International Journal of the Physical Sciences Vol. 6(6), pp. 1501-1507, 18 March, 2011
Available online at http://www.academicjournals.org/IJPS
DOI: 10.5897/IJPS11.057
ISSN 1992 - 1950 ©2011 Academic Journals

Full Length Research Paper

Bactericidal activity of various brands of cephradine against *Staphylococcus aureus*

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Accepted 27 January, 2011

A retrospective eight-year study (January 1998 to December 1999 and January 2008 to December 2009) was under taken in a Government Hospital in Islamabad, Pakistan to evaluate bactericidal activity of various brands of cephradine against *Staphylococcus aureus.* The maximum resistance of the bacterium was observed for ampicillin, penicillin and cotrimaxazole. Where as, the minimum resistance was exhibited against vancomycin. One hundred and fifty five clinical isolates were further tested for minimum inhibitory concentration against various brands of cephradine. The high level of bacterial resistance to different cephradine brands was observed for these isolates after incubation of 18 to 24 h. Majority of the isolates had MIC 50 to 90 in response to 64 to 512 µg/ mL of various brands of cephradine. While, 512 and above 1024 µg/mL of antibiotic had shown the maximum bactericidal activity. The study suggested that the presence or absence of antibiotic-resistant subpopulations among *S. aureus* isolates and their selection during treatment should be considered when therapy is advised.

Key words: *Staphylococcus aureus,* cephradine, minimum inhibitory concentration, minimum bactericidal concentration.

INTRODUCTION

Staphylococci inhabit the nasopharynx and skin of most healthy people and prevail our homes and hospitals, but few belong to virulent strains and they are infectious and frequently become invasive and progressive coagulase-positive *Staphylococcus aureus. Staphylococci* can cause inflammation in any body site. Coagulase-negative *Staphylococci* (*Staphylococcus epidermis and Staphylococcus Saprophyticus*) are less virulent and infect mainly previously damaged tissues and hosts (Frobe*s* et al., 2002). *S. aureus* isolates from human sources confirms that they are normal inhabitants of skin and mucus membranes and are opportunistic pathogens (Mahmood et al., 2001).

Staphylococci can produce disease both through their ability to multiply and spread widely in tissues. *S. aureus* produces a large number of proteins that specially bind to human plasma or the extracellular matrix. Some strains are capable of producing a highly heat stable protein or toxin that causes illness in humans (FDA, 2003).

The emergence of pathogens with increased resistance to available antibacterial has led to development of modern antibiotics with improved activity for these resistant isolates (Polk, 1999). We report the antimicrobial susceptibility and the selection of antibiotic resistance among these isolates, as well as efficacy of various brands of cephradine as cephalosporins are

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prescribed in majority.

MATERIALS AND METHODS

The study was conducted at Pakistan Institute of Medical Sciences, Islamabad, during January 1998 to December 1999 and January 2008 to December 2009. The sensitivity patterns of *S. aureus* isolated from various clinical samples brought to microbiology laboratory were determined against commonly used antibiotics by disc diffusion method.

Identified morphological and biochemical characterization of bacterial strains

Isolated colonies, after purification were initially Gram-stained by using Bergey's manual of determinative bacteriology (9^{th} edition). The isolates were biochemically characterized and identified up to species level by performing various biochemical tests.

1. β-Hemolysis
2. Coagulase test
3. Catalase test
4. DNase test
5. Manitol salt agar (Collin et al., 1998).

Disc diffusion (Bauer-Kirby) susceptibility test

The disc diffusion test was done for each Gram-positive cocci on Mueller-Hinton agar (CM337-OXOID) as growth medium (Bauer et al., 1966). The presence of turbidity in broth cultures was adjusted according to 0.5 McFarland standards to obtain standardized suspension by adding sterile saline against a white background with contrasting black line (Connie and Geogre, 2000).

Determination of minimum inhibitory concentrations (MIC) by agar dilution method

Agar dilution method was used to determine the minimum inhibitory concentrations (MIC), or the lowest concentration of antimicrobial agent required to inhibit the microorganism. Serial two-fold dilution concentrations of cephradine active pharmaceutical ingredient (API) and their standard powder (SP) and their brands in form of injectables of cephradine with the information such as potency, manufacturer/suppliers and expiry dates (market brands), were obtained from local market.

Stock solutions of all antimicrobials (SP) were prepared by calculating the (appropriate weight) with the help of following formula:

$$\text{Weight of powder (mg)} = \frac{\text{Volume of solvent (mL) x Concentration (mg/mL)}}{\text{Potency of powder (mg/g)}}$$

Whereas, stock solutions of different brands, were prepared according to their labeled potencies/concentrations and instructions to reconstitute. Stock solutions were dispensed in aliquots and stored immediately at - 70°C.

Four to five well isolated colonies of various *S. aureus* isolates from a blood agar plate were inoculated in tube containing 5 mL of tryptone soya broth (CM129-OXOID) and incubated at 35°C until it achieved or exceeded the turbidity of 0.5 McFarland standard. After incubation, if turbidity was exceeded McFarland adjusted standard against 0.5 McFarland standard using sterile saline to give the density equivalent to them it was approximately 10^8 cfu/mL. Then it was diluted accordingly to give 10^4 cfu/mL and of 10µL of inoculum was transferred on Mueller-Hinton agar plates containing various concentrations of antibiotics with the help of micropipette. Mueller-Hinton agar plates containing antibiotics was prepared as by sterilized Mueller-Hinton agar was cooled to 50°C in a water bath, after autoclaving.

A dilution series of antimicrobial agents was prepared depending upon antimicrobial agent. Nineteen millilitres of molten agar was added to each container. The containers were mixed thoroughly, and agar was poured into pre-labeled sterile Petri-plates on a level surface. The Petri-plates were allowed to set at room temperature and dried so that no drops of moisture remain on the surface of the agar. Plates were marked with concentration and for orientation. Using micropipette, inoculum of 10^4 cfu/mL was transferred to the series of agar plates, including a control plate without antimicrobial agent and inoculum spots were allowed to dry at room temperature. Plates in inverted position were placed in an incubator at 35°C for 18 h. Control strains were also run with the isolated strains of *S. aureus* (NCCLS, 1993b).

The MIC50 and MIC90 values were determined by applying regression analysis method as described by Somasegaran et al. (1982). These values were used for further statistical analysis.

Determination of minimum bactericidal concentration

The minimum bactericidal concentration (MBC) of an antibiotic is the concentration of antibiotic that kills at least 99.9% of a standardized bacterial inoculum. Inoculam of isolates and control *S. aureus* were sub-cultured on blood agar plates and incubated overnight at 35°C. Five colonies from the agar plates were inoculated in a tube containing 3 mL of Mueller-Hinton broth (CM405-OXOID) and turbidity was adjusted to match 1.0 McFarland standard (approximately 10^8 cfu/mL). An amount of 0.1 mL of the turbid broth was transferred into 10 mL of Mueller-Hinton broth and incubated in shaking water bath at 35°C until turbidity appeared. Two-fold serial dilutions of the antibiotic were prepared in 2 mL of Mueller-Hinton broth.

Using micropipette 0.1 mL of diluted inoculum was dispensed into the tubes that contained serial dilutions of antibiotic, which makes the final inoculum size approximately 2.5 x 10^5 cfu/mL. The tubes *S. aureus* were then incubated for 20 h at 35°C.

After overnight incubation, tubes without growth were vortex vigorously for 15 s to re-suspend any bacteria that might have adhered to the walls of the tube, and were reticulated for an additional 4 h.

After the 4 h of additional incubation, visually clear tubes were again vortex and 0.1 mL from each tube was spread across the surface of the blood agar plates with the help of sterile bent glass rods. The plates were then incubated overnight at 35°C. For determination of the MBC, the number of colonies that grow on each plates after overnight incubation were counted and compared to the number of cfu/mL in the original inoculum, which was determined by counting a dilution of the quantitative subculture that yields between 20 to 200 colonies after incubation for the same length of time that the *S. aureus* subcultures were incubated. The concentration, at which the Petri-plates each isolate showed either no growth or reduction of 99.9% from inoculum, was considered as the MBC of the specific antimicrobial agent (NCCLS, 1993a).

Statistical analysis

For calculations of MIC90 p-value among the standard powder and various brands of antibiotics were calculated by Instat-2 program for calculating one-way-anova, non-parametric and Tuckey test for determining the statistical significance difference at 5% level of

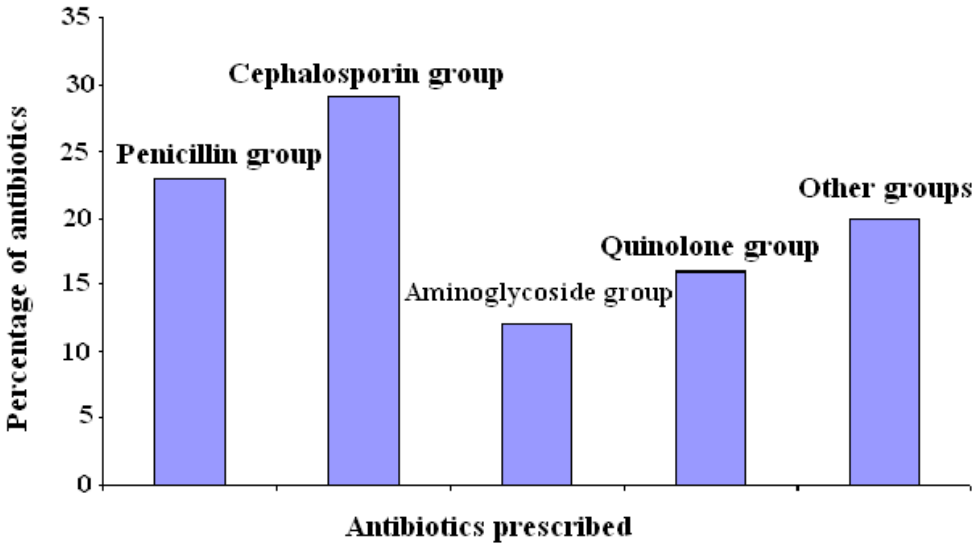

Figure 1. Antimicrobial therapy of hospitalized patients during 1998-1999.

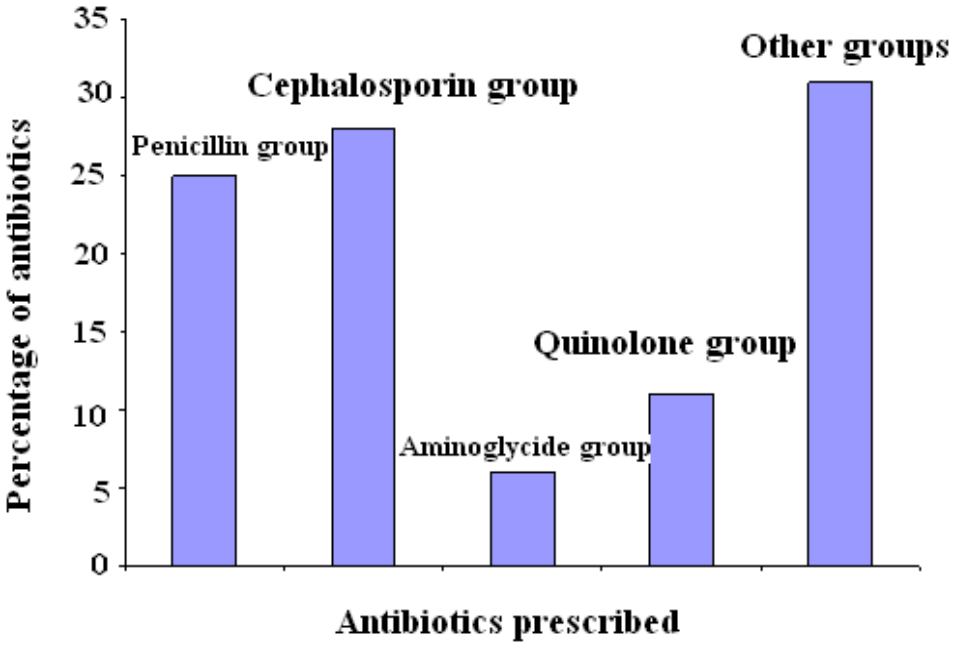

Figure 2. Antimicrobial therapy of hospitalized patients during 2008-2009.

significance.

RESULTS

The study conducted to determine the clinically significant *S. aureus* isolates, during a period of eight years in microbiology laboratory, Pakistan Institute of Medical Sciences (PIMS), Islamabad, Pakistan, its identification and sensitivity study, as well as bactericidal activity of various brands of cephradine against clinical isolates of *S. aureus*. During the first two years the maximum antibiotic group was prescribed cephalosporin (29%), penicillin (23%), other group (20%), quinolone (16%) and aminoglycoside (12%). Whereas in succeeding two years the maximum prescribed group of antibiotics were others (31%), cephalosporins (28%), penicillin (25%), quinolone (11%) and aminoglycoside (6%) (Figures 1 and 2).

Table 1. Percentage resistance of five groups of antibiotics against *staphylococus aureus* during 1998-1999 and 2008-2009

Antibiotic group prescribed	Percentage resistance during 1998-1999	Percentage resistance during 2008-2009
Penicillin	63	53
Cephalosporin	36	50
Aminoglycoside	26	48
Quinolone	16	25
Others	40	30

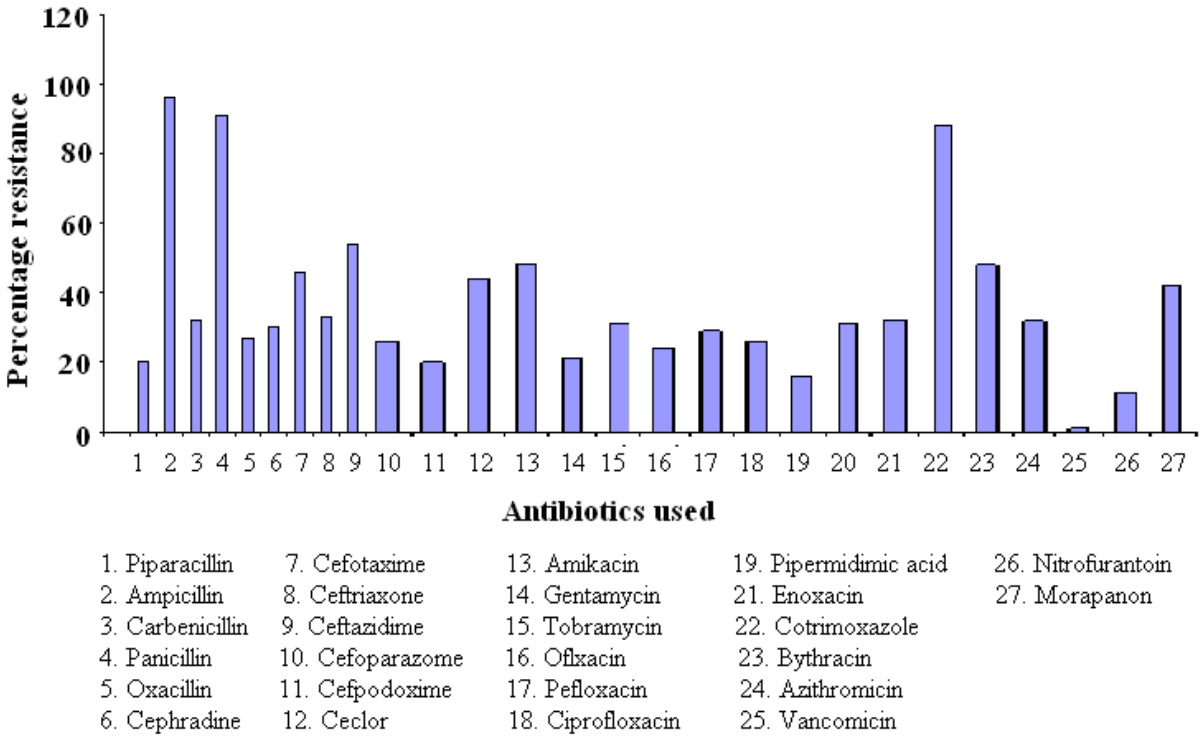

Figure 3. Antibiotic resistance pattern of *staphylococus aureus* against various antibiotics during 1998-1999.

Sensitivity study

The susceptibilities of *S. aureus* clinical isolates from pus, urine and blood were tested against various antibiotics by disc diffusion method. Different antibiotics representing the major groups were used to assess susceptibility pattern of *S. aureus* isolated during 1998 and 1999 and 2008 to 2009.

In this study, among the commonly used group of antibiotics, penicillin group was found to be most resistant towards *S. aureus* were 63% (penicillin), 40% (other group), 36% (cephalosporin), 26% (aminoglycoside) and 16% (quinolone) during 1998 to 1999. Whereas during 2008 to 2009, 53% (penicillin), 50% (cephalosporin), 48% (aminoglycoside), 30% (other group) and 25% (quinolone) (Table 1). The most ineffective antibiotic was ampicillin (95%) isolates of *S. aureus* were resistant, penicillin (90%) followed by cotrimaxazol (80%), ceftriazone (57%), cephradine and ceclor (50%), erythromycin (48%), meropenam (40%) and gentamycin (30%). Whereas vancomycin was most effective with more than 95% isolates sensitive during 1998 to 1999 (Figure 3).

This study demonstrated that among the most commonly used antibiotics during the period of 2008 to 2009, exhibited the highest resistance towards penicillin with resistance rate of penicillin (93%), ampicillin (91%), cotrimaxazol (77%), tobramicin (58%), cefaclor (54%), amikacin (50%), erythromycin, tetracycline, nitrofurantoin, lincomycin and cefixime (44%). Moreover, vancomycin was found to be sensitive for more than 93% isolates (Figure 4).

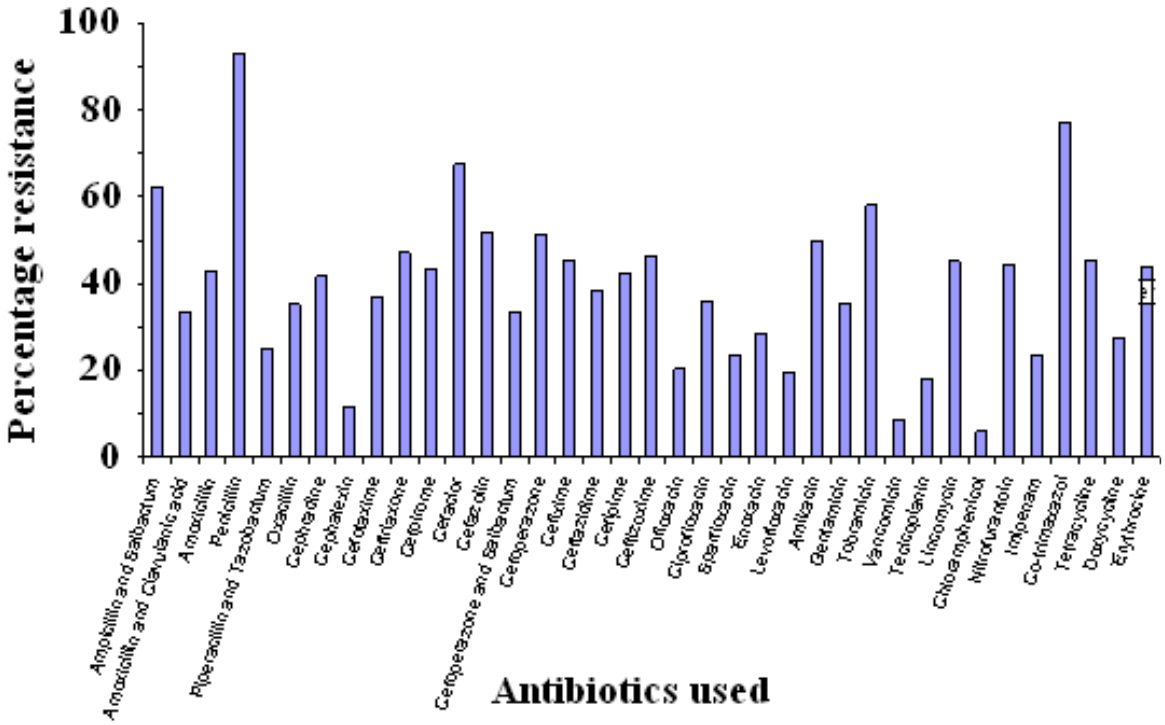

Figure 4. Antibiotic resistance pattern of *staphylococus aureus* against various antibiotics during 2008-2009.

MIC of cephradine

The comparison of MIC50, MIC90 and statistical analysis of MIC90 cehpradine standard powder with four brands of cephradine against 155 clinical isolates of *S. aureus* are summarized in Table 1, MIC90 of 25 isolates against standard powder was 2048 µg/ml and only 3 isolates were inhibited at the same MIC (2048 µg/ml) for velosef. While most of the isolates had MIC90 range of cefatil, monosef and cefrinex lied in the range of 4 to 1024 µg/ml. The MIC50 range of cefatil, monosef and cefrinex lied within the range of 4 to 512 µg/ml. While for the standard powder MIC90 range remained within 4 to 1024µg/ml. For most of the isolates, which were inhibited in case of velosef, cefatil, monosef and cefrinex range was 4 to 512 µg/ml. The highest break point of cephradine lied at 8 µg/ml. Five isolates were inhibited at break point with standard powder, 6 isolates were inhibited to velosef and 6 for monosef, 2 isolates with cefatil and 3 for cerfrinex. Standard powder inhibited 97% of the isolates at MIC90 while cefatil inhibited 99% of the isolates above the break point. Velosef and monosef inhibited 96% of isolates and cefrinex inhibited 98% of the isolates at MIC90 above the break point level.

The *S. aureus* 543, *S. aureus* 5, *S. aureus* 571, *S. aureus* 112, *S. aureus* 227, *S. aureus* 395, *S. aureus* 380, *S. aureus* 524, *S. aureus* 159, *S. aureus* 310, *S. aureus* 44 and *S. aureus* 60 ($p > 0.05$) for MIC90 were not having significant variation among the standard powder and four brands of cephradine. The MIC90 concentrations do not vary significantly among the standard powder and four brands for rest of the isolates (Table 2).

The MBC values of cephradine standard powder and four brands lied within 32 to ≥1024 µg/ml which was two to three fold of the MIC90 values of cephradine standard powder and its four brands. Standard powder, velosef, cefatil, monosef and cefatil had MBC values 32 to 1024 µg/ml. It was two or three fold higher than the MIC90 values. Table 2 showed that the most of the isolates of *S. aureus* were killed at ≥1024 µg/ml with standard powder and velosef, cefatil, monosef and cefrinex. The percentage of isolates had bactericidal activity ≥1024 µg/ml for standard powder, velosef, cefatil, monosef and cefrinex as 87, 86, 83, 78 and 77%, respectively (Table 3).

DISCUSSION

S. aureus is recognized as an important bacterial pathogen contributing towards hospital infection, globally. *S. aureus* causes localized infection spreading into the blood stream (Espersen, 1995). Despite the use of potent antibiotic still high mortality exist in case of *S. aureus* infection. Antimicrobial resistance prolongs the duration of hospitalization, thereby increasing the cost of patient care problems, worldwide. The selection and spread of resistant organisms in developing countries, which can often be traced to complex socioeconomic behavioral antecedents and misuse of antibiotics by health

Table 2. MICs of various brands of cephradine against clinical isolates of *Staphylococus aureus.*

Brands	MICs	No. of isolates at the following MICs (µg/ml)													
		0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Standard powder	MIC90	0	0	0	0	1	4	2	2	7	9	32	39	34	25
	MIC50	0	0	0	1	4	2	2	7	9	34	35	36	25	0
Velosef	MIC90	0	0	0	0	0	6	2	4	6	9	59	51	15	3
	MIC50	0	0	0	0	6	3	3	6	9	64	46	15	3	0
Cefatil	MIC90	0	0	0	0	0	2	5	5	7	28	69	35	4	0
	MIC50	0	0	0	0	2	6	4	6	29	70	34	4	0	0
Monocef	MIC90	0	0	0	0	0	6	2	1	3	16	51	75	1	0
	MIC50	0	0	0	0	6	2	1	3	16	51	75	1	0	0
Cefrinex	MIC90	0	0	0	0	0	3	4	1	8	85	28	25	1	0
	MIC50	0	0	0	0	3	4	1	8	85	28	25	1	0	0

Table 3. MBCs of various brands of cephradine against clinical isolates of s*taphylococcus aureus*

Brands	No of isolates at the following MBCs (µg/ml).												
	0.5	1	2	4	8	16	32	64	128	256	512	1024	>1024
Standard powder	0	0	0	0	0	0	2	4	3	4	7	12	123
Velosef	0	0	0	0	0	0	6	0	5	5	6	22	111
Cefatil	0	0	0	0	0	0	2	2	5	3	14	39	90
Monocef	0	0	0	0	0	0	2	6	1	4	21	35	86
Cefrinex	0	0	0	0	0	0	4	2	2	6	21	68	52

professionals, unskilled practitioners, and the general public, poor drug quality, unhygienic conditions resulting in spread of resistant bacteria, as well as inadequate surveillance (Schwartz, 1999).

The antibacterial activity of cephradine standard powder and its four brands was determined for 155. Isolates of *S. aureus*, at MIC ≤8 µg/mL, 5 isolates were inhibited and maximum numbers of resistant isolates were found in range of 256 to 1024 µg/mL with standard powder. In case of velosef, cefatil and monosef, majority of isolates were inhibited at MIC, value of 256 to 512 µg/mL, while maximum number of isolates, were inhibited at 128 to 256 µg/mL of these antibiotics. Cephradine is a first generation antibiotic, and is being used more frequently as compared to second and third generation cephalosporins. Among 155 isolates, 96% strains had MIC above the break point of cephradine (NCCLS) with standard powder, velosef and cefrinex whereas 98% resistant isolates in case of cefatil and monocef had lower MIC90. This shows comparatively less resistance development against cephradine.

Ravaerinoro and Terrien (1996) noted resistance against cefazolin (first generation cephalosporin) in MRSA against first generation cephalosporin and various brands of other generation. Range and MIC90 ranged from 64 to 256 µg/mL against MRSA. In the current study, percent distribution of isolates with a similar MIC90 range were 30, 48, 67, 45 and 78% against standard powder, velosef, cefatil, monocef and cefrinex, respectively, which is fairly large percentage resistance. The study carried by Mahmood et al. (2001) showed 29% resistance in *S. aureus* isolates against cephalexin (first generation cephalosporin). This study further highlighted the development of resistance in *S. aureus* against cephalosporin in Pakistan. Lower rate of resistance in *S. aureus* MRSA to cephradine (128 µg/mL) as compared to the current study has been reported by James and Reeves (1996).

The MIC50 and MIC90 value co-relate with one another, as MIC50 was just one dilution lower than MIC90 when assessed for the isolates. Most of the MIC90 values had non-significant difference among the MICs of standard powder and its four brands. Very few isolates had statistically significant differences in MIC90 of standard powder and its four brands ($p < 0.05$). More than 96% resistance to cephradine among these isolates

was seen. James and Reeves (1996) studied resistance in methicillin resistant *S. aureus* against cephradine, which was comparable to present study.

MBC values of cephradine and all the brands were 2 to 3 two-fold dilution higher than MIC against all isolates. Various authors have reported same pattern of MBCs as in the present study, their study showed that MIC and MBC could not be assumed to be in the same range (Visser et al., 1991; Brumfitt and Hamilton-Miller, 1992; Connie and George, 1995; Zaoutis et al., 1999).

Cephradine is a semi-synthethetic cephalosporin that can be given both orally as well as injection (Dollery et al., 1991). Cephradine is being used for more than thirty years. Cephalosporins group was used in previous antimicrobial therapy and was prescribed by doctors, which includes cefaclor, cephradine, cefoperazone, cefotaxine, ceftazidine and ceftriazone. This excessive use of cephalosporins in chemotherapy of hospitalized and out door patients has led to an increase in resistance among *S. aureus*.

The misuse of these antibacterial are often called 'miracle drugs' which are becoming increasingly in-effective in curing and controlling bacterial infections. Clinicians are trying to tackle the problem of increasing drug resistance by using a combination of antibiotics, and counseling patients regarding the deleterious effects of over and misuse of antibiotics (Trakulsomboon et al., 2001).

In many developing countries, the availability and use of antibiotics are poorly controlled, which results in a high rate of resistance particularly to the older antibiotics (Kunin, 1993). The high cost of the few remaining second line antibiotics makes them an unrealistic choice where they are most needed.

Conclusion

The study suggested that, the presence or absence of antibiotic-resistant subpopulations among *Staphylococcus aureus* isolates and their selection during treatment should be considered when therapy is advised.

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