Journal of Antimicrobial Chemotherapy (1989) **24**, 387–395

How predictable is development of resistance after β-lactam therapy in *Enterobacter cloacae* infection?

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Certain non-fastidious Gram-negative bacilli, notably *Enterobacter cloacae*, although classified as susceptible by usual in-vitro susceptibility testing, often become resistant in patients treated with newer β-lactam antibiotics. Here various in-vitro tests were carried out together with an animal model allowing the quantification of resistance that emerges after short term therapy. Mice were challenged (10^8 cfu plus talcum) intraperitoneally with one each of four strains of *Ent. cloacae*. Two hours later, a single β-lactam dose was administered subcutaneously. The following day, the peritoneal bacterial population was analysed by using antibiotic-containing gradient plates. Development of resistance after therapy varied according to the compound considered. Imipenem (50 mg/kg) produced no resistance, and piperacillin (200 mg/kg) only a few, while resistance occurred frequently after therapy with aztreonam (50 mg/kg), ceftazidime (50 mg/kg), cefotaxime (50 mg/kg) and cefpirome (50 mg/kg). MICs increased by at least 16-fold when resistance developed. No simple correlations were found between these in-vivo results and initial MICs, killing kinetics, frequency of resistant variants within the bacterial populations before therapy, initial MIC of these variants or antibiotic concentrations assayed in peritoneal fluid 60 min after dosing. The most reliable predictive in-vitro test appeared to be the determination of resistance emerging in broth containing at least 16 times the MIC of the antibiotic tested. Such a test is unlikely to be used on a routine basis. When a β-lactam compound seems appropriate for treating an Enterobacter infection, it may be advisable to avoid drugs that are prone prone to produce resistance in experimental or clinical infections, whatever the results of conventional in-vitro susceptibility tests.

Introduction

During recent years, the development of bacterial resistance after therapy with newer β-lactam antibiotics has been a concern (Sanders & Sanders, 1985). Bacterial species possessing this capacity include mainly non-fastidious Gram-negative aerobic bacilli, notably *Enterobacter cloacae*. These organisms are often classified as susceptible with the conventional susceptibility testing, causing 'very major' errors (Sanders, 1984) potentially harmful for the patients. In our laboratory, we have developed a murine model to detect and quantify resistance emerging after short-time therapy (Pechère *et al.*, 1986). In this model, we have shown that resistance could occur after a single administration of a β-lactam compound but the rate of resistance varied according to the compound considered (Marchou *et al.*, 1987*b*). Ceftriaxone, a third generation cephalosporin, appeared to be the most prone to select resistance in *Ent. cloacae*

infections, followed by carumonam, a monobactam. No resistance occurred after therapy with either cefepime (previously BMY 28142) a newer cephalosporin, or SCH 34343, a new penem. Since these observations may help the clinician, we carried out experiments with six other currently used β-lactam compounds and four strains of *Ent. cloacae*. The in-vivo results were matched with various in-vitro tests carried out with the same strains and the same antibiotics.

Materials and methods

Antibiotics, strains and culture media

Aztreonam, ceftazidime, cefotaxime, cefpirome, imipenem and piperacillin were kindly supplied by their respective manufacturers in the form of powder of known potency, from which fresh working solutions were prepared immediately before use. Four clinical isolates of *Ent. cloacae*, strains 218, 219, 895 and 908, previously described by Marchou *et al.* (1987*a*) were studied. Strains were maintained in skim milk and stored at −70°C. At the start of the study, the strains were thawed and plated on blood agar to check purity. Mueller-Hinton broth and agar (Oxoid Ltd, Basingstoke, Hampshire, England) were used for all precultures, in-vitro tests, colony counts and antibiotic-containing gradient plates. The incubation temperature was 35°C.

Susceptibility testing

Antimicrobial activity was determined by two methods. The first was a microdilution technique for determination of MICs (Thornsberry *et al.*, 1983). The second uses antibiotic-containing gradient plates (Bryson & Szybalski, 1952) as described previously (Michéa-Hamzehpour *et al.*, 1987). This method allowed the definition of two levels of antibiotic activity. The first level was read at the boundary concentration. The boundary is the relatively sharp limit separating the confluent growth at lower antibiotic concentrations from the zone of higher antibiotic concentrations, where only single colonies grow. The second level of antibiotic activity, called the no-growth concentration in this article, was the minimal concentration inhibiting all visible growth.

Killing effect and emergence of resistance in vitro

Strains of *Ent. cloacae* were grown overnight in broth and inocula were diluted in order to provide approximately 3×10^7 cfu/ml and added to broth (final volume 5 ml) containing 16 times the MIC of the drug to be tested. Cells were subcultured immediately and after 6 and 12 h of incubation. For viable counts, a portion (0·1 ml) was sampled from each vial, diluted serially into 0·9% NaCl and plated on antibiotic-free agar. Colonies were enumerated after 24 h of incubation. For determination of boundary concentrations, another portion (0·1 ml) of bacterial culture was transferred to antibiotic-free broth and incubated overnight. Then 0·1 ml of this culture was spread uniformly over the antibiotic-containing gradient plates. After incubation for 24 h, bacterial growth was examined as described in the susceptibility testing section.

Frequency of resistant variants

Portions (0·1 ml) of an overnight broth culture were uniformly spread on antibiotic-free agar (after proper dilution) and antibiotic-containing agar (undiluted inoculum).

Antibiotic concentrations were four and eight times the corresponding MICs. Colonies were counted after 24 h of incubation.

Animal model

Swiss ICR female mice, weighing 20 to 30 g, were conditioned for one week after receipt from the breeders and kept in conventional cages with free access to water and antibiotic-free chow. Inoculum was prepared from an overnight broth culture and diluted with 0·9% NaCl. One ml of this overnight diluted culture containing approximately 10^8 cfu and 125 mg of sterile talcum (magnesium hydropolysilicate) was injected intraperitoneally to establish peritonitis. Talcum was used as a foreign body in order to obtain lethal sepsis in all control mice (Pechère *et al.*, 1986). Two hours after bacterial challenge, a single dose of antibiotic was administered subcutaneously. Twenty-two hours later, mice were killed by hyperanaesthesia and peritoneal fluid was sampled and plated after appropriate dilutions on antibiotic-free agar for colony counts, whereas the rest of the sample was placed in antibiotic-free broth to allow overnight growth for further susceptibility testing. Data were compared from treated and untreated control animals. A significant shift towards resistance was defined as an increase of the boundary concentration by at least four-fold.

Antibiotic assay

Drug concentrations in peritoneal fluid of uninfected mice were determined by a microbiological assay, as previously described (Michéa-Hamzehpour *et al.*, 1986).

Results

Susceptibility testing before therapy

Before therapeutic exposure, the four strains of *Ent. cloacae* were susceptible to the antibiotics tested (Table I). On a concentration basis aztreonam and cefpirome were the most potent drugs. All strains exhibited a similar susceptibility profile to β-lactam antibiotics. MICs as determined by microdilution and boundary concentrations as assessed on antibiotic-containing gradient plates were equivalent within one dilution. Single colonies grew beyond the boundary concentration on gradient agar for all drugs except imipenem. The no-growth concentrations varied from strain to strain, isolate 895 showing the highest values.

Frequency of resistant variants

Actual inoculum size in these experiments was (mean ± S.D.) $1{\cdot}15 \pm 0{\cdot}33 \times 10^9$ cfu. Similar growth ($0{\cdot}46$–$3{\cdot}20 \times 10^6$ colonies) was observed on plates containing aztreonam, ceftazidime, cefotaxime or piperacillin (Table I). Resistant variants were less frequent on cefpirome agar, while imipenem agar yielded no growth. Plates seeded with strain 895 showed higher frequency of variants with all antibiotics except on ceftazidime agar, where strain 219 appeared to contain more variants. The MICs of resistant variants were remarkably stable after several subpassages in antibiotic-free agar or broth.

Table I. Susceptibility testing and frequency of resistant variants of *Ent. cloacae* parent strains

Drug	Strain	Concentration (mg/l) determined by microdilution MIC	antibiotic gradient boundary	antibiotic gradient no-growth	Frequency ($\times 10^{-6}$) resistant variants 4 × MIC	Frequency ($\times 10^{-6}$) resistant variants 8 × MIC
Aztreonam	218	0·03	0·125	8	1·46	1·09
	908	0·03	0·06	12	1·10	0·95
	219	0·03	0·06	32	0·61	0·52
	895	0·04	0·06	48	2·70	2·40
Ceftazidime	218	0·25	0·50	32	1·14	0·78
	908	0·25	0·50	8	1·09	0·80
	219	0·25	0·25	64	2·83	2·67
	895	0·25	0·50	96	1·40	1·20
Cefotaxime	218	0·125	0·50	64	1·25	0·97
	908	0·25	0·25	32	1·04	0·83
	219	0·25	0·25	96	0·81	0·74
	895	0·50	0·50	128	3·20	2·80
Cefpirome	218	0·06	0·06	2	0·13	0·02
	908	0·03	0·03	2	0·80	0·10
	219	0·06	0·06	4	0·33	0·12
	895	0·06	0·06	8	1·80	0·71
Imipenem	218	0·5	0·8	0·8	<0·01	<0·01
	908	0·5	0·8	0·8	<0·01	<0·01
	219	0·5	0·5	0·5	<0·01	<0·01
	895	0·75	1·0	1·0	<0·01	<0·01
Piperacillin	218	2	4	140	0·95	0·65
	908	1	2	75	0·99	0·97
	219	2	4	192	0·47	0·46
	895	4	8	256	1·23	1·03

In-vitro killing studies

Actual inoculum size was (mean ± S.D.) $3{\cdot}3 \pm 0{\cdot}89 \times 10^7$ cfu/ml. Viable counts decreased by more than 2 logs under all test conditions (Table II). Imipenem was the most bactericidal compound (3·8–4·5 $\log_{10}$/ml decrease after 6 h, compared to 2·9–3·4 in the case of the five other antibiotics). Generally less viable cells were found after 12 h (except in four cases). After 24 h of incubation, regrowth was observed in all cases (data not shown).

Emergence of resistance in vitro

Increase in the boundary concentration was measured after 12 h of drug exposure in the vials used for killing studies. Subcultures were made into antibiotic-free broth, then on antibiotic-containing gradients. The decrease in susceptibilities was impressive with aztreonam, ceftazidime, cefotaxime and cefpirome, and notably less striking with piperacillin (Table II). No resistance developed when imipenem was tested.

Table II. Killing studies and susceptibility testing by antibiotic-containing gradient agar of *Ent. cloacae* parent strains (inoculum $3{\cdot}3 \pm 0{\cdot}89 \times 10^7$ cfu/ml) after 6 and 12 h of incubation in Mueller-Hinton broth containing 16 × MIC of each of six antibiotics

Antibiotic	Strain	Log_{10} cfu/ml decrease after 6 h	Log_{10} cfu/ml decrease after 12 h	Fold increase of boundary concentration after 12 h
Aztreonam	218	2·85	3·22	500
	908	2·32	2·83	600
	219	3·38	3·19	>600
	895	2·97	4·08	>600
Ceftazidime	218	3·00	3·17	120
	908	3·05	4·14	200
	219	2·85	3·88	>600
	895	2·50	4·16	>600
Cefotaxime	218	3·01	3·07	200
	908	3·45	2·53	500
	219	4·28	2·25	>600
	895	2·95	4·58	>600
Cefpirome	218	2·86	3·46	32
	908	3·39	3·51	>600
	219	3·89	4·53	>600
	895	3·84	3·55	>600
Imipenem	218	4·53	6·15	1
	908	4·26	4·97	1
	219	4·39	5·78	1
	895	3·83	4·55	1
Piperacillin	218	3·14	3·72	50
	908	2·99	3·83	40
	219	2·71	2·96	75
	895	3·06	3·74	35

Animal studies

Thirty-six infected, but untreated, mice were used as controls. Severe peritonitis was observed in all cases. At autopsy, the peritoneal fluid was purulent and contained $3{\cdot}49 \pm 1{\cdot}19 \times 10^{10}$ cfu/ml. Since the number of peritoneal bacteria at the time of death clearly surpassed the injected inoculum ($1{\cdot}51 \pm 0{\cdot}46 \times 10^8$ cfu/ml), an actual infection occurred in all cases.

One hundred forty four infected mice were treated. Therapeutic activity was assessed by comparing the number of peritoneal viable counts in control and treated animals (Table III). All treatments produced antibacterial effect, which varied according to the drug. The most efficient antibiotics were aztreonam, ceftazidime, cefpirome and imipenem. Cefotaxime and above all, piperacillin produced less killing ($P < 0{\cdot}05$) compared to the four others by student's *t* test). Development of resistance after therapy varied according to the compound considered. Imipenem produced no resist-

Table III. Bacterial population analysis of peritoneal fluid after a single dose therapy in mice challenged with *Ent. cloacae* (inoculum: $1{\cdot}5 \pm 0{\cdot}46 \times 10^8$ cfu). Six mice were used in each row

Treatment (mg/kg)	Strain	Log_{10} cfu decrease[a] after therapy (mean ± S.D.)	Number of mice with acquired resistance (fold increase of boundary concentration)
Aztreonam	218	2·5 ± 1·9	1 (250)
	908	2·0 ± 0·6	4 (250–800)
	219	3·4 ± 2·2	3 (> 500)
	895	1·3 ± 0·6	2 (500)
Ceftazidime	218	2·3 ± 1·3	3 (125–200)
	908	4·1 ± 1·7	2 (250)
	219	2·9 ± 1·5	5 (250–640)
	895	4·2 ± 1·8	4 (500)
Cefotaxime	218	1·0 ± 0·8	6 (125–250)
	908	1·6 ± 1·5	6 (250–500)
	219	1·4 ± 1·2	5 (400–1000)
	895	0·6 ± 0·4	6 (300–500)
Cefpirome	218	2·2 ± 1·4	5 (16–250)
	908	2·5 ± 0·8	4 (64–250)
	219	1·2 ± 0·7	3 (32–140)
	895	1·4 ± 1·5	4 (64–500)
Imipenem	218	1·9 ± 0·9	0
	908	2·3 ± 1·3	0
	219	2·9 ± 1·1	0
	895	2·3 ± 1·1	0
Piperacillin	218	0·8 ± 0·6	1 (32)
	908	0·3 ± 0·1	0
	219	0·2 ± 0·1	1 (32)
	895	0·3 ± 0·1	0

[a]As determined in peritoneal fluid after comparison with control animals.

ance, and piperacillin only a little, while more resistance occurred frequently after therapy with the four other drugs. Analysis by the chi square test applied to these results (with three degrees of freedom to take into account the data of each strain separately) showed that the difference between imipenem and piperacillin on the one hand, and the four other drugs on the other hand, was significant ($P < 0{\cdot}05$), but not the differences between aztreonam, cefotaxime, ceftazidime and cefpirome or between imipenem and piperacillin.

Antibiotic assays

Antibiotic concentrations in peritoneal fluid of non-infected mice 60 min after subcutaneous administration are presented in Table IV. All these concentrations were well above the initial MICs of the organisms causing the infection.

Table IV. Antibiotic concentration in peritoneal fluid of non-infected mice 60 min after β-lactam subcutaneous administration

Antibiotic	dosage (mg/kg)	Antibiotic concentration in peritoneal fluid[a] (mg/l)
Aztreonam	50	36·4
Ceftazidime	50	57·8
Cefotaxime	50	34·4
Cefpirome	50	35·7
Imipenem	50	12·3
Piperacillin	200	51·8

[a]Mean of three determinations.

Discussion

The present study confirmed that development of resistance depended on the β-lactam administered to the animal. Imipenem and piperacillin, as cefepime (ex BMY 28142) and SCH 34343 in previous studies (Marchou *et al.*, 1987*b*) were less prone to produce resistance in *Ent. cloacae* infection than the other compounds tested. The mechanisms by which *Enterobacter* spp. can escape the lethal effects of potent antibiotics such as third-generation cephalosporins is complex. *Ent. cloacae* possesses the ampC gene (Lindberg & Normark, 1988) encoding for β-lactamases falling into class I of the Richmond & Sykes' classification (Richmond & Sykes, 1973). The most convincing indication that β-lactamases are associated with resistance was provided by transfer of the chromosomal *bla* gene from a stably derepressed mutant of *Ent. cloacae* into *Escherichia coli*, leading to acquisition of β-lactam resistance by the recipient *Esch. coli* (Seeberg *et al.*, 1983). *Enterobacter* populations are heterogenous with regard to β-lactamase production. The wild type cells produce basal low levels of enzymes, which can be greatly increased by induction (Lindberg & Normark, 1987). Linked to the inducibility is the ability to undergo mutation to high level constitutive β-lactamase production. Such mutants occurred at high frequency (10^{-5} to 10^{-7}) and appeared to be highly resistant to penicillins (including piperacillin), and the cephalosporins tested; they exhibited decreased susceptibility to aztreonam, but remained susceptible to imipenem. Whether the resistance is caused by inducible wild type cells or by constitutive hyperproducer mutants has been a matter of controversy, but several lines of evidence favour the second hypothesis (Livermore & Young, 1987; Marchou *et al.*, 1987*a*). Consistently β-lactamase production by all four *Ent. cloacae* strains used in our study was inducible (Marchou *et al.*, 1987*a*), and that did not correlate the emergence of resistance in the mice. However, limited penetration of the antibiotic into the bacterial cell is an important consideration for the understanding of resistance. Several studies have shown that relative impermeability of the outer membrane acts synergistically with enzymatic inactivation to produce resistance (Marchou *et al.*, 1987*a*; Sawai, Yamaguchi & Hiruma, 1988). In our study, this interactive system probably accounted for the performance of imipenem, whose compact molecular structure (MW 299) endowed it with the highest penetration rate among all β-lactams tested in reconstituted proteoliposomes with porins of *Esch. coli* (Yoshimura & Nikaido, 1985) or in intact cells of *Esch. coli*, *Ent. cloacae* and *Citrobacter freundii* (Hashizume, Yamaguchi &

Sawai, 1986). The inability of piperacillin to select resistance in the mouse does not fit the model however, as this drug is rapidly hydrolysed by *Ent. cloacae* β-lactamase (unpublished personal results) and exhibited a slow relative permeability rate (Yochimura & Nikaido, 1985).

The question of resistance developing during therapy is even more knotty since, besides bacterial characteristics, pharmacokinetics and host factors also contribute. The prediction of successful therapy which is the major issue of susceptibility testing appears here specially difficult. It has been well recognized (Sanders, 1984) that conventional tests fail to detect these types of resistance. MICs were consistently poor predictors of response of infection in-vivo. The MICs of the resistant variants, as determined by the no-growth concentrations, together with the antibiotic concentrations obtained in the peritoneal fluid, were thought to be associated with further emergence of resistance in the mouse (Marchou *et al.*, 1987*b*). Unfortunately this assumption did not hold in the present study, as eloquently illustrated by the piperacillin and the cefpirome results (Tables I and III).

Also disappointing was the frequency of resistant variants. For instance, in aztreonam experiments conducted with strains 219 and 895 respectively, the number of colonies growing on agar plates containing four or eight times the MIC did not correlate accurately with the murine trials.

In a study dealing with a few human cases of meningitis caused by *Enterobacter* and *Serratia* spp. (Eng *et al.*, 1987) the extent to which an isolate was killed by cefotaxime or latamoxef in a 6-h in-vitro incubation showed good correlation with the clinical outcome. However, once again, the present study cannot support this view, as compounds showing similar killing effects against a specific strain did not necessarily display different resistant rates in the animal.

Finally, the most reliable in-vitro test in this study was the determination of the emergence of resistance in broth containing 16 times the MIC of the drug as shown in Table II. Piperacillin and imipenem performed better, both in-vitro and in-vivo. One must admit however that such a test, although easy to perform in a research laboratory, would be difficult to carry out on a routine basis. On practical grounds, a pragmatic attitude is probably relevant. When a β-lactam antibiotic seems indicated for treating an *Ent. cloacae* infection recognized as such, it is probably advisable to select a molecule which is less likely to produce resistance, as shown in the murine model. Imipenem, piperacillin, as well as cefepime and SCH 34343 (Marchou *et al.*, 1987*b*) belong to this list.

Acknowledgements

This work was supported by the Fonds National Suisse de la Recherche Scientifique (grant 3,079-087). We are indebted to Lasta Kocjančić and Anneliese Kahr for their helpful technical assistance.

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(Received 20 February 1989; accepted 1 May 1989)