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CEPHALOSPORIN RESISTANCE AND β-LACTAMASE PRODUCTION IN CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS* IN KARACHI

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ABSTRACT:

WHO recommends surveillance program for monitoring antibiotic resistance. The present study is a step in this direction. More than 100 clinical isolates of *Staphylococcus aureus* were collected from different hospitals in Karachi and *in vitro* studies were carried out by Agar dilution method using different generation of cephalosporins.

The result showed high resistance to majority of these antibiotics with increase in the MIC's while only ceftriaxone and ceftazidime were the most potent one showing significant antimicrobial activity. For the detection of β-lactamase iodometric method was used which showed that 80% strains produced β-lactamase which clearly depict the misuse of these agents.

INTRODUCTION

Cephalosporins were first obtained from *Cephalosporium acremonium* in 1948 by Brotzu from the sea near a sewer outlet off the Sardinian coast. Crude filters from cultures of this fungus were found to inhibit the in vitro growth of *Staphylococcus aureus*. Culture fluids in which the Sardinian fungus was cultivated were found to contain three distinct antibiotics, which were named cephalosporins P, N and C, 7-amino cephalosporinic acid, and with the addition of side chain, it became possible to produce semisynthetic compound with antibacterial activity very much greater than that of parent substance (Abraham, 1962; Flynn, 1972).

β-Lactamase Production:

Antibiotic resistance is of current concern. It is a major factor that derives changes in the pattern of antibiotic prescribing and is the most important stimulus to the development of new antibiotics. The use of antibiotics in humans include therapeutic and prophylactic prescribing. The latter largely restricted to short term preoperative use of one or more doses. Since most prescribing is therapeutic in nature, there is a need to ensure that antibiotics are administered appropriately and in manner that is likely to minimize the potential for antibiotic resistance.

β-Lactamase production by far the most important and most widespread mechanism of resistance, this type of resistance has spread from country to country in both hospitals and community acquired infection. It has been passed between bacterial species and even genera. Moreover, it has extended to cover more and more β-lactam agent including third generation cephalosporins. β-Lactam antibiotics, such as penicillins, cephalosporins and cephamycins, all have a similar core structure consisting of a β-lactam agents including third generation cephalosporins (Thornsberry, 1988; Wiedemann *et al*., 1989; Nord, 1988).

For most bacterial species the incidence of β-lactamase production throughout the world are remarkably similar. Incidence of β-lactamase production in *Staphylococcus aureus* has consistently been reported to be over 80% in all parts of the world (Parker, 1990). McBride in 1989 reported that in South Africa 90% strains of *Staphylococcus aureus* were producing β-lactamase. Another study by Chong in 1993 also showed 90% production of staphylococcal β-lactamase in U.S.A. In Middle East the β-lactamase production level was very high and a study in 1988-89 showed that almost 75% strains were producing β-lactamase (Shibli, 1992). De Villiers in 1989 observed 81% strains of *Staphylococcus aureus* were resistant due to β-lactamase production. β-Lactamase production in Mexico was 83% (Rodriguez, 1992). Another study by Mutere in Kenya 1992 showed 90% strains of *Staphylococcus aureus* were producing β-lactamase. In India 74% strains of *Staphylococcus aureus* produced β-lactamase (Eke, 1987). Forsgren *et al*. in 1994 found 80-90% strains of *Staphylococcus aureus* produced β-lactamase. Barry in 1990 reported that over 92% of the *Staphylococcus aureus* produced β-lacamase enzymes.

Present study gives the excellent information about the resistance pattern of Gram positive isolates and Beta-Lactmase production in Pakistan. This information will be helpful for the physician and consultants in prescribing antibiotics.

EXPERIMENTAL

Agar Dilution Susceptibility Test:

To determine the MIC for one or more bacterial isolates, the study drug may be incorporated into a liquefied agar medium (45-50°C), which is then mixed, poured into petri dishes and allowed to solidify (Barry, 1976; Snyder *et al*., 1976). A series of petri plates are prepared with increasing concentration of the drug and with the aid of a multiple inoculum replicator (Steers *et al*., 1959) as many as 11 different strains can be spot inoculated on to each plate. After overnight incubation, the MIC end point is read as the lowest concentration that completely inhibits growth, disregarding a single colony or faint haze or growth (Barry, 1976; Ericson, 1971; Washington, 1985).

Preparation of Antimicrobial Plates:

- Dilutions of antimicrobial agents are prepared in sterile double distilled water or other appropriate diluents at a concentration 10 times that desired in the final test (Barry, 1976; Washington, 1985).
- The Agarmedium is then prepared in flask or tubes and allowed to cool in a 50°C water bath.
- Sufficient volumes are prepared to fill each 9 cm petri plates with 20 to 25 ml of Agar.
- The diluted antimicrobial solutions are added to the melted and cooled medium in a ratio of 1 part antimicrobial agent to 9 part medium (2 ml of drug to 18 ml of Agar for each petri plate).
- The medium is then mixed by gently inverting the tube or flask several times. The contents are then poured into the appropriate number of petri plates.
- The plates are then set aside on a flat horizontal surface and allow to harden undisturbed.
- For reference the Agar plates should be prepared on the same day that the tests are to be performed. However for most other purposes, the antimicrobial plates can be refrigerated in a sealed plastic bag for at least 1 week without a significant loss of antimicrobial activity (Ryan *et al*., 1970).

Inoculation of Test Plates:

Apply an inoculum (1-2 ml) of each organism to the surface of each antimicrobial plates with the help of a replicating device containing 11 wire loops, one for the standard and 10 for the clinical isolates. The inoculum should be applied as a spot that covers a circle about 5-8 mm in a diameter and each spot should contain about 10^4 viable cells (Ericson, 1971; Barry, 1976; NCCLS, 1990).

Incubation of Test Plates:

The inoculated plates are allowed to stand undisturbed until the spot of inoculum have absorbed completely. The plates are then inverted and allowed to incubate at 37°C for 16 to 24 hours.

Examine plate for the presence or absence of growth. The lowest concentration of each antimicrobials that inhibit growth (ignore single colony or faint inoculum haze) is considered the MIC (Wentworth, 1987).

β-Lactamase Test:

Indometric assays:

Iodine is reduced by reaction with penicillioic acid and products of the hydrolysis of β-lactam ring of cephalosporins. Reduced iodine is lost from a starch-iodine complex, causing reduction in the blue colour of this complex (Cattin, 1975).

Paper Strip-Iodine Starch Assay:

Reagents: Solutions of 1 g benzyl penicillin G and 0.2 g soluble starch in 100 ml distilled water. Immerse 1 × 5 cm strips of Whatman No. 3 filter paper into the starch penicillin solution and air dry for 2 hours. Also required is a Gram Iodine solution.

Procedure: Place strips in a petri dish and moisten with Gram's iodine solution to cause a purple colour. To this add about 10 colonies of the organism to be tested to the center of the strip. A positive reaction is indicated by the test spot turning white.

RESULTS AND DISCUSSION

Microbial drug resistance is an inescapable consequences of the use of antimicrobial agents. The rate at which resistance occurs among microbial populations is often driven by the overuse and abuse of antimicrobial agents in many clinical settings. β-Lactam antibiotics are among the most frequently prescribed antibiotics worldwide, and as such their use is subject ot the problems associated with microbial resistance. Previous work has shown that resistance to β-lactam antibiotics is rising among the clinical isolates of Gram-positive organism especially *Staphylococcus aureus*. The present work consist of 110 clinical isolates of *Staphylococcus aureus* which are tested by Agar dilution method against 19 β-lactam agents and their combination. Agar dilution is a satisfactory and proven method of susceptibility testing. The role of the Agar dilution susceptibility test is one of aiding the selection of an appropriate antimicrobial agent for treating a specific infection (Berttina, 1987).

Minimum inhibitory concentration is the most common measure for bacterial sensitivity. MIC reflects only result after overnight incubation and do not account for interactions occurring

between the time of inoculation and the final reading (Lorian, 1991). Because of the ease of assessing the MIC, it is common practice to use MIC data to describe the antibacterial activity of a drug toward a bacterium or the sensitivity of a bacterium toward a drug (Table 1).

For the detection of β-lactamase, Iodometric method was employed (Table 2).

80% strains of *Staphylococcus aureus* produce β-lactamase. It is in confirmation with the work of Parker (1990).

First Generation Cephalosporins:

These drugs were very active against Gram positive cocci, including Pneumococci, group A hemolytic Streptococci and *Staphylococcus aureus* (Katzung, 1992).

Cefazolin is a first generation cephalosporin and is active against *Staphylococcus aureus* with MIC ranging from 0.25-5 μg/ml (Lambert and O'Grady, 1991) (Table 1). Lentnek *et al*. in 1985 reported excellent activity of cefazolin against *Staphylococcus aureus*. Igari *et al*. in 1988 observed that from 1984 onwards there was a decrease in number of resistant strains of *Staphylococcus aureus* in response to cefazolin.

Tanaka *et al*. and Otsuki *et al*. in 1987 and 1986 reported good antibacterial activity of cefazolin against *Staphylococcus aureus*. European study group in 1987 reported MIC of cefazolin ≤ 0.5-1 μg/ml against staphylococci.

The present study does not comply with the previous data. More than 78% strains of *Staphylococcus aureus* are resistant to cefazolin with increase in the MIC_{50} which is >4 μg/ml (Table 1).

Cephalexin, Cephradine, Cefatrizine have the same antibacterial activity having MIC's 2, 2 and 0.25 μg/ml (Table 1). Cephradine was active against *Staphylococcus aureus* including β-lactamase producing but not methicillin resistant, while cephalexin was relatively resistant to staphylococcal β-lactamase but has a variabl activity against β-lactamase producing and no useful activity against methicillin resistant strains (Lambert and O'Grady, 1991). Takenouchi *et al*. in 1994 also indicated that resistance to cephalexin against *Staphylococcus aureus* was due to β-lactamase production.

During the present study cephalexin shows that it is the most active agent among the three with more than 45% strains show resistance, while the cephradine and cefatrizine exhibit poor antibacterial activity with more than 93% and 95% strains of *Staphylococcus aureus* are resistant to these two drugs (Table 1).

Cefadroxil is another first generation cephalosporin having activity closely resembles to that of cephalexin. Hartstein *et al*. in 1977 showed that its activity was similar to that of cephalexin (Lambert and O'Grady, 1991). In present study more than 90% strains of *Staphylococcus aureus* are resistant to cefadroxil that clearly differentiate cephalexin and cefadroxil antibacterial spectra (Table 1).

Second Generation Cephalosporins:

The present study include cefaclor, ceforanide and cefuroxime. The activity of cefaclor is less against staphylococci and streptococci and is less resistant to staphylococcal β-lactamase than cephalexin. The MIC of cefaclor ranges from 0.5-1 μg/ml (Lambert and O'Grady, 1991).

Baurenfeind *et al.* in 1990 reported intermediate antibacterial activity of cefaclor against *Staphylococcus aureus*. Takenouchi *et al*. in 1994 showed that staphylococcal resistance against cefaclor was due to β-lactamase production. The result of the present study also shows that more than 93% strains of *Staphylococcus aureus* are resistant to cefaclor having MIC_{50}, >32 μg/ml (Table 1).

Ceforanide was broadly active against Gram positive and Gram negative microorganism (Dollery, 1991). The present antibacterial work exhibit that more than 57% strains are resistant against ceforanide (Table 1). The MIC_{50} and the MIC_{90} ≥16 μg/ml and >16 μg/ml. This means that ceforanide is better than other 1st generation cephalosporins except cephalexin against *Staphylococcus aureus*.

Cefuroxime was active against *Staphylococcus aureus* including penicillin resistant strains, but less so against methicillin resistant strains (Lambert and O'Grady, 1991). Hugbo *et al*. in 1992 observed that cefuroxime was highly sensitive against *Staphylococcus aureus*. European study group reported MIC of cefuroxime ≤0.5-1 μg/ml against staphylococci.

The present study of cefuroxime antibacterial activity shows high *Staphylococcus aureus* resistance with 98% strains are resistant to cefuroxime (Table 1).

Third Generation Cephalosporins:

Present antibacterial activity comprises five drugs which include cefotaxime, ceftizoxime, ceftazidime, cefixime and ceftriaxone. Cefotaxime was highly resistant to staphylococcal β-lactamases and has a good activity against Gram positive and Gram negative bacteria (Neu *et al*., 1979). Garcia *et al*. in 1992 observed high activity of cefotaxime against *Staphylococcus aureus*. Another study by Willke *et al*. in 1987 showed that cefotaxime was the most effective third generation cephalosporins as compared to that of ceftizoxime and ceftriaxone but Barriere *et al*. in 1985 noted that ceftizoxime was more active than cefotaxime against *Staphylococcus aureus*. Both cefotaxime and ceftizoxime have the MIC ranging from 2-4 μg/ml (Lambert and O'Grady, 1991). During the present study cefotaxime is more active with more than 31% of *Staphylococcus aureus* are susceptible while against ceftizoxime more than 76% strains are resistant (Table 1).

Ceftriaxone have activity in vitro similar to that of ceftizoxime and cefotaxime having MIC 4 μg/ml (Gillman, 1991; Lambert and O'Grady, 1991). Deguchi *et al*. in 1992 reported elevated MIC of ceftriaxone against *Staphylococcus aureus* isolated from 1987 to 1990. Another study by Eltahawy *et al*. in 1988 shown that ceftriaxone and ceftazidime were more active than augmentin by inhibiting 90% of the *Staphylococcus aureus*. Frenkel *et al*. in 1988 also observed high antibacterial activity of ceftriaxone against *Staphylococcus aureus* by eradicating 97% of the test strians.

The present result shows that ceftriaxone inhibit nearly 92% strains of *Staphylococcus aureus* and it is the most active β-lactam agent against *Staphylococcus aureus* (Table 1). MIC_{50} and MIC_{90} is ≤ 2 μg/ml and ≤ 16 μg/ml respectively. Xerri *et al*. in 1985 reported that there was no resistance of *Staphylococcus aureus* against ceftazidime and some of the strains are inhibited below the MIC level. Present study shows that there is an increase in resistance by *Staphylococcus aureus* against ceftazidime with more than 37% strains show resistance (Table 1).

Table 1
Population distribution of Mic of β-Lactam antibiotics
for 110 *Staphylococcus aureus* isolates Cefazolin

0.13	**0.25**	**0.5**	**1**	**2**	**4**	**>4**	**% Resistant**
8	2	4	10	8	6	72	78.18
Cephalexin							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
11	19	21	9	11	4	35	45.45
Cefadroxil							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
1	1	3	5	5	7	88	90.90
Cephradine							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
0	4	2	1	3	9	91	93.63
Cefaclor							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
0	0	3	4	12	2	89	93.63
Ceforanide							
0.5	**1**	**2**	**4**	**8**	**16**	**>16**	**% Resistant**
3	21	17	6	2	5	56	57.27
Cefuroxime							
0.5	**1**	**2**	**4**	**8**	**16**	**>16**	**% Resistant**
0	0	2	0	0	0	108	98.18
Cefatrizine							
0.25	**0.5**	**1**	**2**	**4**	**8**	**>8**	**% Resistant**
0	1	0	4	2	4	99	95.45
Ceftizoxime							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
1	5	4	8	9	19	64	83.63
Cefotaxime							
1	**2**	**4**	**8**	**16**	**32**	**>32**	**% Resistant**
16	8	2	9	25	44	6	68.18
Ceftriaxone							
2	**4**	**8**	**16**	**32**	**64**	**>64**	**% Resistant**
69	7	13	12	5	2	2	8.18
Cefixime							
2	**4**	**8**	**16**	**32**	**64**	**>64**	**% Resistant**
0	4	4	3	3	11	85	90.00
Ceftazidime							
2	**4**	**8**	**16**	**32**	**64**	**>64**	**% Resistant**
5	6	28	30	14	19	8	37.27

Acquisition of resistance was defined as increase in MIC of atleast 4-fold (Pour *et al*., 1994).

Note: Upper line of each column indicates the concentration of each drug.
Lower line indicates the number of isolates susceptible to that concentration of antibiotics.

Table 2
β-Lactamase Test (Iodometric Assay)

Strain No.	β-Lactamase	Strain No.	β-Lactamase
1	+	56	+
2	+	57	+
3	–	58	+
4	+	59	+
5	+	60	+
6	+	61	+
7	+	62	+
8	+	63	+
9	+	64	+
10	+	65	–
11	–	66	+
12	+	67	–
13	+	68	+
14	+	69	+
15	+	70	+
16	–	71	+
17	–	72	+
18	+	73	+
19	+	74	+
20	+	75	+
21	–	76	+
22	–	77	–
23	+	78	–
24	+	79	–
25	+	80	–
26	–	81	+
27	+	82	+
28	+	83	+
29	+	84	+
30	–	85	+
31	+	86	+
32	+	87	+
33	+	88	–

Strain No.	β-Lactamase	Strain No.	β-Lactamase
34	–	89	+
35	+	90	+
36	+	91	+
37	+	92	+
38	+	93	+
39	+	94	–
40	+	95	+
41	+	96	+
42	+	97	+
43	+	98	+
44	+	99	+
45	+	100	–
46	–	101	–
47	+	102	+
48	+	103	+
49	+	104	+
50	+	105	–
51	+	106	+
52	+	107	+
53	+	108	+
54	+	109	–
55	+	110	+

+ = β-lactamase present
– = β-lactamase absent

Cefixime is an orally active broad spectrum antibiotic which showed good activity against Gram negative microorganism but *Staphylococcus aureus* were less susceptible having MIC ranging from 4-16 μg/ml (Lambert and O'Grady, 1991). Schatz *et al*. in 1996 reported no activity of cefixime against *Staphylococcus aureus*. Baurenfiend *et al*. in 1990 also showed inactivity of cefixime against *Staphylococcus aureus*. Motohiro *et al*. in 1986 compared cefixime with other β-lactam agents and showed cefixime was the least potent as compared to the other agents. The present study also exhibits inactivity of this compound against *Staphylococcus aureus* with only 10% strians are susceptible (Table 1). The present research work concludes that ceftriaxone is the most active third generation cephalosporin and cefixime is the least active.

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