown a MBC of 32µg/ml amoxicillin. Whereas 4µg/ml ampicillin MBC had observed against isolates1, 2, 28 and conversely isolate17, 22, 36, 47 and 56 were shown 48 µg/ml MBCs. Chloramphenicol 40µg/ml MBC was showed by 11, 41 and 60 isolates. But erythromycin has shown MBC of 24µg/ml against isolate 15. Tetracycline, 32µg/ml MBC against isolates12, 4 and 54 was the highest, followed by 24µg/ml against 10, 21, 29 and 36 isolates. MBC of (3µg/ml) vancomycin against isolates 6 and 32 and 4µg/ml against isolate 31 were found.



	AMC		AMP		CAPH	ERY		TET		VAN	
MIC	32	24	4	48	40	0.5	16	2	32	4	3
No. of Isolates	3	2	4	2	3	7	4	4	3	1	1
MBC	32		4	48	40	24		32	24	3	4
No. of Isolates	4		3	5	3	1		3	4	2	1

Figure1: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Resistance Patterns of Isolates for Tested Antibiotics

In this study, inhibition zones were evaluated as per CLSI to determine sensitivity, intermediates and resistance of isolates as shown in the figure 2 below. From tested antibiotics vancomycin 58(96.7%) had shown the highest sensitivity followed by tetracycline 47(78.3%) sensitivity. Similarly, highest intermediate isolates were recorded to chloramphenicol 10(16.7%). However, there were no any intermediate isolates against amoxicillin and vancomycin 0(0%). Whereas, more than half of isolates 31(51.7%) were found to be resistance to amoxicillin. Meanwhile chloramphenicol and tetracycline had shown equal resistance 9(15%).

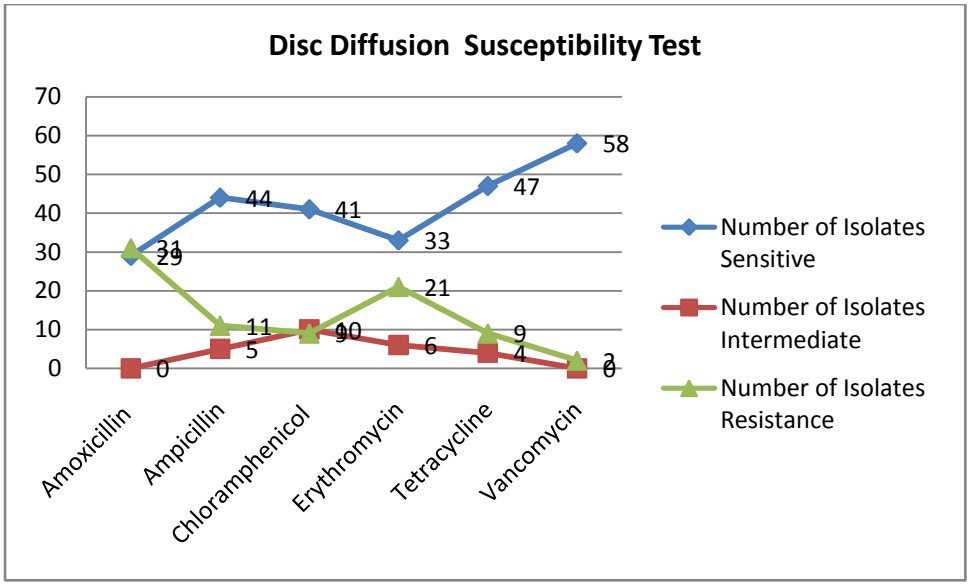


Figure 2: Resistance patterns of isolates for tested antibiotics

Determination of Susceptible and Resistant Isolates in Specimens

In this study as shown in figure 3, blood specimen 18 (72%) has shown the highest resistance from all clinical specimens. Though resistance of 8 (66.67%) against wound specimen, 4(66.67%) against urine specimen and 11 (64.71%) against drainage fluid specimen were revealed.

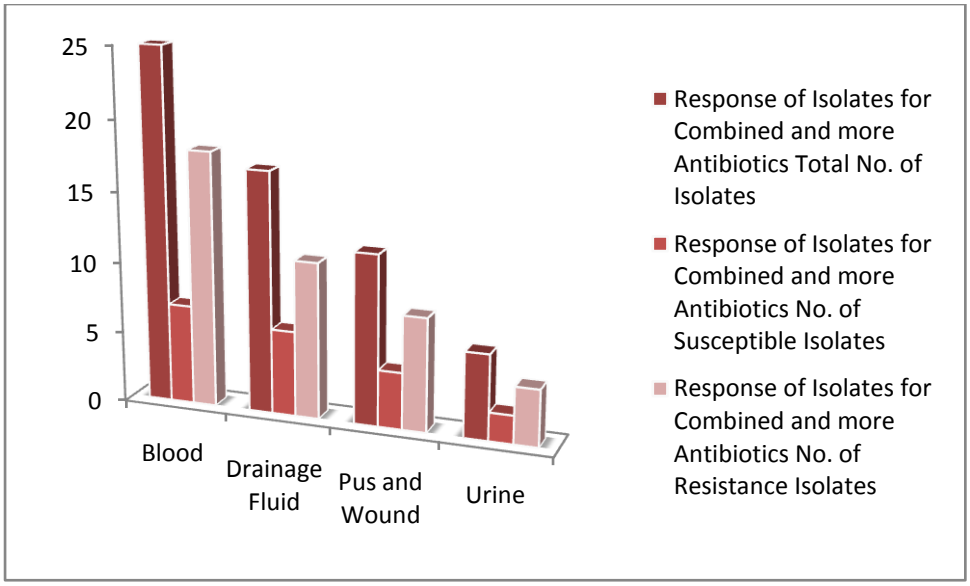


Figure 3: Antibiotic susceptibility and resistance determination result in different specimens

Determination of Susceptibility and Resistance Isolates across in Demographic Status

The prevalence of resistance and susceptibility isolates across various Demographicstatuswasassessed (Table 1**)**. In this study, 12 (20%), 24 (40%) and 24 (40%) were found to be neonates, children and adults respectively. Incidence of resistant isolates in neonates 10 (83.33%) were higher than children 18 (75%) and adults 13 (54.17%). In this work, 36 (60%) and 24 (40%) were males and females correspondingly and resistance of 28 (77.78%) males and13 (54.17%) females had been found. Moreover, based educational status, susceptibility and resistance prevalence were evaluated and resistance of 15 (71.43%) and 29 (74.35%) were found from educated and illiterate respectively. lastly self-employed 35 (71.43%) and government employer 6(54, 55%) resistance were also shown.

Table 1: Determination of susceptible and resistance isolates in different demographic status.

Types Demographics		Total no. of Isolates No (%)	No. of Susceptible Isolates No (%)	No. of Resistance Isolates No (%)
Age	Neonate≤ 28 days	12(20%)	2(16.67%)	10(83.33%)
	Children 1 Month-18 yrs.	24(40%)	6(25%)	18(75%)
	Adult (19-60 yrs.)	24(40%)	11(45.83%)	13(54.17%)
Sex	Male	36(60%)	8(22.22%)	28(77.78%)
	Female	24(40%)	11(45.83%)	13(54.17%)
Educational Status	Educated	21(35%)	6(28.57%)	15(71.43%)
	Illiterate	39(65%)	13(33.33%)	29(74.35%)
Employer	Government employer	11(18.33%)	5(45.45%)	6(54.55%)
	Self-employer	49(81.67%)	14(28.57%)	35(71.43%)

DISCUSSION

S. aureus is a widespread cause of infection in hospitals and is most responsible to infect new born babies, surgical patients, old, malnourished persons and patients with diabetes and other chronic diseases both in developed and developing countries (Nimmo *et al*.,2008). Today *S. aureus* has incredible genetic adaptability, which able to get an advantage to live in the presence of antibiotics. Consequently,*S. aureus*became multidrug resistance to different class of drugs (Adebayo *et al.*, 2006). Currently, drug resistance *S. aureus* increasingly reported that makes treatment of serious infection complicated(Daniyan and Sani, 2011)

The highest frequency of *S. aureus isolates* (40%) in the present study was observed in (1 month -18yrs) age group and (40%) adults (19-60) age groups followed by (20%) neonate's ≤ 28 days. This indicates that, (60%) frequency of *S. aureus*was seen under the age of 18 yrsand less. This value is in line with prevalence of 40.4% by (Dagnachew *et al.*, 2013), 53.75% (Alebachew *et al.*, 2012), 60%(Kristin A., *et al*, 2012), 60% (Emmanuel and Magaji, 2011), 64% (Idighri *et al.*, 2012)and 67.9% (Ndip, *et al.*, 2012). It is believed that neonates and children's immunity is not good enough to cope up the bacterial infections; this may be making them vulnerable and easily infected by every opportunity especially when hospitalized. Children are more active than fully developed individuals at time theircommunication with their playmates and while playing for a time and expose with different objects. In this course of action, they become a target to different bacteria such as *S. aureus*. However, this result is lower than the study in Ethiopia 90.3% by Legesse*etal.*, (2012).This may be due to socio-demographic differences that were included in the present study and study in Ethiopia by (Legesse *et al.*, 2012). Because this study was small scale and didn't include representative of each regional state and zones in each regional state.

In the present study, male 36(60%) have shown higher infection prevalence than 24(40%) female infection prevalence and resistance of 28 (77.78%) males and 13 (54.17%) females were seen. This result was agreed with the earlier report of62% male and 38% female (Carvalho *et al.*, 2005), 52.8% male and 47.2% female (Sebastian *et al.*, 2012)and 54% male and 46% female (Chih-Chun *et al.*, 2012). But this result was disagrees with other studies where male47% and female 53%(Dagnachew *et al.*, 2013, 51.3% female and 48.75% male(Kristin A., *et al*, 2012). As far as this study and previous study, still it is not clearly understood why males were more infected than females.

In this study, blood specimen showed the highest infection rate as well as resistance rate 18 (72%) from all clinical specimens. But a study in Nigeria by (Carvalho *et al.*, 2005)and (Tula *et al.*, 2013)shows wound and urine infection was highly prevalent. It may be associated with*S. aureus* infection increased communicability rate by blood during hospitalization and this increment may be due to the inclusion of neonates in this study than the previous study.

Gradually, through time,*S.aureus*buildsup antibioticresistance very rapidly and successfully.In the present study, prevalence of susceptible *S.*

aureus to vancomycin antimicrobial agent was 58 (96.67%). This result strongly agrees with vancomycin 100% (Shahid *et al.,* 2013)and 99% (Patrick *et al*., 2013). Though vancomycin was the first most effective antibiotic drug in this study, study in Mekele, Ethiopia by Legesse*etal.*, (2012) indicated as the second most effective next to ciprofloxacin. This is due to its lack of self-assurance for treatment of each and every one infection caused by *S. aureus* since its pharmaceutical and pharmacokinetic properties. It is accessible scarcely as injectable form. In addition, it has a low absorbency to central nervous system (CNS) so that it can't be used for CNS infections caused by *S. aureus*(Rang *et al*., 2003).

Similarlyin this study, tetracycline showed 78.9% killing efficacy. This result is in agreement with 98% (Haythem *et al.*, 2012), 80%(Simon *et al.*, 2010), 79% (Tekalign and Ketma 2013), 75% (Tewlde *et al.*, 2013), 70%(Legesse *et al.*, 2012), 70.1% (Opere *et al.*, 2013), 68% (Durgadas and Alem, 2009)and 66% (Idighri *et al.*, 2012).But strongly disagrees with susceptibility study in Eritrea 22% (Durgadas and Alem, 2009), Nigeria 31% (Daniyan and Sani, 2011), Nigeria 30% (Akindele *et al.*, 2010)andNigeria (Olaside *et al.*, 2012). There may be associated with countriesirregular use of antibiotics without prescriptions. In the meantime there are no any regulatory policies in developing countries such as Eritrea and Nigeria, left the commonly used antibiotics totally unsuccessful in the treatment of *S. aureus* infections (Obiazi *et al.*, 2007).

In this study, high level of resistance to ampicillin 57(95%) result were consistent with previous report of (Idighri *et al.*, 2012) 90%, (Carvalho *et al.*, 2005) 85% and 79.5%,(Tekalign and Ketma, 2013)76.3% and (Durgadas and Alem 2009)73%.The increased resistance may be linked with previous exposure of *S. aureus* isolates for antimicrobial drugs, which may be the cause forenhanced bearing of resistance. This may happened throughunreasonable prescription, presence of antibiotics without prescription and widespread traditional self-medication which ultimately associated with inadequate dosage and failure to comply with treatment (Yanhong and Wei 2009).In contrast, the lowprevalence of resistance to ampicillin were reported (Tewlde *et al.*, 2013) 48.1%, (Durgadas and Alem, 2009) 26%, this could be time of research conducted and high regional variations of same country and different countries holding people with various background of knowledge, personal hygiene and demographics. It was encouraging to note that vancomycin showed highest susceptibility against all clinical isolates. However out of sixty tested isolates in the present study, 2(3.28%) did not show any sensitivity for this antibiotics which needs a continuous follow-up.

CONCLUSION

Most of *S. Aureus*infected individuals developedantimicrobial resistance property. The prevalence of multiple antimicrobial drug resistance is alarmingly increased. Continuous investigation of antimicrobial drug sensitivity pattern for S. *aureus*is good for selecting appropriate antimicrobial therapy. The result found in the present study confirms that vancomycin and tetracycline are suggested as first line antimicrobial drugfor effective treatment of *S. aureus*infections.However, further antimicrobial test should be conducted with other antimicrobial agent to select

effective antimicrobial agent for *S. aureus* treatment.

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