ENTEROBACTERIA, β -LACTAM ANTIBIOTICS AND β -LACTAMASES IN CLINICAL PRACTICE

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THE resistance of enterobacteria to penicillins and cephalosporins is usually associated with β -lactamase production. Three main types of enzyme have been described (Jack and Richmond, 1970): penicillinase (Sawai, Mitsuhashi and Yamagishi, 1968), cephalosporinase (Fleming, Goldner and Glass, 1963) and wide-spectrum β -lactamase (Datta and Kontomichalou, 1965; Marshall *et al.*, 1972). Some of these enzymes can be inhibited by β -lactam antibiotics. Antibiotic resistance may be mediated by R factors and so be transferable between bacterial species.

We have studied 1050 strains of enterobacteria collected in 3 years by a laboratory serving a cardio-thoracic hospital and a rural general practice. The study began as an epidemiological survey, but it was soon noted that many strains were ampicillin resistant—a reflection of the widespread use of this antibiotic. Therefore the resistance of the strains to four β -lactam antibiotics was assessed. Later, 325 strains that were resistant to 100 μ g per ml of at least one of the four were examined for β -lactamase production. The types of enzyme produced were determined by substrate specificity and inhibitor profiles (O'Callaghan and Muggleton, 1967; O'Callaghan and Morris, 1972), and possible correlations between species, antibiotic resistance and enzyme production were investigated. Several previously reported enzyme-producing strains were also studied for comparison. Finally some of our strains were examined to see how often resistance was mediated by R factors.

METHODS

Organisms were isolated on horse-blood or MacConkey agar and examined in batches for basic biochemical characteristics and for growth in the presence of ampicillin, cephaloridine, carbenicillin and cephalexin each at a concentration of 100 μ g per ml. Resistant strains were cloned and stored at -70°C ; they were subsequently re-tested with 500 μ g per ml and 100 μ g per ml of each of the antibiotics and identified according to Cowan and Steel's (1965) tables. Fermentation and sensitivity tests were performed in microtitration trays and other tests in Replidishes. Subcultures were made with multi-point inoculators (Newsom, 1974).

Production of β -lactamase, and the substrate profiles of the enzymes, were determined by the membrane-filter technique of Knox and Smith (1961). Antibiotics were incorporated into agar at $\times 5$ – $\times 10$ their minimal inhibitory concentrations for the Oxford staphylococcus, which was used as indicator. The concentrations used were: benzylpenicillin 0.4 μ g per ml; ampicillin 1.25 μ g per ml, carbenicillin 2 μ g per ml, methicillin 2.5 μ g per ml, cloxacillin 2.5

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μg per ml, cephaloridine 0.1 μg per ml and cephalixin 5 μg per ml. Substrate destruction was recorded qualitatively. The method measured only constitutive enzyme.

Inhibition of β -lactamase. This was assessed by measuring the increase in inhibition zone produced by a 25- μg cephaloridine-sensitivity disk (Oxoid) impregnated with 200 μg of the inhibitors cloxacillin, methicillin or their cephalosporin analogues 5/1, and 291/1, compared with that produced by a control 25- μg cephaloridine disk. Agar plates were seeded with the strains under test and the control and "inhibitor" disks were applied.

Intracellular enzymes. These were prepared by ultrasonic disintegration of cells, and their capacity to degrade cephaloridine in the presence or absence of each of the four inhibitors was monitored by a recording spectrophotometer (Pye-Unicam SP 800) set at a fixed wavelength of 255 nm (O'Callaghan, Muggleton and Ross, 1969).

TABLE I

Resistance to β -lactam antibiotics of strains of enterobacteria isolated over a three-year period*

Species	Number of strains		
	sensitive	resistant	examined
<i>Enterobacter</i> and <i>Citrobacter</i> spp.	25	113	138
<i>Proteus morgani</i> and <i>P. vulgaris</i>	0	35	35
<i>P. mirabilis</i>	135	29	164
<i>Escherichia coli</i>	307	91	398
<i>Klebsiella</i> spp.	23	153	176
Others	108	31	139
Total	598	452	1050

* Strains with the ability to grow in 100 μg per ml of ampicillin, carbenicillin, cephaloridine or cephalixin were regarded as resistant.

Resistance-transfer factors. A variety of *Escherichia* and *Klebsiella* strains were examined for resistance transfer by a modification of Datta's (1962) method. Non-lactose-fermenting variants of *Escherichia coli* strain K12 resistant either to streptomycin or nalidixic acid were used as recipients.

Reference strains. *Enterobacter cloacae* strains P99 (Fleming *et al.*, 1963) and 214 (Hennessey, 1967), *Klebsiella aerogenes* strain 1082E (Marshall *et al.*, 1972), *Esch. coli* strains TEM (Datta and Kontomichalou, 1965) and D31 (Burman, Nordström and Boman, 1968) and *Proteus morgani* no. NCTC235 (O'Callaghan and Muggleton, 1967) were included in the survey for comparison.

RESULTS

Four hundred and fifty strains (of 1050) grew in 100 μg per ml of at least one of the four β -lactam antibiotics. *Enterobacter*, *Klebsiella*, *P. vulgaris* and *P. morgani* strains were virtually all resistant, but most strains of *Esch. coli* and *P. mirabilis* were sensitive (table I). Cowan and Steel's tables were adequate for the classification of resistant strains, although all the *Klebsiella* strains (of

which 45% were indole positive) were regarded as *K. aerogenes*. The citrobacter strains were all of type 1. One hundred and eight strains could not be identified on the initial biochemical results and were not studied in more detail.

Some resistant strains were not examined further, either because there were too few isolates to be representative of a species or because a clear enzyme pattern had already been established. Multiple isolates from the same patient were also omitted. β -Lactamase production was therefore studied in 325 strains whose origin is recorded in table II. *Enterobacter* and *P. vulgaris* were found

TABLE II

The origin and distribution of resistant organisms according to location of patient and type of specimen

Species	Number of strains tested	Percentage of strains tested	Percentage of strains isolated from location*							Percentage of strains isolated from			
			1	2	3	4	5	6	7	urine	sputum	wound	other sites
<i>Enterobacter</i> and <i>Citrobacter</i> spp.	88	27	50	10	5	6	21	7	1	11	61	13	15
<i>Klebsiella</i> spp.	111	34	29	9	4	12	31	9	6	26	52	12	10
<i>Escherichia coli</i>	72	22	24	0	12	15	20	29	0	47	36	7	10
<i>Proteus morgani</i> and <i>P. vulgaris</i> .	25	8	32	0	5	27	22	7	7	26	42	20	12
<i>P. mirabilis</i>	29	9	14	35	7	14	10	20	0	25	30	40	5
Any	325	100	32	10	6	12	23	14	3	48	26	14	12

* Key to location: 1. open-heart-surgery ward; 2. surgical ward; 3. cardiac ward; 4. female chest ward; 5. male chest ward; 6. general practice; 7. necropsy specimens.

only in hospital patients, whilst *Esch. coli* and *P. mirabilis* were the commonest species among the general-practice patients. The ratio of sputum : urine as a source of resistant strains was 2:1 in hospital and 1:5 in general practice. Many resistant strains (42%) came from the surgical wards, in which the nose and throat of the patients were screened for acquisition of Gram-negative rods, after open-heart surgery; 23% came from the male medical ward from patients with chronic bronchitis.

Patterns of resistance to the four antibiotics are correlated with enzyme production in the figure; while substrate-destruction and enzyme-inhibition profiles (including those of the two cephalosporin analogues) are summarised in tables III and IV. There was a general correspondence between the resistance pattern and the generic grouping of the organisms.

Strains of Enterobacter and Citrobacter. Eighty-eight strains were predominantly cephalosporin resistant, even at 500 μ g per ml. Three-quarters of these produced a β -lactamase that destroyed cephaloridine, cephalixin and

benzylpenicillin but was susceptible to all four inhibitors (table IV). Several active enzyme-producing strains destroyed methicillin and ampicillin, but none attacked cloxacillin or carbenicillin (table III). Only two strains produced enzymes as active as those of strains P99 and 214 (table III).

Strains of Klebsiella. All the *klebsiella* strains grew in 100 μ g per ml of ampicillin, carbenicillin or both; but the indole-positive and indole-negative strains showed differences.

FIGURE—Patterns of resistance to, and destruction of, β -lactam antibiotics by resistant strains of enterobacteria: Cb=carbenicillin; A=ampicillin; Ce=cephaloridine; Cx=cephalexin; P=penicillin; M=methicillin; Cl=cloxacillin.

TABLE III
Substrate destruction patterns of the β-lactamases of enterobacteria that were resistant to β-lactam antibiotics

Substrate-destruction pattern*							Numbers of strains with stated substrate profile				Previously reported strains with similar substrate profiles
Cx	Ce	P	A	M	Cb	Cl	<i>Klebsiella</i> spp.	<i>Escherichia coli</i>	<i>Enterobacter</i> and <i>Citrobacter</i> spp.	<i>Proteus mirabilis</i> and <i>P. vulgaris</i>	
+	+	+	+	+	+	+	13	2†	0	0	<i>K. aerogenes</i> 1082E
							7†	6†	0	0	
							0	2†	0	0	<i>Esch. coli</i> RGN238
+	+	+	+	+	+	+	1	2†	0	0	
							7	4†	0	0	<i>Esch. coli</i> TEM
							7	2	0	0	
							22	7	0	0	
							12	0	0	0	
							4	17	4	0	
							7	0	0	0	
							0	1	16	0	<i>P. mirabilis</i> 235
+	+	+	+	+	+	+	0	3	48	4	
+	+	+	+	+	+	+	0	2	2	0	
+	+	+	+	+	+	+	0	7	1	1	<i>Esch. coli</i> D31
+	+	+	+	+	+	+	0	0	2	0	<i>Ent. cloacae</i> P99 and 214
Other patterns							6	7	5	6	
No detectable enzyme							25	10	10	6	
Any pattern							111	72	88	25	29

* Cx = Cephalaxin; M = methicillin; Ce = cephaloridine; Cb = carbenicillin; P = penicillin; Cl = cloxacillin; A = ampicillin.
† Producers of R-factor-mediated β-lactamase.

TABLE IV
Profile of inhibition of enterobacterial β -lactamases by β -lactam antibiotics

Species	Serial numbers of previously reported strains with similar inhibition profiles	Inhibition* of β -lactamase by				Total number of strains of the stated species	Number of strains with the stated inhibition profile
		cloxacillin	methicillin	cephalosporin 5/1	cephalosporin 291/1		
<i>Enterobacter cloacae</i>	P99, 214	+	+	+	+	60	60
<i>Ent. aerogenes</i>		+	+	+	+	14	14
<i>Citrobacter freundii</i>		+	+	+	+	14	14
<i>Proteus morgani</i>	235	+	+	+	+	15	15
<i>P. vulgaris</i>		+	+	+	+	10	2
<i>P. mirabilis</i>		+	+	+	+	14	1
<i>Escherichia coli</i>	D31	+	+	+	+	48	7
<i>Klebsiella</i> spp. (indole positive)	1082E	—	—	—	—	36	36
<i>Klebsiella</i> spp. (indole negative)		—	—	—	—	30	25
<i>P. mirabilis</i>		—	—	—	—	14	10
<i>Esch. coli</i>	RGN238	—	—	—	—	48	20
<i>Klebsiella</i> spp.† (indole negative)		+	+	—	—	30	3
<i>Klebsiella</i> spp. (indole negative)		—	+	—	—	30	2
<i>P. mirabilis</i>		+	+	—	—	14	2
<i>Esch. coli</i> †		+	+	—	—	48	16
<i>Esch. coli</i>	TEM	—	+	—	—	48	5

* + = Inhibited; — = not inhibited.

† Producers of R-factor-mediated β -lactamase.

Forty-nine indole-positive strains were nearly all isolated from hospital patients, and were active enzyme-producers. Thirteen grew in 500 μ g per ml of each of the four antibiotics and produced a strain 1082E- like enzyme that was unaffected by the inhibitors.

Some indole-negative strains came from general practice patients. They were less active; 45 of 62 strains yielded a detectable enzyme, but only six degraded cephaloridine. Resistance patterns were not well correlated with β -lactamase production. The inhibitor compounds had no effect on the majority of strains but three that degraded cephaloridine were inhibited by methicillin and to some extent by cloxacillin. Two of them produced enzymes like those found in certain *Esch. coli* strains containing R factors, which they were subsequently found to be carrying.

Strains of Proteus. There was little correlation between antibiotic resistance and enzyme production except in *P. morgani* (the figure, tables III and IV). The *P. morgani* strains resembled *P. morgani* no. NCTC233 and were cephalosporin resistant. All but two strains produced an enterobacter-like enzyme particularly active against cephalosporins and susceptible to all four inhibitors. Strains of *P. vulgaris* produced weak enzymes that acted only on single but differing substrates, although six strains grew in 500 μ g per ml of one or more of the antibiotics. Methicillin inhibited all these enzymes, but the other inhibitors had variable effects. The *P. mirabilis* strains were predominantly resistant to the penicillins; only three produced detectable enzymes, all of which were different.

Strains of Esch. coli. The resistant *Esch. coli* strains had a greater diversity of resistance, substrate-specificity and inhibitor patterns than did members of the other groups. The cultures were more often resistant to penicillins at the 500 μ g per ml level; but the enzyme patterns varied and were placed into well defined groups by the inhibitor profiles (tables III and IV).

Seven cephalosporin-resistant strains produced a cephalosporinase which—like those of the enterobacter and *P. morgani* strains—was susceptible to all four inhibitors. This enzyme appeared to be chromosomally mediated like that of *Esch. coli* strain D31; resistance could not be transferred to a recipient culture. All seven strains originated in the hospital.

A second group of predominantly penicillin-resistant strains produced enzymes that were inhibited by methicillin and often by cloxacillin. These enzymes resembled that of *Esch. coli* strain TEM. R-factor-mediated resistance was demonstrated in all our strains. This group occurred both in hospital and general-practice patients.

A heterogenous group of 20 strains had a wide range of resistance patterns and produced enzymes that were not inhibited (table IV). Most of these strains produced weakly active enzymes that destroyed only one or two of the penicillin substrates. Resistance was found to be R-factor-mediated in two strains with enzyme profiles like that of *E. coli* strain RGN238 (table III). Five other strains carried R factors; they degraded all the substrates except methicillin and cloxacillin.

Intracellular enzyme preparations. These were made from 22 strains of

Klebsiella, 15 of *Esch. coli* and 7 of *Enterobacter*. The cephaloridine-destruction profiles and the inhibition of cephaloridine degradation by the four inhibitor compounds were identical to those found by the biological methods.

DISCUSSION

All the enterobacteria isolated during 3 years were included in the study, although comparatively few were of clinical importance. Cowan and Steel's tables adequately defined the species except those of *Klebsiella*, which were all classified as *K. aerogenes*. According to Bascomb *et al.* (1971) these organisms would all have been grouped as *K. aerogenes/oxytoca/edwardsii*, but neither classification recognises the indole-positive strains as a separate entity. Indole-positive strains were commonly encountered; unlike those reported by Ørskov (1957), they occurred in urine as well as sputum. These strains, together with enterobacter and *P. vulgaris* strains, were "hospital" organisms; but the time-scale of isolations and the growth of different species from successive patients indicated that single-source infection seldom occurred.

The antibiotic concentrations employed in the sensitivity tests were selected to indicate potential enzyme producers rather than to detect resistance of possible clinical significance. Patterns of resistance did fall into well defined groups, except in *Escherichia* and *Proteus*, where episomal transmission may have occurred. This suggested that the resistance in other species may have been chromosomal.

The use of relatively simple micromethods, and of biological methods of enzyme analysis, made the study feasible. A marked relationship between species, antibiotic resistance and the formation of enzymes of specific types became clear during the survey. Patterns of cephaloridine degradation and its inhibition were similar to those obtained by more complex biochemical methods. Fixed antibiotic levels were used to determine substrate specificity and so the results may have been imprecise for weak enzyme producers such as the *Proteus* spp. Inducible β -lactamases were not considered in this survey, though these have been described, particularly amongst proteus and enterobacter strains (Ayliffe, 1964). A few of our strains were examined after induction and their fundamental enzyme type was unchanged. Possibly, however, induction of all our strains would have resulted in more being recorded as active, particularly amongst the proteus strains. Potentiation of cephaloridine activity was sometimes seen even when enzymic degradation had not been demonstrated. This was probably due to the production of inducible enzyme in the presence of cephaloridine.

Examples of most of the β -lactamases so far reported were found amongst our strains. Inhibitor studies divided the enzymes into three main groups. (1) *Cephalosporinases* were produced by *Enterobacter*, *Citrobacter* and *P. morgani*, and by *Esch. coli* strains with chromosomally-mediated resistance like that of *Esch. coli* strain D31 (Burman *et al.*, 1968). All four inhibitor compounds inhibited these enzymes. (2) Broad-spectrum penicillinases were produced by *Klebsiella* and by some *Esch. coli* strains. These were unaffected by the inhibi-

tors. (3) R-factor-mediated β -lactamases, with a broad spectrum of activity, were produced by *Esch. coli* and a few *Klebsiella* strains. These enzymes were partially inhibited by cloxacillin and completely by methicillin, but differed from cephalosporinase in being resistant to inhibition by the cephalosporin analogues 5/1 and 291/1.

This survey therefore indicated a close relationship between enzyme production and resistance of strains to β -lactam antibiotics. The finding of the distinct enzyme types fitted with the observation that except for the R-factor-mediated enzymes, the β -lactamase produced by an organism is related to its taxonomic group.

SUMMARY

Strains of enterobacteria isolated in a cardio-thoracic hospital and a rural general practice were collected for three years. The organisms were tested for sensitivity to four β -lactam antibiotics, and the β -lactamases from resistant strains were subsequently studied. There was no evidence of single-source infection with most of the strains.

Investigations were carried out on 1050 of the isolates. Eighty per cent. of the *Escherichia coli* and *Proteus mirabilis* cultures were sensitive to the two penicillins and two cephalosporins used and were omitted from further studies. The organisms resistant to 100 μ g per ml of at least one of the four drugs were grouped taxonomically and the β -lactamases from 325 of them were examined. Relationships between the antibiotic resistance of the organisms and the substrate specificities and inhibitor profiles of their enzymes were studied. Seven β -lactam antibiotics were used to determine enzyme substrate-specificities and four for the inhibitor profiles. *Enterobacter*, *Citrobacter*, *P. morgani* and *P. vulgaris* were predominantly resistant to the cephalosporins and produced cephalosporinases that were inactivated by all the inhibitor compounds. *Klebsiella*, *Esch. coli* and *P. mirabilis* were mainly penicillin resistant. *Klebsiella* enzymes attacked penicillins and cephalosporins, but were not inhibited. The β -lactamases from *Esch. coli* strains showed a wide diversity of substrate degradation and inhibitor profiles, partly because of the frequency with which the enzyme activity was R-factor-mediated.

Most of the previously reported β -lactamases from enterobacteria were encountered among our organisms. The resistance patterns and enzyme types of the strains were well correlated with the generic groups of the producer organisms.

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REFERENCES

- AYLIFFE, G. A. J. 1964. Induction of cephalosporinase and penicillinase in proteus species. *Nature, Lond.*, **201**, 1032.
- BASCOMB, S., LAPAGE, S. P., WILLCOX, W. R. AND CURTIS, M. A. 1971. Numerical classification of the tribe Klebsiellae. *J. gen. Microbiol.*, **66**, 279.

- BURMAN, L. G., NORDSTRÖM, K. AND BOMAN, H. G. 1968. The resistance of *Escherichia coli* to penicillins-V. *J. Bact.*, **96**, 438.
- COWAN, S. T. AND STEEL, K. J. 1965. Manual for the identification of medical bacteria, London.
- DATTA, N. 1962. Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*. *J. Hyg., Camb.*, **60**, 301.
- DATTA, N. AND KONTOMICHALOU, P. 1965. Penicillinase synthesis controlled by infectious R-factors in Enterobacteriaceae. *Nature, Lond.*, **208**, 239.
- FLEMING, P. C., GOLDNER, M. AND GLASS, D. 1963. Observations on the nature, distribution and significance of cephalosporinase. *Lancet*, **1**, 1399.
- HENNESSEY, T. D. 1967. Inducible β -lactamases in *Enterobacter*. *J. gen. Microbiol.*, **49**, 277.
- JACK, G. W. AND RICHMOND, M. H. 1970. A comparative study of eight distinct β -lactamases synthesised by Gram-negative bacteria. *J. gen. Microbiol.*, **61**, 43.
- KNOX, R. AND SMITH, J. T. 1961. Use of cellulose acetate membranes for detecting penicillinase-producing organisms. *Nature, Lond.*, **191**, 926.
- MARSHALL, M. J., ROSS, G. W., CHANTER, K. V. AND HARRIS, A. M. 1972. Comparison of the substrate specificities of the β -lactamases from *Klebsiella* 1082E and *Enterobacter cloacae* P99. *Appl. Microbiol.*, **23**, 765.
- NEWSOM, S. W. B. 1974. Easy, economic typing for enterobacteria. In New approaches to the identification of microorganisms, edited by C. G. Heden and H. Illeni, New York.
- O'CALLAGHAN, C. H. AND MUGGLETON, P. W. 1967. The action of cephaloridine with cloxacillin or methicillin against β -lactamase-producing Gram-negative bacteria. *J. gen. Microbiol.*, **48**, 449.
- O'CALLAGHAN, C. H., MUGGLETON, P. W. AND ROSS, G. W. 1969. Effects of β -lactamases from Gram-negative organisms on cephalosporins and penicillins. *Antimicrob. Agents Chemother.*, 1968, p. 57.
- O'CALLAGHAN, C. H. AND MORRIS, A. 1972. Inhibition of β -lactamases by β -lactam antibiotics. *Antimicrob. Agents Chemother.*, **2**, 442.
- ØRSKOV, I. 1957. Biochemical types in the klebsiella group. *Acta. path. microbiol. scand.*, **40**, 155.
- SAWAI, T., MITSUHASHI, S. AND YAMAGISHI, S. 1968. Drug resistance of enteric bacteria. XIV. Comparison of β -lactamases in Gram-negative rod bacteria resistant to α -aminobenzyl penicillin. *Jap. J. Microbiol.*, **12**, 423.