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Susceptibility of Mycobacterium Tuberculosis to β-Lactamase with or without β-Lactamase Inhibitors

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ABSTRACT

Background : *Reemergence of tuberculosis along with multi drug- resistant strains has made both the treatment of affected patients and the progress of eradication programs a real struggle. Most second –line drugs are toxic and expensive and it is necessary to search for effective anti-tuberculosis drugs which are safer and less expensive. Due to their structure and production of β-Lactamase enzyme, mycobacteria are considered as β -lactam resistant.*

Materials and Methods: *we study the effects of β -Lactamase inhibition on the susceptibility of mycobacterium to β -Lactamase, changes in Minimal Inhibitory Concentration (MIC) of four cephalosporins; cephapirin, ceftriaxone, cefotaxime, and cefoperazone in the presence of sulbactam in both sensitive and resistant mycobacteria.*

Results: *β –Lactamase production was assessed with the Nitrosfin method and all strains were β-Lactamase. Resistant strains showed less sensitivity to β -Lactamas and both groups were most sensitive to cephapirin. Equal doses of sulbactam added to the cephalosporins reduced their MICs from zero to 16 times. MIC reduction was more pronounced with ceftriaxone in the sensitive group and with cefoperazone in the resisitant group.*

Conclusion: *Although antimycobacterial effects of β -lactamase such as cephalosporin in combination with β-Lactamase inhibitors, could not be compared with first- line anti TB drugs. We are still hopeful these drugs with the least side effects could be considered as the second- line anti TB drugs in near future.* **(Tanaffos 2002;1(1):28-35)**

Keywords: *Mycobacterium Tuberculosis , β -Lactam, β - Lactamase, Cephalosporin.*

INTRODUCTION

WHO experts had anticipated 88 million new cases of TB and 30 million deaths due to this disease during the 1990s. 8 million of which are AIDS patients(1).

Multi-drug resistance is a growing clinical problem. Specially resistance to isoniazid and rifampin which are the most effective mycobactericidals, is of the utmost importance to both the clinic and public health (2,3).Inspite of producing several new pharmaceuticals for treatment of bacterial, parasitic, fungal, and recently viral diseases, no other new major mycobactericidal drug, except amikacin, has been introduced since early 1970s when rifampin was first marketed (4).

The manufacturing of modern antimycobacterials are costly. Furthermore, with the decreased incidence of tuberculosis in the low-income countries where TB is more prevalent, it is not profitable for the manufacturers to produce these

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drugs. Meanwhile, approval of a new anti TB drug requires at least five years (4). Thus, recognizing anti-mycobacterial effects of the current antibiotics, which are more abundant and less costly, could be one way to address this problem.

At first sight, the general structure of mycobacteria and production of β -Lactamase make the use of β -Lactamse in TB look unjustifiable, whereas report of susceptibility of environmental mycobacterium to this group of antibiotics are documented and their employment against M. tuberculosis seems possible (5). Researchers believe that despite half a century of studies on this subject and practical utilization in the treatment of TB and leprosy, more studies are to be done to find the most effective β-Lactam and its route of administration (4,6,7)

In the present study the activity of a β -Lactamase inhibitor in combination with 4 cephalosporins against 2 groups of mycobacterium tuberculosis was challenged.

MATERIALS AND METHODS

Bacterial strains

A total of 40 strains of M. Tuberculosis non-randomly isolated from the microbial bank of Massih Daneshvari Hospital. All strains were identified with standard antibiogram tests. Twenty were sensitive to all first-line anti-mycobacterial drugs and 20 were MDR which resist to at least isoniazid and rifampin, and at most to all first- line drugs. M.tuberculosis H37-Rv was used as a control in the culture and S.aureus was used to control drug potentials.

Culture media

Susceptibility testing was performed in Middlebrook 7H9 MO178 media with additive supplement of OADC 211-886 (Sigma,Becton: Sigma-Aldrich, Inc. Becton Dickinson and company loveton circle sparks). Culture media were prepared according to the manufacturer's instruction and were distributed in sterile, screw-capped tubes under aseptic conditions.

Antibitiotics

Standard powders of Cephapirin(Sigma; C8270), Ceftriaxone (Sigma C5793), Cefotaxime (Sigma C7912), Cefoperazone (Sigma C4292) were prepared.The proper dose of each antibiotic was dissolved in sterile distilled water on the day of experiment then necessary dilutions were made in broth and filtered into the culture tubes through 0.2 μm pore filters (8,9).

β -lactamase inhibitor

Sulbactam Na powder with the purity of 901.77μg/mg, (SSC015H0,Aurobindo Pharma LTD Plot, Hyderabad 500 038 India) was used.

Needed doses were weighed by considering the purity and dissolved in sterile distilled water. Then there were added to the antibiotics as 1:1 or 2:1 proportions(9).

β-Lactamase substrate

Paper discs containing substrate (nitrosfin,TM)with the formulation of pyridine-2-azo-p-dimethyl aniline, a coloured cephalosporin, were prepared by Becton company.

Bacterial preparation

First, a fresh culture of bacteria was prepared in Lowenstein-Jensen's (L.J) medium. Three to four weeks later, when small colonies appeared on the surface (logarithmic growth phase), a microbial suspension was made in sterile screw-capped tubes containing 3 ml of sterile normal saline and 10-15 glass balls, and it is also homogenized by an electric blender (8,10)

Microbial suspension diluted with normal saline to match the turbidity of a 1-McFarland standard and then diluted to 100 fold in separate tubes of the aforementioned dilution,0.1ml of the above solution was introduced to 4 ml of medium and antibiotics, so that final dilution of 10^5 was achieved; and 0.05

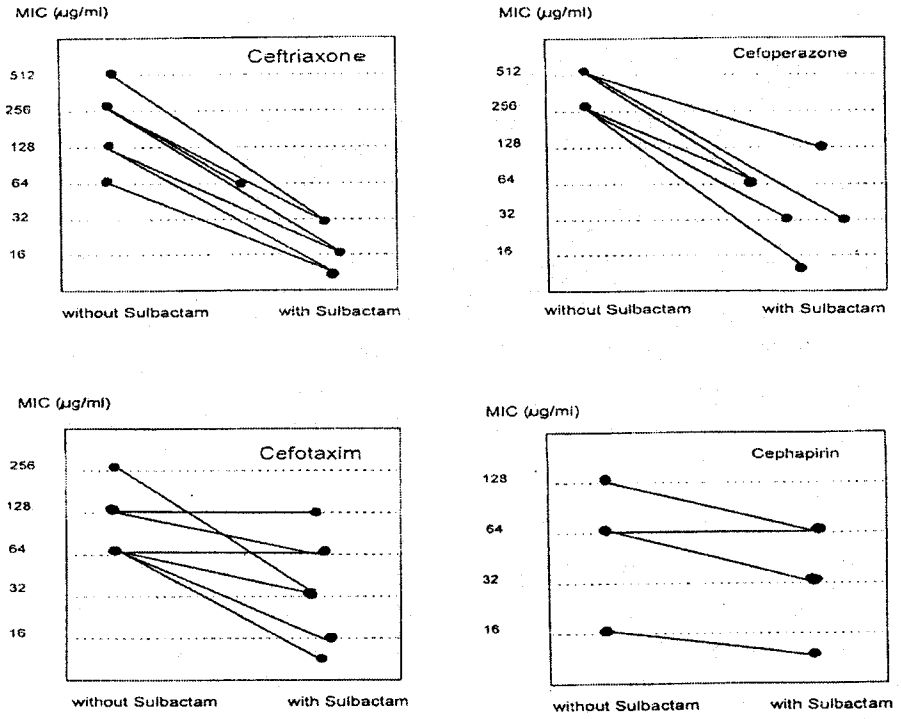

Fig 1-4: MIC reduction in the group of sensitizes to anti-TB drugs after administrating equal doses of sulbactam and cephalosporins

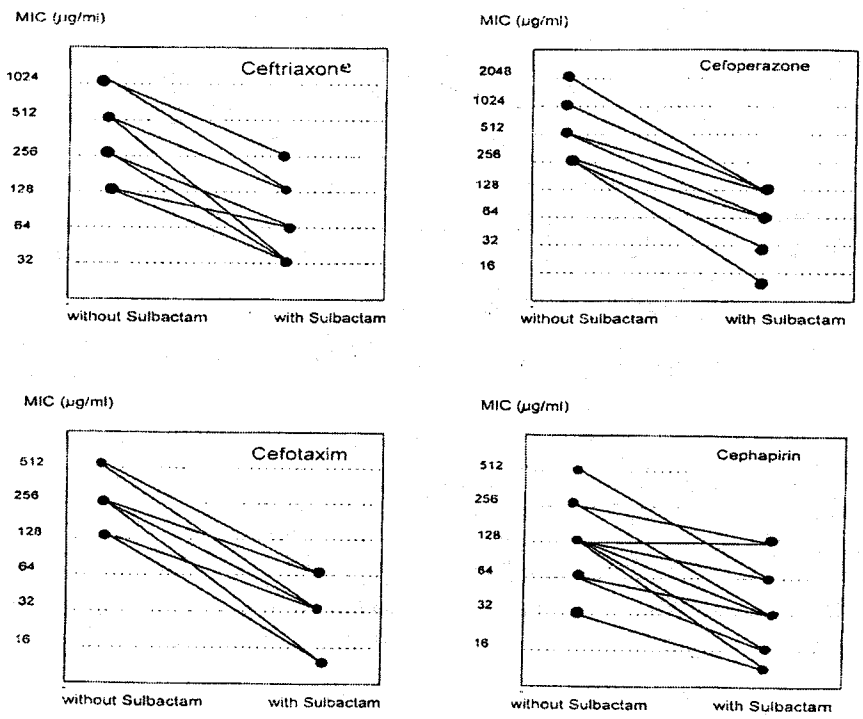

Figure 5-8: MIC reduction in the group of MDRs after administrating equal doses of sulbactam and cephalosporins

ml was inoculated to L.J. medium as a control for the assay of colony forming unit per ml-- CFU/ml(5,8).

Susceptibility testing

The test was performed with the Broth Macro-dilution method and all tests were down duplicate. Dilution range was 16-2048 µg/ml according to the instructions (8,9,11). For each series, there were two control tubes without antibiotics. The supernatant in these control tubes were incubated at 37^0C. Microbial infection was assessed after 72 hours of incubation. There after, in case of any nonspecific turbidity, they were sampled and stained. Tubes were observed 10-15 days later, after obvious growth in control groups was achieved.

β -Lactamase test

0.05 ml of supernatant was transferred in to sterile, screw-capped tubes containing a nitrosfin disc (12). The color change -- from light yellow to purple-- was determined 1h and 24 h of incubation (13,14). S.aureus and E.coli were positive controls and H.influenza and sterile distilled water were negative controls (15).

RESULTS

Beta-Lactamase test

There was no color change after incubation at 37 ^{0}C for 1 h, although all samples and positive controls showed discoloration after 24 h. If the samples were incubated in a refrigerator or at room temperature, the colour began to fade out over time.

Susceptibility testing

Some unpredictable problems like the one with OADC supplement (Becton) caused turbulence in 30 samples and led to repetition of the tests. After 10 to 15 days, the cultures were harvested , based on the visible growth in the medium supplemented with control tubes (7). M. tuberculosis was grown in 0.02 % Tween 80(9) and was shaped small white clumps that were better visible by gently shaking the tubes and transilluminating them, but the presence of a homogenous turbidity was considered as nonspecific reaction and assessed by gram and acid-fast staining. Other samples were randomly checked as above. Table 1 shows ranges of the MIC of antibiotics with and without β-Lactamase inhibitor, and also shows MIC_{50} values in both sensitive and MDRs. The definite MIC_{50} of Cephapirin against MDRs could not be determined. Figures 1-4 illustrate the decrease of MIC in sensitive strains and 5-8 in MDRs respectively.

Table1. MIC range and MIC $_{50}$ of four cephalosporins with/without sulbactam in sensitive M. tuberculosis strains and MDRs

	Sensitives		MDR_{50}	
	Range	MIC_{50}	Range	MIC_{50}
Ceftriaxone	64-512	128	128-102·	256
Ceftriaxone+ Sulbactam	<16-64	<16	32-256	32
Cefoperazone	128-512	256	128-204;	512
Cefoperazone+Sulbactam	16-128	64	<16-128	128
Cefotaxim	32-256	64	32-512	128
Cefotaxim+Sulbactam	<16-128	32	<16-64	16
Cephapirin	16-128	64	32-256	128
Cephapirin+Sulbactam	<16-64	32	<16-128	ND*

*ND: Not Determined.

At first, antibiotic-inhibitor was used as 2:1 in 14 samples but the reduction in MIC was not noticeable, thus, 1:1 proportion like Ampicillin-Sulbactam combination was used in accordance with Gelberts experience (16). The results of 2:1 combination are not presented.

DISCUSSION

The study of the effects of penicillin on tubercle bacillus had been started before the problem of MDR strains was appeared(17,18,19).An unique cell wall structure distinguishes mycobacteria from other bacteria (20). The lipid-rich cell wall is a major factor in the resistance of M.tuberculosis to acids, alkalines, antibiotics and many antiseptics (13). Previous studies revealed that besides a low permeability cell wall, mycobacteria like some other bacteria produce extra and intracellular

penicillinase(21,22,23). Regarding the resistance to penicillin and other β-Lactamase , β-Lactamase enzymes are more effective than a low permeability cell wall and penicillin-binding-proteins (PBPs) (21,24,25,26).Invitro and animal studies have shown a synergistic effect of penicillin G and semisynthetic penicillinase resistant ones like oxacillin and cloxacillin on M.tuberculosis (22,26). Production of cephalosporin and it's derivatives with some degrees of resistance against β -Lactamase were a breakthrough in dealing with penicillin resistance (25,27,28).

The widespread effort to identify more effective β -Lactamase inhibitors lead to recognition of carbapenemes and clavulanic acid of natural origin, and synthesis of sulfones, sulbactam and tazobactam(8).These agents have weak antimicrobial activity, but in combination with penicillins suspectible to β -Lactamase show considerable synergetic effects (29,30).

Regarding the lack of adequate in vitro evaluation of the antimicrobial effects of β -lactamase together with β-lactamase inhibitors against mycobacteria and little success in synthesizing new anti TB drugs on one hand , and the side effects and high cost of second- line drugs on the other hand have offered the opportunity to revise anti-mycobacterial effects of common antibiotics like β-Lactamase inhibitors (5,6,7,9,10,12,14,16,31-38).In the present study, the role of β-Lactamase inhibition on changing the susceptibility of M. tuberculosis against cephalosporins from the groups 1,4 and 6, with variable resistance to β -Lactamase was evaluated in vitro. The dilution range was deliberately chosen to be wide and identical for all of the cephalosporins to detect MIC reduction as precisely as possible. However, MIC reduction in some strains was out of the range and more studies are needed.

Since the activity of β-Lactam- β-Lactamase inhibitor complex is chiefly depended on the inhibitor(15) in the present study sulbactam was adopted. Sulbactam has weak antimicrobial activities and inhibits many β-Lactamases (1,35). In the previous studies, mostly cephalosporins, plus clavulanic acid were used on mycobacteria (14,30) and the only article considering the use of cephalosporins and sulbactam was published by Dr Chen et al. They have determined the MIC of different cephalosporins on 16 strains of sensitive M. tuberculosis. They used proportional method in solid media(9). In the present study Broth Macro dilution method was applied because of it's advantages (8). Meanwhile, 20 sensitive strains were tested as well as 20 resistant strains to make the comparison of their sensitivity to cephalosporins alone or in combination with sulbactam. MICs obtained in this study place M. tuberculosis beside other cephalosporin- resisitant bacteria like pseudomonas and entrococcus (30), however, sensivity was different in different strains(34). This experiment had revealed that cephapirin from group 1 with the highest susceptibility to β-Lactamase was the most effective agent against both sensitive and MDR strains, and this may imply the relation between structure and function of antibiotics. By comparing the results, it can be concluded that MDRs have more resistance to cephalosporins alone and with sulbactam is the highest. This suggests the possibility of factors other than β-lactamase enzymes in general resistance of antibiotics or cross resistance. This was especially prominent with cefriaxone and cefoperazone. Although the MIC reduction which was up to more than 16 times in both susceptible and MDRs is promising. MIC reduction was out of the dilution range from some strains, thus, more precise studies are highly recommended. Future studies will probably lessen the mean susceptibility of M. tuberculosis beyond 16-32 μg/ml. The slope of MIC reduction curve for combinations of cefoperazone or ceftriaxone with sulbactam was steeper in MDRs and MIC curve for cephapirin had a steady slope in susceptible strains.

Sensitive M. Tuberculosis strains studied by Dr Chen et al. were more susceptible to cephapirin than ceftriaxone or cefotaxime, however, the highest reduction in MIC was achieved by the

combination of cefotaxime and sulbactam. In this study combination of these drugs revealed the most decrease in MDRs. It is not necessary to say that bacterial strain and the amount of enzyme production play an important role in treatment of tuberculosis. At this stage it can not be foreseen whether β-Lactamase will play a role in treatment of M.tuberculosis or not, but reemergence of TB especially in developing countries and coming up of MDRs along with scan progress in making new anti-TB drugs make it mandatory to carry out widespread research to enlist more antibiotics as anti tuberculosis drugs.

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سنجش حساسیت مایکوباکتریوم توبرکلوزیس به داروهای بتالاکتام همراه با / بدون مهار کننده بتا لاکتاماز

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سابقه و هدف: افزایش روزافزون گونه‌های سل مقاوم به درمان، عوارض و گرانی داروهای انتخاب دوم، باعث شده است تا تحقیقات آزمایشگاهی معطوف به مطالعه بر روی آثار ضد مایکوباکتریایی آنتی‌بیوتیک‌های در دسترس شود. در تحقیق حاضر، حساسیت مایکوباکتریوم توبرکلوزیس به داروهای بتالاکتام بررسی می‌شود.

مواد و روشها: به منظور بررسی تأثیر مهار آنزیم بتالاکتاماز در میزان حساسیت باکتری، MIC چهار نوع سفالوسپورین شامل سفاپیرین، سفتریاکسون، سفوتاکسیم و سفوپرازون در کنار سولباکتام در دو گروه باکتری حساس و مقاوم به درمان ضد سل، ارزیابی شد.

یافته‌ها: آزمون تعیین حساسیت با روش نیتروسفین در مورد تمامی گونه‌های مورد بررسی، از نظر تولید بتالاکتاماز مثبت بود. گونه‌های مقاوم، حساسیت کمتری به داروهای بتالاکتام نشان دادند. حساسیت گونه‌های مقاوم و حساس به سفاپیرین بیشتر بود. همراهی سولباکتام با سفالوسپورین‌ها، MIC آنها را از صفر تا بیش از ۱۶ مرتبه کاهش داد. در گونه‌های حساس، بیشترین کاهش با سفتریاکسون و در گونه‌های مقاوم، بیشترین کاهش با سفوپرازون دیده شد.

نتیجه‌گیری و توصیه‌ها: وجود اثر ضد مایکوباکتریایی بتالاکتام‌ها مانند سفالوسپورین‌ها به همراه مهار کننده بتالاکتاماز، این امیدواری را در زمینه درمان سل ایجاد می‌کند که این داروها در آینده جایگاهی خاص در میان داروهای انتخاب دوم پیدا کنند.

واژگان کلیدی: مایکوباکتریوم توبرکلوزیس، بتالاکتام، سفالوسپورین