EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF STAPHYLOCOCCI ISOLATED FROM DIFFERENT INFECTIOUS DISEASES

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ABSTRACT

A total of 187 isolates from 470 clinical specimens were collected from three hospitals in El-Minia governorate and identified as 132 *Staphylococcus aureus* strains and 55 coagulase-negative staphylococci (CoNS) strains. Susceptibility of isolates to antimicrobial agents was tested by the agar dilution method. The isolated *S. aureus* strains showed low resistance to vancomycin (1.5%), amikacin (2.3%) and gatifloxacin (3.8%). Vancomycin was the most effective antibiotic against CoNS. The ampicillin-resistant isolates were tested for β-lactamase production where, 61.7% of *S. aureus* and 42.9% of CoNS were positive for β-lactamase enzyme. Beta-lactamase producing strains were screened for their plasmid profile using alkaline lysis method. Some of these strains carried at least one plasmid suggesting plasmid-mediated antibiotic resistance. When cells of these strains were exposed to curing agent ethidium bromide, the production of the β-lactamase was lost. Resistance by efflux was studied by a modified fluorometric assay. Addition of uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) increased norfloxacin accumulation in quinolone resistant *S. aureus* strains, suggesting endogenous energy-dependent efflux. Combinations of ciprofloxacin with four antimicrobial agents against methicillin resistant *S.aureus* (MRSA) strains were investigated using decimal assay for additivity (DAA) technique. Synergistic interaction was observed between ciprofloxacin and oxacillin. ciprofloxacin plus cefepime and gentamicin appeared to be additive, while ciprofloxacin plus erythromycin was antagonistic.

Key words: β-lactamase, CCCP, fluorometric assay, efflux, DAA technique.

INTRODUCTION

In the last decade, the staphylococci have again emerged as the predominant organisms causing infections in the hospital setting. Staphylococci are responsible for a variety of medical problems, including skin and soft-tissue infections, surgical site infections, endocarditis and hospital-acquired bacteraemia. An increasing number of infections are related to medical developments, including the use of joint prostheses, immunosuppressants and catheters (3,8).

Staphylococcus aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as well as community-acquired diseases. The spectrum of staphylococcal infections ranges from folliculitis and furuncles to toxic shock syndrome and

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sepsis (20). Coagulase-negative staphylococci found in the normal skin flora and mucous membranes has recently got attention as a potential pathogen, specifically for nosocomial infections (34,37).

There is a significant increase in the methicillin-resistant staphylococci infections and these bacteria have recently started to gain resistance to many widely used antibiotics (15,19). In spite of the advancements in the antibacterial treatment field, there are still serious difficulties in the treatment of staphylococcal infections. In several countries, vancomycin-resistant staphylococci have been isolated (6,27).

Efflux systems are one of several mechanisms of resistance described for a variety of bacterial species, including *S. aureus*. They protect cells from antibiotics by actively transporting compounds out of the cytoplasm and thereby limit their steady-state accumulation at their site(s) of action. It would be valuable to use bacterial efflux pump inhibitors which is inhibitors of resistance mechanisms, able to potentiate the activity of existing antibiotics (1,22,24).

In this study, the incidence of staphylococcal infections in patients attending El-Minia governorate hospitals was detected. We studied the antibiogram of the isolated strains against different antimicrobial agents as an epidemiological indicator and possible mechanisms of resistance of the isolated strains to different antimicrobial agents. The main mechanisms involved in the bacterial resistance in our study were β-lactamase production, plasmid-mediated antibiotic resistance and efflux mechanism. We also studied some possible combinations for management of MRSA infections.

MATERIALS AND METHODS

Study design: A total of 470 clinical specimens were examined; 100 urine specimens (from patients with urinary tract infection), 195 specimens (from patients with respiratory tract infections), 140 specimens (from patients with skin infections) and 35 specimens (from patients with eye infections). The specimens were collected from different hospitals at El-Minia governorate. The present study was

designed from July 2005 to January 2008. Identification of staphylococci was based on standard laboratory criteria (colony morphology, hemolytic zones, and production of catalase and coagulase) (11).

Antimicrobial Agents: The antimicrobial agents used in this study were obtained from the following manufacturers: ampicillin and chloramphenicol (Chemical Industries Development; CID, Egypt), amoxicillin and cefotaxime (Egyptian International Pharmaceutical Industries Company; EIPICO, Egypt), ampicillin/sulbactam, oxacillin, ciprofloxacin, ofloxacin, norfloxacin, levofloxacin, gatifloxacin, clindamycin and tetracycline from Sigma-Aldrich (St Louis, MO, USA), amoxycillin/clavulanic acid (Beecham Pharmaceuticals, England), cephalexin and cefuroxime (Glaxo Wellcome, Egypt), cefoperazone (Pfizer, Egypt), cefepime and amikacin (Bristol Myers Squibb; BMS, Egypt), gentamicin (Memphis for Pharmaceutical Chemical Industries Co., Egypt), erythromycin (WINLAB, Harborough), vancomycin (Lilly Pharma, Germany), and rifampicin (El Nasr , Egypt).

Determination of antimicrobial susceptibilities: Minimum inhibitory concentrations (MICs) were determined by the two-fold agar dilution method, according to the Clinical and Laboratory Standards Institute (CLSI) (2005) (10) guidelines with Mueller-Hinton agar (MHA). The overnight Mueller–Hinton broth (MHB) cultures of the bacterial strains were diluted with broth corresponding to a final concentration of about 10^7 CFU/ml. Inocula of about 10^5 CFU per spot were applied with an inoculator to the surface of dry MHA plates containing twofold serial dilutions (from 0.25–512 mg/L) of the respective antibiotics. Plates were incubated at 37˚C for 18–24 h and spots with the lowest concentrations of antibiotic showing no growth were defined as the MIC.

Beta-lactamase test: After inoculation of tested isolates on nutrient agar plates and overnight incubation at 37˚C, the plates were overlaid with 1% molten agarose containing 0.2% soluble starch and 1% penicillin and incubated for 15 min at

room temperature; iodine solution was poured onto the agar plates. After 10 s, the residual iodine solution was damped out and the plates were incubated at room temperature until a discolouration zone appeared around β-lactamase producing colonies (35).

Plasmid profiles: Beta-lactamase producing strains were screened for their plasmid profile using alkaline lysis method (2). Agarose gel electrophoresis was performed. The samples were run on 0.8% agarose gel with Tris-Borate (TBE) buffer and ethidium bromide (final concentration 0.5µg/ml). The gel was observed under UV light and a picture was captured using a digital camera.

Curing of β-lactamase: Beta-lactamase producing strains were inoculated in Luria-Bertani (LB) broth containing different concentrations of the curing agent, ethidium bromide ranging from 0.25 to 128 µg/ml and incubated at 37˚C for overnight. The cultures were diluted and subculture was done on nutrient agar plates. The growths obtained were tested for β-lactamase production (7). The plasmid profile of the cured derivatives was also analyzed.

Fluorometric assay: The accumulation of norfloxacin by *S. aureus* cells was determined using the modified fluorescence method (9). Carbonyl cyanide m-chlorophenylhydrazone was added to a parallel set of tubes to a final concentration of 100 µM. The concentration of norfloxacin accumulated was determined on a Perkin-Elmer fluorescence spectrophotometer. The norfloxacin excitation and emission wavelengths used were 277 and 443 nm respectively.

Combination studies: Combinations of ciprofloxacin with oxacillin, cefepime, gentamicin and erythromycin were investigated by the DAA with MRSA strains (32). For the combination decimal mixtures, the mean zone size ($\overline{x}_{comb}$) was calculated from data obtained with combination mixtures, 95% confidence intervals (*t* distribution) were calculated for this mean. Results were considered indicative of synergism if $\overline{x}_{comb} > \overline{x}_{drug\,A}$ and $\overline{x}_{drug\,B}$ and the 95% confidence intervals for $\overline{x}_{comb}$ did not overlap those for $\overline{x}_{drug\,A}$ or $\overline{x}_{drugB}$, antagonism if $\overline{x}_{comb} < \overline{x}_{drug\,A}$ and $\overline{x}_{drug\,B}$ and the 95% confidence intervals did not overlap and all other results were considered additive.

RESULTS

Incidence of staphylococcal species in clinical specimens

Out of the 470 clinical specimens, 187 (39.8%) staphylococcal strains were isolated and identified. Out of the 187 isolates, 132 were *S. aureus* and 55 were CoNS (70.6% and 29.4% respectively). The incidence of staphylococcal isolates was shown in Table 1.

Antibiotic susceptibility and determination of MICs

Tables 2 and 3 show the respective MIC distributions of different antibiotics for staphylococcal isolates. Figures 1 and 2 show whether the bacteria were sensitive, intermediately sensitive or resistant to each antibiotic. The isolated *S. aureus* strains showed lower resistance to vancomycin, amikacin and gatifloxacin. Vancomycin was the most effective antibiotic against CoNS.

Table 1. Incidence of staphylococcal species in clinical specimens

Infection/colonization	Number of samples (n=470)	Number of *S. aureus* isolates (n=132)	%*	Number of CoNS isolates (n=55)	%*
Skin	140	60	42.9	20	14.3
Respiratory tract	195	53	27.2	10	5.1
Urinary tract	100	13	13	21	21
Eye	35	6	17.1	4	11.4

ᵃPercentage was correlated to the number of specimens collected from each type of infection/colonization.

Table 2. Distribution of MICs of different antibiotics among *S. aureus* isolates (132 isolates)

Antibiotic	Break Point[*] µg/ml	MIC in µg/ml											
		≤0.25	0.5	1	2	4	8	16	32	64	128	256	512
β-lactams:													
a. Penicillins:													
Ampicillin	0.25	17	15	13	9	14	12	14	9	13	8	6	2
Amoxycillin	0.25	24	16	6	7	15	18	10	13	9	7	6	1
Oxacillin	2	18	35	32	15	7	8	5	6	2	2	1	1
b.Cephalosporins:													
Cephalexin	16	2	3	5	7	5	8	16	28	19	23	12	4
Cefuroxime	8	3	5	6	8	13	11	17	24	20	14	8	3
Cefoperazone	16	5	5	6	3	21	1	12	14	23	14	7	0
Cefotaxime	8	2	2	6	12	24	22	14	6	28	17	12	1
Cefepime	8	17	28	13	12	3	8	12	23	11	0	0	0
β-lactam β-lactamase inhibitor combinations:													
Amp/Sul[**]	8	8	22	17	10	16	14	6	21	10	8	0	0
Amox/Clav[**]	4	10	15	17	21	26	19	12	8	4	0	0	0
Aminoglycosides:													
Gentamicin	4	11	18	28	32	20	8	7	5	3	0	0	0
Amikacin	16	24	30	43	24	5	3	3	0	0	0	0	0
Fluoroquinolones:													
Ciprofloxacin	1	22	18	35	23	14	7	6	4	3	0	0	0
Norfloxacin	4	10	3	22	6	26	27	16	10	9	3	0	0
Ofloxacin	1	24	21	32	23	16	9	5	1	1	0	0	0
Levofloxacin	1	33	42	20	26	5	3	3	0	0	0	0	0
Gatifloxacin	0.5	68	30	29	1	2	2	0	0	0	0	0	0
Macrolides: Erythromycin	0.5	20	9	12	14	16	25	14	8	6	5	2	1
Lincosamides: Clindamycin	0.5	36	30	21	9	6	10	8	5	4	2	1	0
Tetracyclines: Tetracycline	4	2	15	10	12	5	20	18	15	10	5	7	3
Glycopeptides: Vancomycin	4	21	20	29	25	17	8	10	2	0	0	0	0
Phenicols: Chloramphenicol	8	6	8	10	19	6	25	12	16	10	8	7	5
Ansamycins: Rifampicin	1	14	25	31	26	20	9	5	2	0	0	0	0

[a]Break points of different antibiotics according to CLSI (2005).

Table 3. Distribution of MICs of different antibiotics against CoNS isolates (55 isolates)

Antibiotic	Break Point[*] µg/ml	MIC in µg/ml											
		≤0.25	0.5	1	2	4	8	16	32	64	128	256	512
β-lactams:													
a. Penicillins:													
Ampicillin	0.25	6	5	4	3	6	5	8	6	3	5	1	2
Amoxycillin	0.25	11	11	5	4	2	6	4	4	2	3	1	1
Oxacillin	2	10	15	12	5	2	3	2	2	1	1	2	0
b.Cephalosporins:													
Cephalexin	16	0	0	2	3	2	4	6	10	12	8	5	3
Cefuroxime	8	0	0	3	4	1	8	9	14	9	5	1	1
Cefoperazone	16	0	0	5	7	2	8	5	6	11	9	2	0
Cefotaxime	8	0	0	2	2	3	5	7	8	13	9	3	3
Cefepime	8	6	7	5	3	4	7	5	8	6	4	0	0
β-lactam β-lactamase inhibitor combinations:													
Amp/Sul**	8	2	3	8	9	5	8	4	7	7	2	0	0
Amox/Clav**	4	7	6	5	3	7	9	7	3	4	3	1	0
Aminoglycosides:													
Gentamicin	4	9	12	10	9	7	2	3	2	1	0	0	0
Amikacin	16	16	13	6	11	5	2	2	0	0	0	0	0
Fluoroquinolones:													
Ciprofloxacin	1	0	8	12	13	9	7	3	1	2	0	0	0
Norfloxacin	4	0	4	3	7	3	9	12	8	5	4	0	0
Ofloxacin	1	3	12	10	8	11	6	3	1	1	0	0	0
Levofloxacin	1	13	21	10	8	2	1	0	0	0	0	0	0
Gatifloxacin	0.5	25	20	8	1	0	1	0	0	0	0	0	0
Macrolides:													
Erythromycin	0.5	5	6	8	9	6	7	2	3	3	4	1	1
Lincosamides:													
Clindamycin	0.5	10	12	11	8	5	2	3	2	1	1	0	0
Tetracyclines:													
Tetracycline	4	0	6	8	4	5	7	9	6	5	2	2	1
Glycopeptides:													
Vancomycin	4	12	6	9	11	5	7	4	4	1	0	0	0
Phenicols:													
Chloramphenicol	8	0	5	2	8	6	7	3	9	6	2	5	2
Ansamycins:													
Rifampicin	1	6	8	11	14	10	4	2	0	0	0	0	0

[a]Break points of different antibiotics according to CLSI (2005).
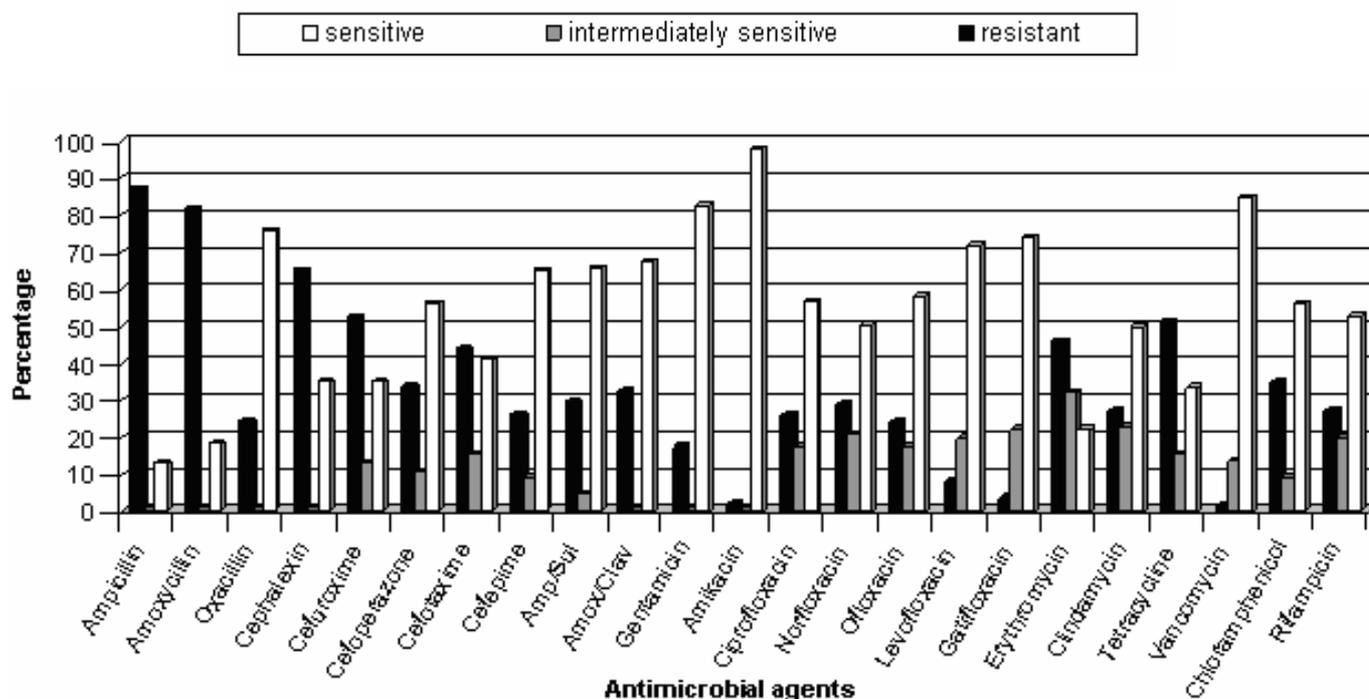
Figure 1. Antibiotic susceptibility ofS. aureus isolates
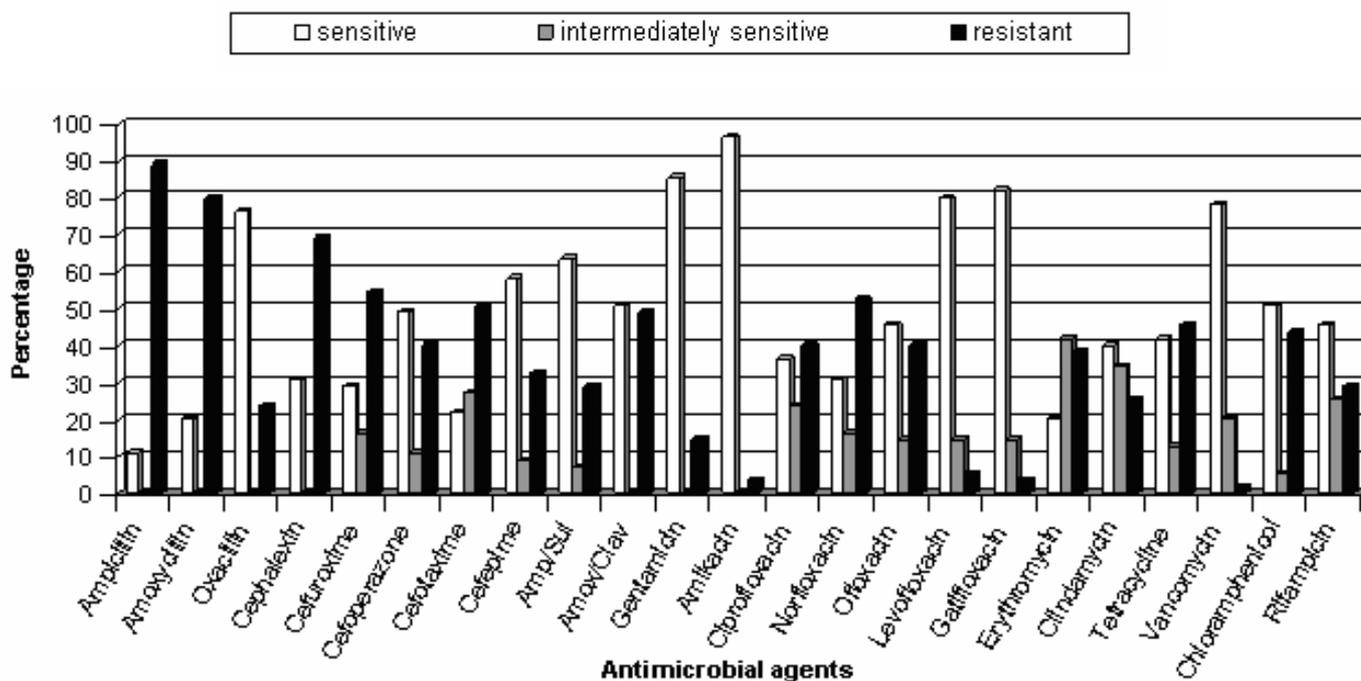

Figure 2. Antibiotic susceptibility of CoNS isolates

Resistance through β-lactamase production

It was found that 61.7% of ampicillin-resistant *S. aureus* strains and 42.9% of ampicillin-resistant CoNS strains were β-lactamase producers (Figure 3).
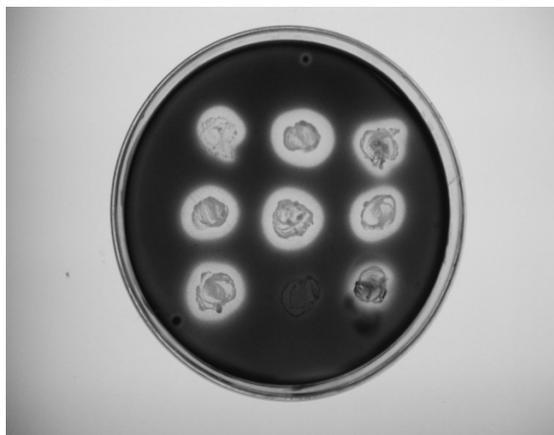

Figure 3. Beta-lactamase production by the isolated staphylococci

Plasmid-mediated antibiotic resistance

Some β-lactamase producing strains carried at least one plasmid and others don't (Figure 4). Results of curing revealed that when these cells were exposed to curing agent ethidium bromide, the production of the β-lactamase was lost (Figure 5). When the plasmid profile of the cured derivatives was analyzed, none of the isolates harbored plasmids. This indicates that β-lactamase production by these strains were plasmid-mediated. On the other hand, strains which were resistant to ampicillin, β-lactamase producers and did not show plasmid bands were not cured and did not loose their enzyme activity. This evidence that β-lactamase production by these strains might be chromasomally mediated.
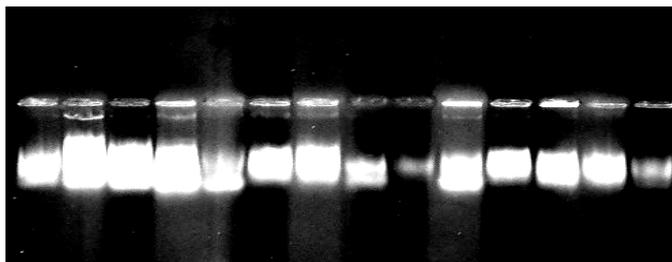
Figure 4. Agarose gel electrophoresis of plasmid DNA of the selected ampicillin-resistant isolates.

Figure 5. Beta-lactamase detection after plasmid curing.

Resistance through the efflux system

A biphasic pattern of accumulation of norfloxacin was observed with an initial rapid phase of accumulation seen within the first 5 minutes. The rapid phase was followed by a plateau phase. Norfloxacin-sensitive strain 1 took up higher amount of norfloxacin than did any of the resistant strains tested (2, 3, and 4) and the addition of CCCP did not affect the accumulation (figure 6). In norfloxacin-resistant strains, norfloxacin uptake was reduced when compared with that in norfloxacin-sensitive strains, and the uptake of norfloxacin by these strains increased rapidly after the addition of CCCP (137%, 191% and 218% for 3 resistant strains) (Table 4). Thus addition of uncoupler CCCP increased norfloxacin accumulation in quinolone resistant *S. aureus*, suggesting endogenous, energy-dependent efflux (Figure 6, 7, 8, and 9).
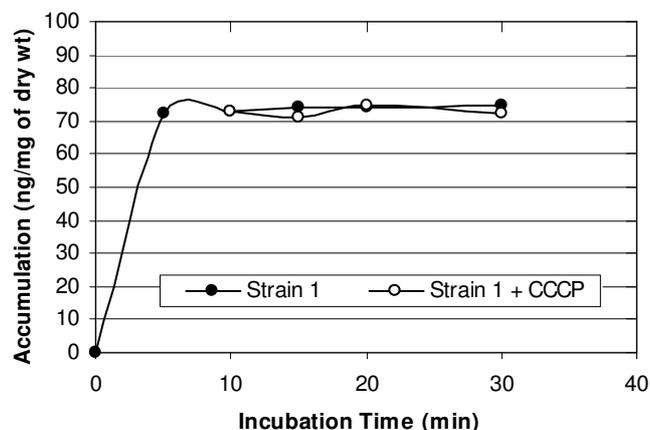

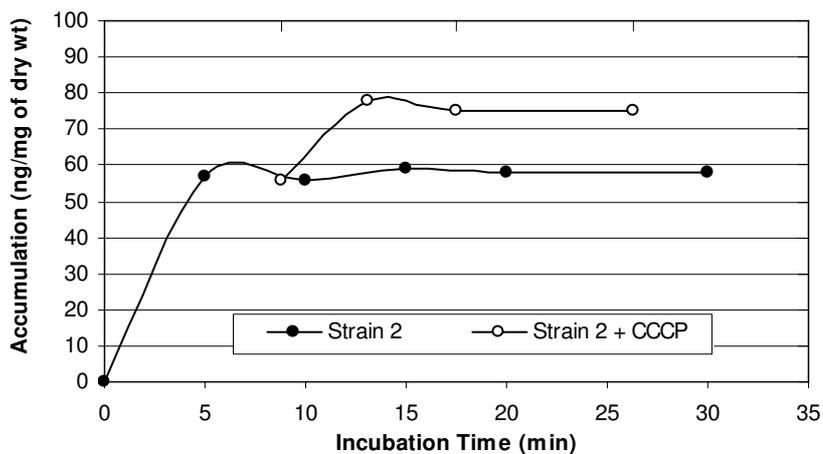**Figure 6.** Norfloxacin accumulation in case of strain 1.

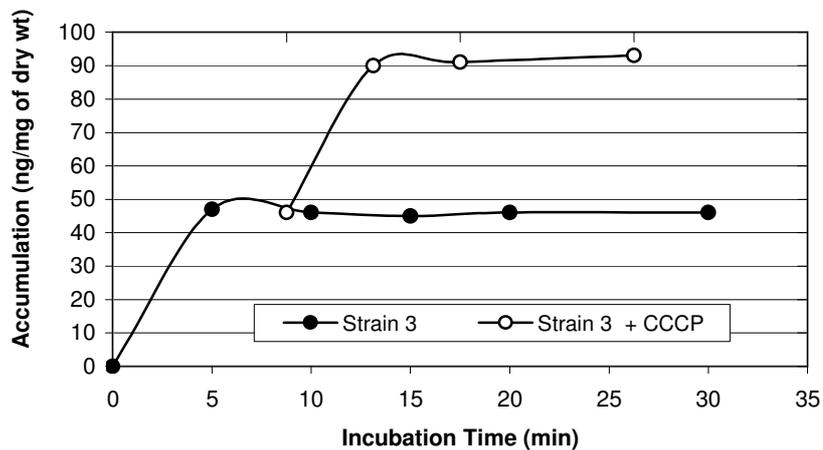**Figure 7.** Norfloxacin accumulation in case of strain 2.

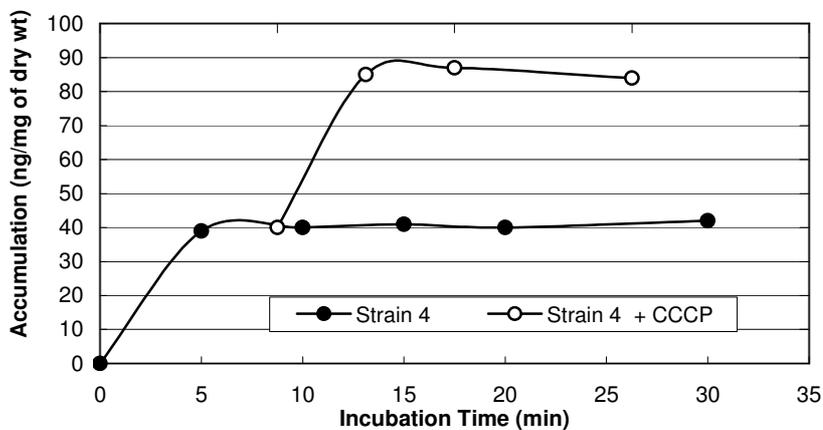**Figure 8.** Norfloxacin accumulation in case of strain 3.

Figure 9. Norfloxacin accumulation in case of strain 4.

Table 4. Concentration of norfloxacin $\pm$ 100μM CCCP accumulated by staphylococcal strains

Staphylococcus aureus strains	MIC (μg/mg)	Susceptibility to Norfloxacin	Accumulation (ng/mg dry cells)		Percentage of increase in accumulation
			- CCCP	+ CCCP	
Strain 1	2	Sensitive	72	71	---
Strain 2	16	Resistant	57	78	137%
Strain 3	128	Resistant	47	90	191%
Strain 4	128	Resistant	39	85	218%

Decimal assay for additivity method

For each pair of antibiotics tested, the selected target size for zones of inhibition, the BEF, and the mean diameters of the inhibition zones (with 95% confidence intervals) obtained are provided in table 5. For each antibiotic the BEF was calculated by using the linear regression equation for the standard dose-response curve, similar to that illustrated in figure 10 for ciprofloxacin.

Interactions between ciprofloxacin and oxacillin, cefepime, gentamicin and erythromycin were shown in figures 11, 12, 13 and 14.
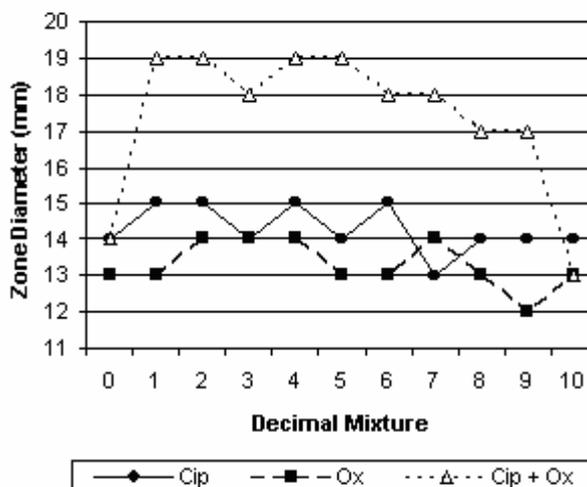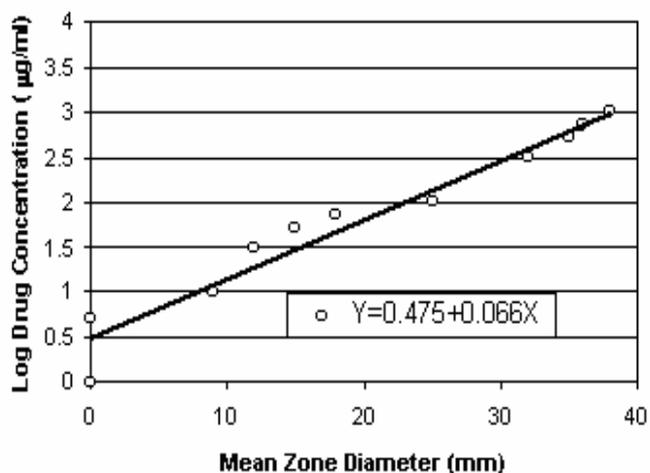

Figure 11. Synergy between ciprofloxacin and oxacillin.

Figure 10. Standard dose-response curve generated in disk diffusion assays with *S. aureus* strain and ciprofloxacin.
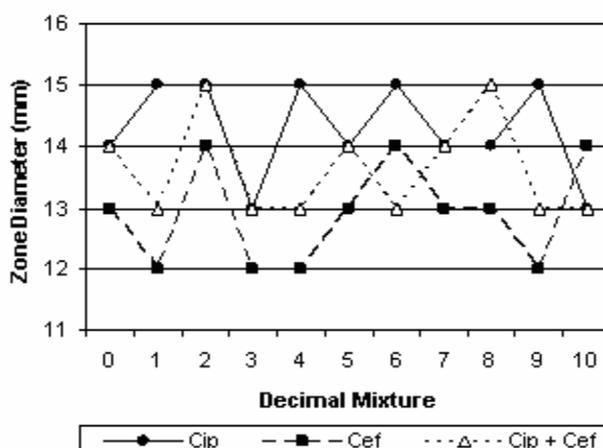

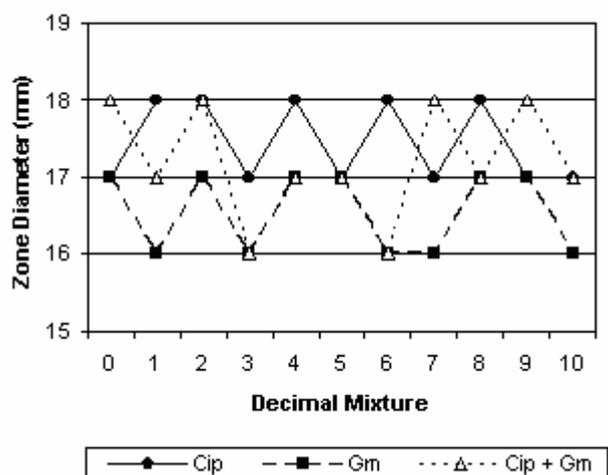**Figure 12.** Additivity between ciprofloxacin and cefepime.

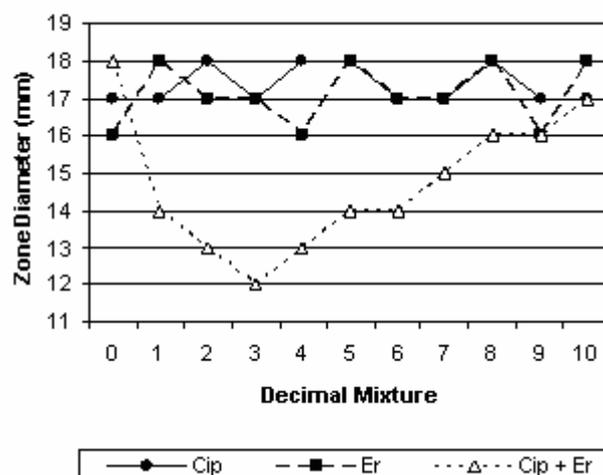**Figure 13.** Additivity between ciprofloxacin and gentamicin.

Figure 14. Antagonism of ciprofloxacin by erythromycin.

Table 5. Interactions between pairs of antibiotics with MRSA strain

Antibiotics	Target zone diameter (mm)	BEF (µg)	Mean diameter of zone of inhibition attained (mm)	95% CI[a] (mm)	Type of interaction
Ciprofloxacin	14	25.06	14.3	13.8-14.8	Synergism
Oxacillin	14	309.03	13.3	12.6-14.0	
Ciprofloxacin+Oxacillin			18.2	17.4-19.0	
Ciprofloxacin	13	21.53	14.3	13.8-14.8	Additive
Cefepime	13	918.33	12.9	12.4-13.4	
Ciprofloxacin+Cefepime			13.7	13.1-14.3	
Ciprofloxacin	18	46.03	17.5	17.0-18.0	Additive
Gentamicin	18	67.14	16.5	16.2-16.8	
Ciprofloxacin+Gentamicin			17.1	16.6-17.6	
Ciprofloxacin	18	46.03	17.4	17.1-17.7	Antagonism
Erythromycin	18	74.64	17.1	16.6-17.6	
Ciprofloxacin+Erythromycin			14.1	13.2-15.0	

[a]Confidence interval.

DISCUSSION

Staphylococcal infections are a common and significant clinical problem in medical practice (30). In the present work, the incidence of staphylococci was studied among the clinical specimens isolated in Minia. Out of the 470 clinical specimens, 187 (39.8%) staphylococcal strains were isolated and identified. Out of the isolated staphylococci, 70.6% were *S. aureus* and 29.4% were CoNS. *Staphylococcus aureus* was more prevalent than CoNS in skin, respiratory and eye infections, while CoNS were the most common isolates in urinary tract infections.

Our study revealed high activity of vancomycin and amikacin towards staphylococci. The effectiveness of vancomycin against staphylococci is corroborated by data from other groups (5,12,18).

In order to define the main mechanisms used by staphylococci to resist antibiotics, we tested for β-lactamase

production and for possess of plasmid and efflux-mediated resistance. Liang *et al*. (21) reported that one of the major mechanisms of resistance to β-lactams was the expression of ß-lactamases, such as penicillinase and cephalosporinase. We observed high levels of β-lactamase production in staphylococcal isolates (61.7% in *S. aureus* and 42.9% in CoNS). High levels of β-lactamase production in staphylococci had been reported elsewhere (4).

Other mechanisms of staphylococcal resistance to β-lactams were reported; such as intrinsic mechanism, which is not due to drug inactivation, and accounts for methicillin-resistance; and tolerance, in which there is a dissociation of the inhibitory and killing actions of beta-lactam antibiotics (31).

Beta-lactamase producing staphylococcal strains were screened for their plasmid profile, results of the screening revealed that some strains were carrying at least one plasmid suggesting plasmid-mediated antibiotic resistance and others did not. Plasmid-mediated antibiotic resistance was reported in several studies (14,25). Results of β-lactamase curing were compatible with other studies (7,23). Also, Shakibaie *et al*. (33) reported that the β-lactamase-cured cells did not exhibit any enzyme activity.

In this study the efflux mechanism in *S. aureus* strains was measured by a modified fluorometric assay. Addition of uncoupler CCCP increased norfloxacin accumulation in quinolone resistant *S. aureus* strains. Numerous reports involved fluorimetric method to study the efflux mechanisms and the effect of CCCP on the accumulation (28,29). Kaatz *et al*. (17) and Aeschlimann *et al*. (1) had shown increased activity or accumulation of the hydrophilic quinolones, norfloxacin and ciprofloxacin, in the presence of efflux inhibitors. Takenouchi *et al*. (36) demonestrated an increase in norfloxacin accumulated by *S. aureus* strains in the presence of CCCP.

Our study revealed that, using DAA technique with MRSA strain, synergistic interaction was observed between ciprofloxacin and oxacillin, ciprofloxacin plus cefepime and gentamicin appeared to be additive, while ciprofloxacin plus erythromycin was antagonistic. Combinations of fluoroquinolones with other antimicrobial agents against *S. aureus* strains have been previously investigated (13,16). Also the validity of DAA had been demonstrated (26,32).

CONCLUSION

This study examined the incidence of staphylococci, and its susceptibility patterns to different antibiotics. Drug resistance mediated by β-lactamases, plasmid and efflux pumps were important modes of cellular resistance to antimicrobial agents in our study. It would be valuable to use efflux pump inhibitors. Also, combination of antimicrobials which potentiate the activity of the existing antibiotics may be useful to decrease the resistance rate.

ACKNOWLEDGEMENT

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