HEAVY METAL RESISTANCE AND ANTIBIOTIC RESISTANCE IN HOSPITAL ISOLATES OF *STAPHYLOCOCCUS AUREUS* FROM KARACHI

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Abstract

One hundred and five hospital strains of *Staphylococcus aureus* isolated from in-patients and out-patients were examined for sensitivity to heavy-metal ions i.e. cadmium and mercury. Strains were also tested for resistance to antibiotics and the nucleic-acid-binding compounds, ethidium bromide. In the hospital strains of *S. aureus*, resistance to cadmium ion at 50µg/mL was found to be 77% and resistance to mercury at 10µg/mL was found 20%. No strain was found to be resistant to mercury at 50µg/mL. Mercury resistance was found to be low as compared to cadmium. A high number of ethidium bromide resistance isolates were found which have been classified as multi-drug resistances as well on antibiotic resistance. Majority of these strains were resistance to more than three different classes of antibiotics and antibiotype KTPG was most prevalent. The curing studies showed the association of cadmium resistance to plasmid but not for the ethidium bromide resistance. Resistance found to be frequently cured among the tested strains was kanamycin followed by gentamicin. As the heavy metal resistance microorganisms has significant role in the detoxification of polluted environment but the spread of this resistance to the multidrug resistance *S. aureus* is alarming.

Introduction

Staphylococcus aureus is a Gram-positive bacteria acting as one of the main pathogens associated with skin infections, wound infections and more serious outcome such as septicaemia, urinary tract infections, osteomyelitis or endocarditis (Duffy, *et al.*, 2013; Taylor, 2013). Antibiotic resistance of staphylococci is a major public health concern since the bacteria can be easily circulated in the environment. Multiple drug-resistant *S. aureus* have been frequently recovered from foodstuffs, water and biofilms, nasal mucosa of humans (Stefani and Goglio, 2010).

Although resistance phenotype determination is of paramount importance for clinical isolates, the tolerance to antimicrobial substances, even when these are below the resistance/susceptibility breakpoints, may represent a selective advantage for the organism in the environment. It has been hypothesized that in the environment, bacteria may face different types of chemical aggressions, capable of selecting positively or negatively antibiotic tolerance. Presumably, bacteria surviving in hospital wastewater, where antibiotic residues or heavy metals may be discharged, many different challenges than those surviving in a water supplying system, where the organic content and chemical contamination is absent or at trace levels. Such environmental pressures may also be responsible for the prevalence of specific groups of organisms able to deal with the environmental conditions imposed (Faria *et al.*, 2009). The combined resistance to heavy metals was also reported by Aktan *et al.*, (2013).

Researchers have shown that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Reyes *et al.*, 1999).

Plasmids mediating penicillinase production by *Staphylococcus aureus* frequently carry determinants for resistance to heavy-metal ions such as cadmium, arsenate and mercury (Richmond and John, 1964; Novick and Roth, 1968). Resistance to all three metal ions may be associated with multiple antibiotic resistances (Dyke *et al.*, 1970). The high frequency of heavy-metal ion resistance in S. aureus may follow co-selection of heavy-metal ion resistance with penicillin resistance as a result of penicillin therapy (Nakahara *et al*., 1977). Although a number of reports described prevalence of multidrug resistant *S. aureus* (Siddiqi, *et al.*, 2002; Perwaiz *et al.*, 2007; Hafiz *et al.*, 2002; Hakim *et al.*, 2007; Butt *et al.*, 2004) but there are no reports available for frequency of heavy-metal ion resistance in the hospital isolates of S. aureus from the Pakistan.

This study reports the frequency of heavy-metal resistance and antibiotics resistance in recent hospital isolates of S. aureus from the Karachi, Pakistan.

Materials and Methods

Strains, culture media, and growth conditions: The clinical isolates of *Staphylococcus aureus* studied were isolated from various clinical specimens received at the diagnostic laboratories in Karachi. Total numbers of

identified *Staphylococcus aureus* strains were one hundred and five. *Staphylococcus aureus* American type culture collection (ATCC) 29737 was used as a control for the antimicrobial susceptibility pattern. Staphylococci were identified according to growth characteristics in mannitol-salt agar (MSA) and coagulase reactions. Blood agar base (BA); nutrient agar (NA), Mannitol Salt Agar (MSA), Muller Hinton Agar (MHA), Brain Heart Infusion broth (BHI)and DNase agar were purchased from Oxoid and DNAase agar was purchased from Merck. All isolates were stored on BHI (Oxoid) slopes at 4^{o}C and stock cultures were maintained at -35^{o}C in 40% glycerol.

Biochemical tests: Isolated strains were biochemically identified by conventional tests i.e. catalase and tube coagulase as described by Bannerman *et al.*, (2003).

Heavy metal resistance: Heavy metal resistance of *Staphylococcus aureus* isolates were determined by agar dilution method. Different concentrations of heavy metal were added in MHA plates. Concentrations for Cadmium chloride, Mercuric chloride, Ethidium bromide and CTAB were as follows 50µg/mL, 10 and 50µg/mL, 20µg/mL and 12µg/mL respectively. Range of concentrations covered those usually employed in similar studies done to find metal resistance in *Staphylococcus aureus* (Udo and Grubb, 1990). Cultures were inoculated on metal supplemented MHA plates and were incubated at 37^{o}C for 2 days. Plates containing media with no added metal were inoculated in the same manner to serve as controls. Control strains used for all assays included *S. aureus* ATCC 29737. All tests were carried out in duplicate. After incubation growth corresponds to, no growth refers to sensitive to that metal ion concentration.

The antibiotic susceptibility test: Antibiotic susceptibility was determined by agar dilution method using Muller Hinton Agar (Oxoid). MIC levels were monitored according to the guideline of Clinical Laboratory Standards Institute. 2006).

The antibiotics used were penicillin, gentamicin, kanamycin, amikacin, tobramycin, ciprofloxacin, levofloxacin, sparofloxacin, and getifloxacin, tetracycline, lincomycin, erythromycin, rifampicin, mupirocin and vancomycin. Incubate the MIC plates at 37^{o}C for 18-20 hours. The MIC value for the organism is the antibiotic concentration of the first plate showing ≥ 99% inhibition of growth.

Plasmid curing and loss of phenotypic resistance: Plasmid curing was carried out by growing isolates at higher temperature i.e. at 43^{o}C on BHI agar plates for 24 hours as described by Ashesov (1966). The grown colonies were subsequently screened for loss of the resistance. To confirm the marker curing, antibiotic assay was performed by replica plate technique. Plasmid curing studies were performed on cadmium and ethidium bromide resistant isolates. The strains also showing antibiotic resistance to aminoglycoside class including kanamycin, gentamicin, amikacin, and tetracycline.

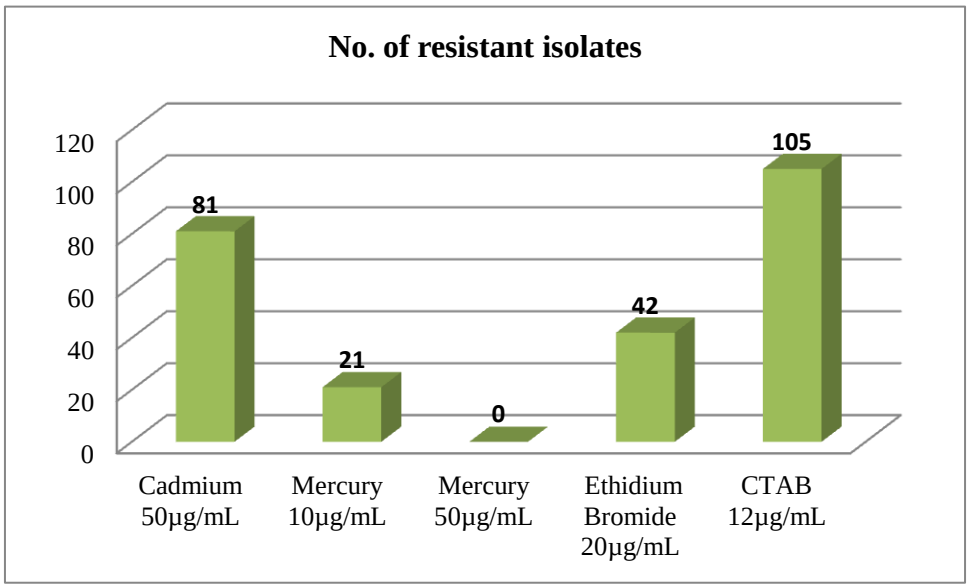

Fig. 1. Number of S. aureus isolates resistant to cadmium, mercury, ethidium bromide and CTAB.

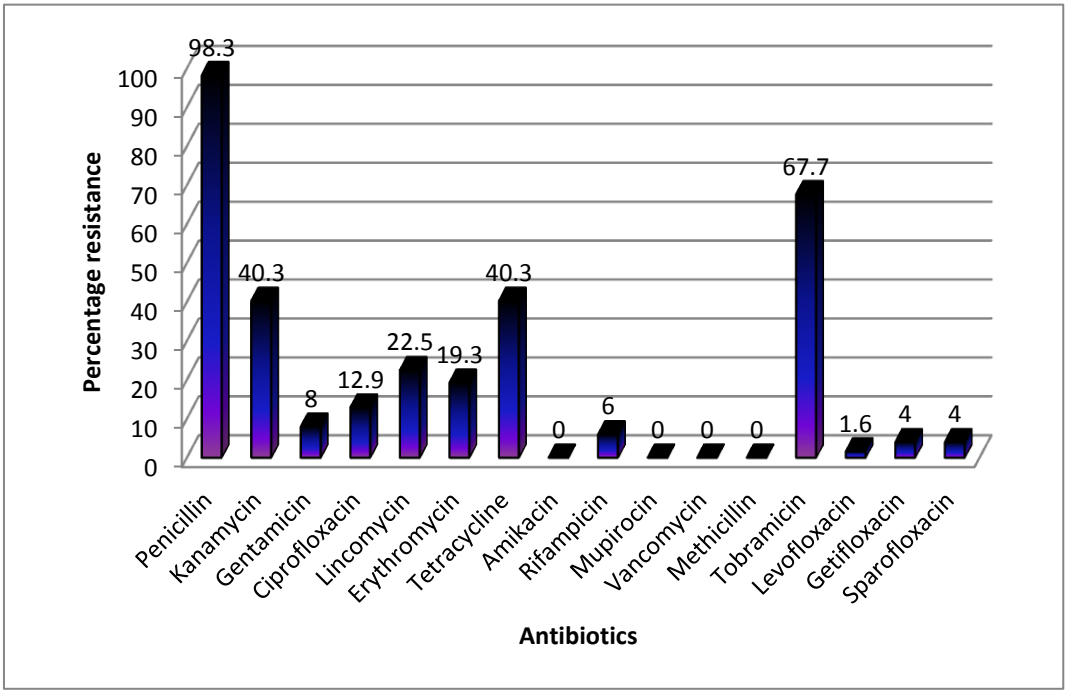

Fig. 2. Antibiotic resistance pattern of Staphylococcus aureus determined by miniumal inhibitory concentration method.

Table.1 Effect of Plasmid curing on drug resistance pattern and on heavy metal resistance of the S. aureus isolates.

Strain No	Antibiogram		Marker cured
	Pre-Curing	Post-Curing	
EM005	KTP Cad	KTP	Cad
EM006	KTP Cad Eb	TP Eb	K Cad
EM007	KTP Cad Eb	KTP Eb	Cad
EM008	KTPG	KTP	G
EM010	KTP Cad	TP Cad	K
EM017	KTP Cad Eb	P Cad Eb	K T
EM020	TPG Cad	T P Cad	G
EM031	KT	T	K
EM035	KTP Cad Eb	TP Cad Eb	K
EM039	KTPG Cad	TP Cad	K G
EM051	KTP Cad Eb	TPG Cad Eb	K
EM074	KTP GA Eb	KTPG Eb	A
EM079	KPG	K P	G
EM095	KTP Cad	TP Cad	K
EM097	KP Cad	P Cad	K
EM099	KTPG	KTP	G

KEY: A= Amikacin, K =Kanamycin, T= Tetracycline, P= Penicillin, G= Gentamicin, Cad = Cadmium resistance, Eb= Ethidium Bromide

Results and Discussion

Heavy metal and other resistance: A total of one hundred and five strains of *Staphylococcus aureus* isolated from in-patients and out-patients were identified on the basis of morphological, cultural and biochemical characteristics according to the Bannerman *et al.*, 2003). All the strains were tested for their resistance against certain heavy metal ions, *i.e.* Hg^{2+} & Cd^{2+}. Fig.1 showed 77% of *Staphylococcus aureus* from hospital were resistance to cadmium ions at the concentration of 50 µg/mL, Our result showed relatively low resistance to Cd^{2+} as compare to several studies where the frequency of cadmium resistance in strains of *S. aureus* has been reported to exceed 80% (Dyke *et al.*, 1970; Kondo *et al.*, 1974 ; Rosdahl and Rosendal, 1980). Explanations for the high frequency of cadmium resistance include co-selection of cadmium resistance by penicillin therapy because determinants for penicillinase synthesis are usually found between genes coding for cadmium and arsenate resistance in penicillinase plasmids (Novick *et al.*, 1979; Shalita *et al.*, 1980). Another explanation involves selection of cadmium resistance by environmental cadmium. All of the *S. aureus*, included in this study were found to be 100% resistant to benzyl penicillin.

The majority of the mercury resistant strains *i.e.* 21 out of 105 were showing this resistance phenotype at mercuric chloride concentration of 10µg/mL whereas all the strains were found to be sensitive to 50 µg/mL of mercuric chloride. Mercury resistance was found to be low as compare to cadmium, *i.e.* only 20% of the strains were resistance. Hospital populations of staphylococci have been shown to have a large fraction of mercury-resistant strains (Parker and Jevons, 1963) characteristics likely to promote survival in the hospital environment (Hall, 1970). Mercury resistance has been associated, however, with resistance to penicillin and tetracycline (Green, 1962; Moore, 1960).

The 40% of the strains were found to be resistant to a nucleic acid binding compound *i.e.* Ethidium bromide. Ethidium bromide resistance has been reported as a characteristic of Australian MRSA strains (Townsend *et al.*, 1985). However we found ethidium bromide resistance in the 42 isolates included in this study. Resistance to Cetrimonium bromide, (CTAB) that is an amine based cationic quaternary surfactant, was found to be 100% at the concentration of 12µg/mL is comparable to the Udo and Grubb, 1990).

Antibiotic susceptibility test: In the present study, the antibiotic resistance profiles of *Staphylococcus aureus* isolates done by MIC agar dilution method showed that these clinical isolates were resistant to multiple antibiotics. It is noted that none of the strain is found susceptible to all antibiotics tested however, a total of 104 *S. aureus* were resistance to penicillin G. Penicillin was proved to be the least effective antibacterial agent, in addition, 57.1% were resistant to kanamycin and 52.3% offered resistance to tetracycline and tobramycin, 21.9% to sparofloxacin, and resistance to lincomycin, ciprofloxacin, gentamicin and erythromycinwas found to be 34-35%. All the collected strains were entirely susceptible to vancomycin and mupirocin and the percentages of isolates resistant to amikacin, levofloxacin, getifloxacin, rifampicin and fusidic acid was less than 15%, these results are presented in Fig. 2.

Plasmid profiling and curing: A total of 60 isolates were selected for the Plasmid curing., the isolates were classified into three subgroups, first only antibiotic resistance strains, second antibiotic and cadmium resistance strain and third antibiotic, cadmium and ethidium bromide resistant strains. The most prevalent antibiotype was KTPG among the strains, so therefore we selected the kanamycin, tetracycline, penicillin and gentamicin resistant isolates for plasmid curing. Table #1 represent the result of curing on the heavy metal and drug resistance of 16 representative *S. aureus* isolates. Most of the strains were cured only for antibiotic resistance marker. 15% kanamycin resistance strains were cured and gentamicin resistance curing was observed in 16.6% of tested strains. One of the tested strains *i.e.* EM074 cured for amikacin resistance which has a biotype of KTPGAEb.

Only 26.6% of the tested strains cured for mostly for drug marker by this elevated temperature method as Durve *et al* (2013), found that ethidium bromide (100 µg/ mL) was a much efficient agent for curing the plasmid. Cadmium resistance was cured in only three strains (EM005, EM006 and EM007) and no ethidium bromide resistant strains were cured. EM006 strains showed the simultaneous loss of cadmium plus kanamycin resistance during curing study (Durve *et al.*, 2013).

Conclusion

The following conclusion can be drawn from the present study.

- The high percentage of cadmium, ethidium bromide and mercury resistance further to the already serious problem of the management of infection caused by multi-drug resistant *S. aureus.*
- Due to the generous use of antibiotics and metal containing disinfectants (especially mercury), the hospital discharge waste water can lead the resistant strains to the environmental reservoir.
- Knowledge of the gene associations on plasmid between antibiotic and heavy metal resistance will defiantly need to improve in current area of study.

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