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INOCULUM SIZE EFFECT OF SOME CLINICAL ISOLATES OF STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI ON ACTIVITY OF SOME ß-LACTAM, AMINOGLYCOSIDES AND FLUOROQUINOLONES

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ABSTRACT

The effect of inoculum size of five clinical isolates of Staphylococcus aureus and Escherichia coli on the activity of six antibiotics was studied. Cefoperazone and cefotaxime exhibited a large inoculum size effect with the studied strains. Amikacin, gentamicin, ofloxacin, and ciprofloxacin exhibited a rapid bactericidal activity and a minimal inoculum size effect. The inoculum size markedly affected the antibacterial activity of all the studied antibiotics against the clinical isolates studied. This inoculum size effect was more pronounced towards Escherichia coli than Staphylococcus aureus.

INTRODUCTION

Ofloxacin and ciprofloxacin are fluoroquinolone antibiotics that have a high activity against Staphylococcus aureus and Escherichia coli. Cefotaxime and cefoperazone are cephalosporin antibiotics that resist ß-lactamases produced by resistant bacterial isolates[1], while gentamicin and amikacin are aminoglycoside antibiotics with high activity against Escherichia coli [2]. Although the clinical importance of the inoculum size effect towards antibiotics is yet unclear, it is of major importance in laboratory susceptibility testing[3-4].

The dependence of susceptibility results on the inoculum size has been shown to be particularly important, especially for Gram negative bacteria with cephalosporin antibiotics[4-5]. In that setting, cephalosporin antibiotics which did not rapidly kill the organism but caused the organisms to form filamentous structures, were shown to have a high inoculum size effect[5]. No evidence is known for the presence and the extent of the inoculum size effect for ciprofloxacin and ofloxacin with Staphylococcus aureus and Escherichia coli, which are generally regarded as highly susceptible to those antibiotics[6].

This study aimed to test for and evaluate the inoculum size effect as well as the killing rate of the tested antibiotics using two different inocula of the tested clinical isolates.

EXPERIMENTAL

Bacterial strains :

A total of 10 clinical isolates were used. Five of each of Staphylococcus aureus and Escherichia coli were isolated and identified[7] for subsequent use throughout this work.

Antibiotics :

Cefoperazone (Pfizer Pharmaceutical Company), Cefotaxime, gentamicin, and ofloxacin (Hoechest- Roussel Pharmaceutical Company), and ciprofloxacin and amikacin (Microbiology Research Laboratories, Faculty of

Pharmacy, Mansoura) were used in this study.

Susceptibility tests :

Antibiotic susceptibility testing was performed using standard broth dilution technique with Mueller-Hinton broth with a final inoculum of $5x10^5$ and $5x10^7$ CFU/ml in tubes of 2 ml volume[8]. Using inoculum of $5x10^5$ CFU/ml, growth was defined as the detection of turbidity after 18 hours incubation at 37°C. At inoculum $5x10^7$ CFU/ml, growth was defined as any increase in turbidity over that of the original inoculum after the addition of 10% formalin which stops the increase of bacterial population. The degree of inoculum size effect was determined by the ratio of the MIC obtained at the higher inoculum ($5x10^7$ CFU/ml) to the MIC obtained at the lower inoculum ($5x10^5$ CFU/ml)[10]

Time-kill curve studies :

The antibacterial activities of the six studied antimicrobial agents were determined using concentrations equal to four times their MICs obtained with the inoculum size ($5x10^5$ CFU/ml). Samples were withdrawn after 0,4,8,12 and 24 hours of incubation and plated on agar plates. After incubation for 24 hours at 37°C, the CFU/ml of the withdrawn samples were calculated[9].

Optical density studies :

During time-kill curve studies, the contents of tubes containing high inocula of bacteria were subjected to optical density measurements at 580 nm at 0,4,8,12 and 24 hours of incubation, using Spekol 11 visible spectrophotometer (DDR) with a 1 cm cell pathway.

RESULTS

Degree of inoculum size effect :

Two inocula, $5x10^5$ and $5x10^7$ CFU/ml, from ten clinical isolates (five of Staphylococcus aureus and five of Escherichia coli) were used in this study. The MICs for both inoculum sizes, as determined by broth dilution method, are shown in Table 1. Higher MIC was determined with the higher inoculum size ($5x10^7$ CFU/ml) over that of the lower inoculum size ($5x10^5$ CFU/ml). This was observed for the two studied cephalosporin antibiotics (cefoperazone and cefotaxime). As a result, higher inoculum size effects were observed over that determined for the other antibiotics. The highest inoculum size effect was recorded with cefoperazone against Escherichia coli strain No. 1 and No. 3 (MIC ratio 16). On the other hand, the tested aminoglycosides and fluoroquinolones antimicrobial agents had inoculum size effects ranging from 1 to 8.

Time-kill curve studies :

The rates of killing of two strains (strain No. 1 of both Staphylococcus aureus and Escherichia coli) were determined at different time intervals using the two inoculum sizes of $5x10^5$ and $5x10^7$ CFU/ml. Cefoperazone and cefotaxime killing rates were shown to be decreased by increasing the inoculum size from $5x10^5$ to $5x10^7$ CFU/ml (Fig. 1-4). Generally, both of the antibiotics have lower killing effect than the other antimicrobial agents.

Amikacin, gentamicin, ofloxacin, and ciprofloxacin showed rapid bactericidal activities with no detectable inoculum effects. Among these antibiotics, amikacin and gentamicin showed the highest bactericidal effects against Escherichia coli clinical isolate studied using both inoculum sizes (Figs. 1-4).

Optical density studies :

Cefotaxime and cefoperazone produced the highest optical densities especially when using Escherichia coli as test organism rather than Staphylococcus aureus. Gentamicin and amikacin showed a minimal increase in optical

Table (1): Inoculum size effect of the tested antibiotics on Staphylococcus aureus and Escherichia coli isolates.

Strain No (CFU/ml)		CPZ		CTX		AK		GM		OFL		CIP	
		MIC	IE	MIC	IE	MIC	IE	MIC	IE	MIC	IE	MIC	IE
Staph. aureus:													
1	5×10^5	2		1.0		1/4		0.12		1/4		1/4	
	5×10^7	16	8	4.0	4.0	1/4	1.0	0.12	1.0	1/4	1.0	1/4	1
2	5×10^5	16		4		1/2		1/4		1/2		1/4	
	5×10^7	64	4	16	4	1/2	1.0	1/4	1.0	1/2	1.0	1/4	1
3	5×10^5	1		1/2		1/4		0.12		0.2		0.2	
	5×10^7	8	8	2	4	1/4	1.0	0.12	1.0	0.2	1.0	0.2	1
4	5×10^5	16		4		1/2		1/4		1/2		1/4	
	5×10^7	128	8	32	8	1/2	1.0	1/4	1.0	1/2	1.0	1/4	1
5	5×10^5	4		1		1/4		0.12		1/4		0.2	
	5×10^7	16	4	4	4	1/4	1.0	0.12	1.0	1/4	1.0	0.2	1
E. coli :													
1	5×10^5	4		8		1/4		0.2		0.3		1.5	
	5×10^7	64	16	64	8	1/2	2	0.2	1.0	0.6	2.0	1.5	1
2	5×10^5	32		32		1/2		0.2		0.6		1.5	
	5×10^7	128	4	128	4	1	2	0.4	2.0	0.6	1.0	3.0	2
3	5×10^5	2		4		1/4		0.2		0.3		1.5	
	5×10^7	32	16	32	8	1/4	1	0.2	1.0	0.3	1.0	1.5	1
4	5×10^5	8		4		1/2		1/4		0.6		1.5	
	5×10^7	32	4	32	8	1/2	1	1/4	1.0	1.2	2.0	3.0	2
5	5×10^5	4		8		1/4		0.2		0.6		1.5	
	5×10^7	32	8	32	4	1/4	1	0.4	2.0	0.6	1.0	1.5	1

MICs: The Minimal Inhibitory Concentrations measured in μg/ml.

IE: Inoculum Size Effect which is the ratio of the MIC determined at 5×10^7 CFU/ml to the MIC obtained at the standard inoculum (5×10^5 CFU/ml).

CPZ: cefoperazone, CTX: cefotaxime, AK: amikacin, GM: gentamicin, OFL: ofloxacin, and CIP: ciprofloxacin.

Table (2): Optical density measurements of Staphylococcus aureus (No. 1) and Escherichia coli (No. 1) during time-kill study with the tested antibiotics.

Antibiotic/test organism	O.D at 580 nm				
	0.0 hr	4.0 hr	8.0 hr	12 hr	24 hr
Cefotaxime:					
Staph. aureus	0.24	0.32	0.38	0.51	0.53
E. coli	0.26	0.35	0.43	0.55	0.55
Cefoperazone:					
Staph. aureus	0.27	0.29	0.38	0.52	0.51
E. coli	0.28	0.30	0.48	0.58	0.80
Gentamicin:					
Staph. aureus	0.27	0.28	0.28	0.29	0.30
E. coli	0.28	0.30	0.32	0.34	0.34
Amikacin:					
Staph. aureus	0.28	0.29	0.29	0.30	0.30
E. coli	0.26	0.29	0.30	0.32	0.32
Ciprofloxacin:					
Staph. aureus	0.28	0.30	0.32	0.34	0.36
E. coli	0.29	0.31	0.35	0.39	0.40
Ofloxacin:					
Staph. aureus	0.26	0.31	0.33	0.36	0.40
E. coli	0.27	0.32	0.36	0.40	0.43

Staph. aureus: Staphylococcus aureus (clinical isolate No. 1).
E. coli: Escherichia coli (clinical isolate No. 1).
0.0 hr, 4.0 hr, 8.0 hr, 12 hr, and 24 hr are the time intervals in hours.

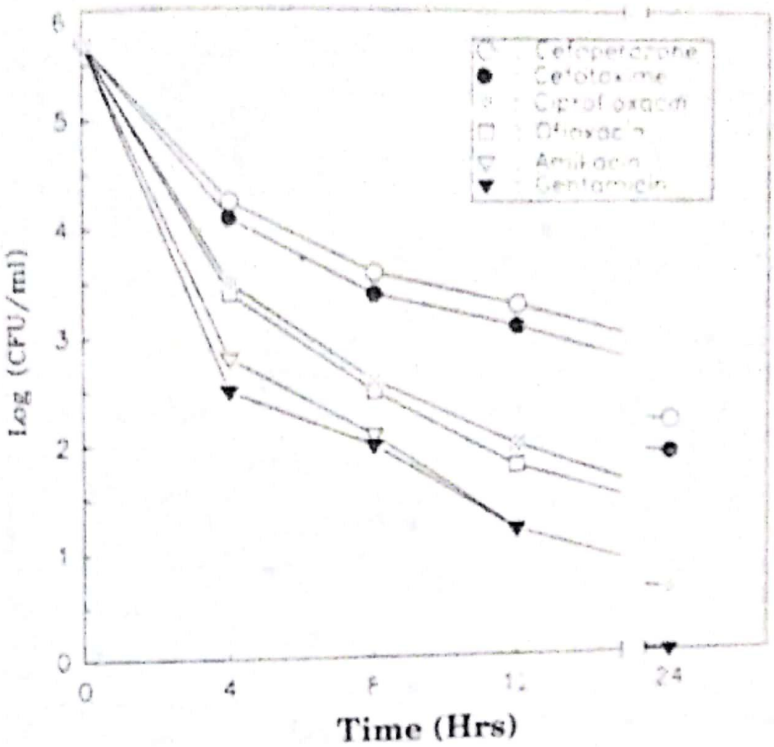

Fig. (1): Time-kill curve of tested antibiotics for 5 x 10^5 CFU/ml of Staphylococcus aureus No. 1.

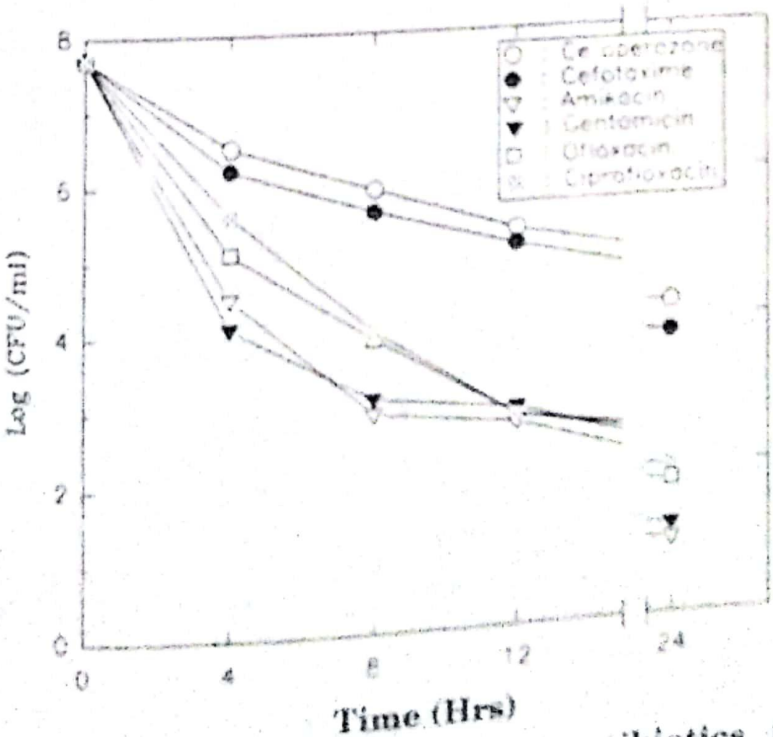

Fig. (2): Time-kill curve of tested antibiotics for 5 x 10^7 CFU/ml of Staphylococcus aureus No. 1.

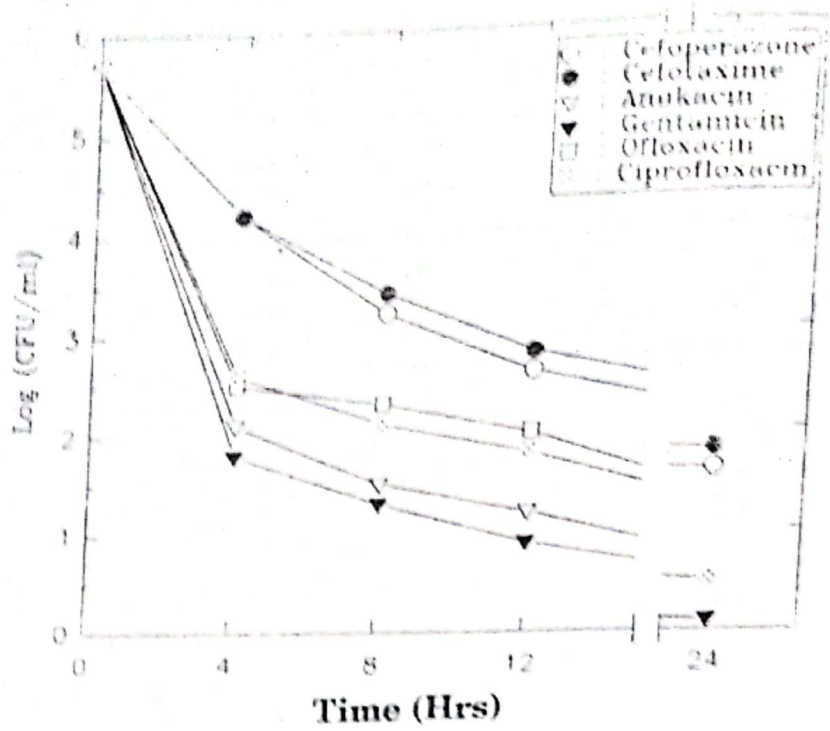

Fig. (3): Time-kill curve of tested antibiotics for 5×10^5 CFU/ml of E. coli No. 1.

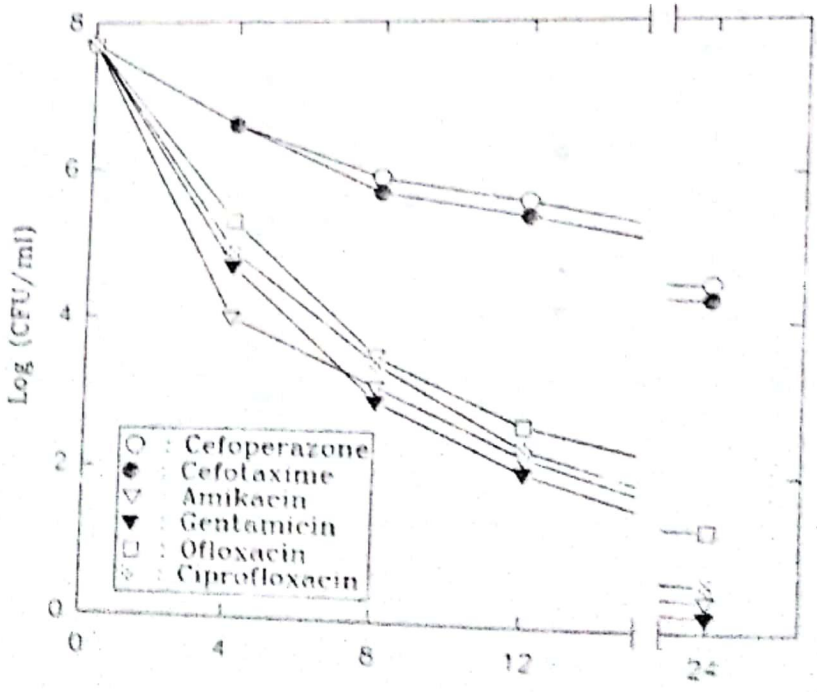

Fig. (4): Time-kill curve of tested antibiotics for 5×10^7 CFU/ml of E. coli No. 1.

density when using Escherichia coli as test organism and an increase in optical density when using Staphylococcus aureus. However, ciprofloxacin and ofloxacin exhibited a slight increase in optical densities (Table 2).

DISCUSSION

Although the clinical importance of the inoculum size is yet unclear, it is of great importance in the study of the in vitro antimicrobial activity[3]. The dependence of susceptibility tests on inoculum size was observed with a group of antibiotics rather than another group regardless the test organism[10]. With gentamicin, amikacin, ciprofloxacin, and ofloxacin, the inoculum size effect was minimal, comparing with cefotaxime and cefoperazone, which was significantly higher (Table 1).

This finding may be clinically important, since development of resistant strains has been reported when ß-lactam antibiotics were used as a single agent in the treatment of serious bacterial infections [11].

The study of inoculum size effect on susceptibility and bactericidal activity of the antimicrobial agents has received some but not enough attention. Therefore, the explanations for the observed inoculum effect have not yet been clearly defined. One of the previous explanations is that the organism may be less susceptible when present in a population of larger numbers. The reduced susceptibility may in part be due to the combined production of ß-lactamases[3&12], or alternatively because of the occasional emergence of resistant variants which would shift the susceptibility results towards the resistant side [13].

The evidence to support the former explanation could be found with ß-lactam antibiotics against Staphylococcus aureus [14]. However, the two ß-lactam antibiotics studied (cefotaxime and cefoperazone), which exhibited a pronounced inoculum size effect are ß-lactamase-stable. These drugs were reported to exist as intact and in their original potency despite the growth of Pseudomonas aeruginosa[4]. Hence, the second suggestion (combined production of ß-lactamases and occasional emergence of resistant variants) seems to be more realistic. It is likely to explain our results observed with cefotaxime and cefoperazone since no difference could be traced between susceptible and resistant strains of both Staphylococcus aureus and Escherichia coli as shown in Table 1.

The inoculum size effect of the ß-lactam antibiotics on Escherichia coli isolates may also be attributed to the increase in bacterial mass in presence of the antibiotics and in absence of any marked bactericidal activity (Fig. 2&4). The absence of noticeable inoculum size effect with gentamicin, amikacin, ofloxacin, and ciprofloxacin against both Staphylococcus aureus and Escherichia coli tested strains may be due to their rapid bactericidal activities as shown (Figs. 1-4) and confirmed by the minimal or no optical density.

In conclusion, the inoculum size effect was found to be significantly high when the antibiotics exhibited a slow bactericidal activity (cefotaxime and cefoperazone). When the antibiotic showed a rapid killing rate, no inoculum size effect was detected as shown throughout this study with gentamicin, amikacin, ofloxacin, and ciprofloxacin. Accordingly, a higher inoculum size effect may be expected with antibiotics of slower bactericidal activity while minimal inoculum size effect may be associated with rapid acting bactericidal drugs.

REFERENCES

1- Irvani, A.; Gina, S.W.; Bruce, R.N. and Richard, G.A.: **Antimicrob. Agents Chemother.**, 27 : 449-451 (1985).

2- Buesing, M., and James, H.J.; **Antimicrob. Agents Chemother.**, 25 : 283-585 (1984).

3- Budger, R.R. and Washington, A.; **Antimicrob. Agents Chemother.**, 17 : 393-396 (1980).

4- Corado, M.; Landesman, S.H. and Cherubin, C.E.; **Antimicrob. Agents Chemother.**, 18 : 893-896 (1980).

5- Eng, R.H.; Smith, S.M. and Cherubin, C.; **Antimicrob. Agents Chemother.**, 26 : 42-47 (1984).

6- Hirschhorn, L. and Harold, N.; **Antimicrob. Agents Chemother.**, 30 : 143-146 (1986).

7- Blair, J. Lennette, E.H. and Traunt, J.P.; **Manual Clinical Microbiology.** American Society for Microbiology. Bethesda MD. p. 300 (1970).

8- Jones, R.N.; Barry, A.L.; Gavan, T.L. and Washington, J.A.; Susceptibility tests : microdilution and macrodilution broth procedures, p. 972-977. In : E.H. Lennette, A. Balows, W.J. Hausler, Jr., and H.J. Shadomy (eds), **Mannual of Clinical Microbiology,** 4th ed. American Society for Microbiology, Washington D.C. (1985).

9- Schoenknecht, F.D.; Sabath, L.D. and Thornsberry, C.; Susceptibility tests : Special tests, p. 1000-1008. In : E.H. Lennette, A. Balows, W.J. Hausler, Jr., and H.J. Shadomy (eds), **Mannual of Clinical Microbiology,** 4th ed. American Society for Microbiology, Washington D.C. (1985).

10- Robert, H.K.; Charles, C.; Sharon, M.S. and Frank; **Antimicrob. Agents Chemother.**, 28 : 601-606 (1985).

11- Simon, G.L.; Snydham, D.R.; Tally, F.P. and Gorbach, S.L.; **Antimicrob. Agents Chemother.**, 18 : 167-170 (1980).

12- Sabath, L.D.; Garner, C.; Wilox, C. and Finland, M.; **Antimicrob. Agents Chemother.**, 8 : 344-349 (1975).

13- Gwynn, M.N. and Rolinson, G.N.; **Infection**, 8 : 73-80 (1980).

14- Chapman, S. and Steigbiegel, R.; **J. Infect. Dis.**, 147 : 1078-1082 (1983).

دراسة تأثير الحجم المحقون لبعض العترات الإكلينيكية من المكور العنقودى الذهبى والايشريشيا كولاى على نشاط بعض المضادات الحيوية البيتالاكتامية والامينو جليكوزيدية والفلوركينولية

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تناولت هذه الدراسة تأثير الحجم المحقون لخمس عترات اكلينيكية من المكور العنقودى الذهبى وكذلك خمس عترات اكلينيكية من الايشريشيا كولاى على فعالية ستة مضادات حيوية.

وقد وجد أن للحجم المحقون من هذه العترات المختبرة تأثيراً واضحاً على نشاط المضادات الحيوية البيتالاكتامية (السيفوبيرازون والسيفوتاكسيم)، أما المضادات الحيوية من مجموعة الفلوركينولون (الاوفلوكساسين والسيبروفلوكساسين) والامينوجليكوزيدية (الاميكاسين والجنتاميسين) فقد أظهرت تأثيراً قاتلاً سريعاً وكان للحجم المحقون من البكتريا المختبرة تأثيراً طفيفاً على نشاط هذه المضادات الحيوية محل الدراسة. وقد أظهرت النتائج بصفة عامة أن تأثير الحجم المحقون لعترات الإيشريشيا كولاى على المضادات الحيوية المختبرة أكبر من تأثيرهم لعترات المكور العنقودى الذهبى.