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Susceptibility of Recent Isolates of *Pseudomonas aeruginosa* to Gentamicin, Polymyxin, and Five Penicillins, with Observations on the Pyocin and Immunotypes of the Strains[1]

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The susceptibilities of recently isolated strains of *Pseudomonas aeruginosa* to gentamicin, polymyxin B, carbenicillin, ampicillin, penicillin G, and two newer penicillins were tested with the inocula-replicating technique by using undiluted and 10^{-3} dilutions of the cultures. With either inoculum, polymyxin B was the most active agent, and a comparison with previous data from this laboratory showed that the susceptibility of *P. aeruginosa* to this antibiotic had not changed over the past 20 years. Gentamicin was nearly as active as polymyxin, all but 2 of the 141 strains tested with the diluted inoculum being inhibited by 6.25 μg/ml or less. AB-2288, an agent resembling carbenicillin, was four times more active than carbenicillin or BLP-1654; the last two were equally active against the 10^{-3} inoculum. A more marked inoculum effect was noted with the penicillin analogues tested, the increase in minimum inhibiting concentration with the undiluted culture being eightfold for carbenicillin and at least 16-fold for AB-2288 and BLP-1654. Pyocin typing and serotyping failed to demonstrate any clearly predominating types.

In recent years *Pseudomonas aeruginosa* has assumed an important role as a causative organism in intrahospital epidemics and as an increasingly frequent cause of death from bacterial sepsis (4, 5). These infections have been difficult to treat because of their resistance to most of the commonly used antibiotics and the necessity to resort to more toxic agents. Until quite recently, the polymyxins (polymyxin B and colistin) were the only agents which had useful in vitro activity against most strains of of *P. aeruginosa*. However, the advent of gentamicin and carbenicillin has made available less toxic agents with greater antibacterial activity against these infections.

Strains of *P. aeruginosa* recently isolated from specimens of infected materials submitted to the bacteriology laboratory of the Boston City Hospital have been collected on several occasions in the past and tested for susceptibility to available and potentially useful antibiotics (7, 15, 24). Additional collections of contemporaneous strains have been included during studies of the activity of individual antibiotics, particularly new ones while under clinical trial (3, 10, 20). Other chemically related antibiotics such as the aminoglycosides (12), tetracyclines (23), and broad spectrum penicillins (22) were included in some of these studies.

In this paper, we present data on the comparative susceptibility of recent clinical isolates of *P. aeruginosa* to polymyxin B, gentamicin, carbenicillin, ampicillin, penicillin G, and two newer penicillins; one of them, BLP-1654, resembles ampicillin and the other, AB-2288 (also designated BRL-2288), resembles carbenicillin. Also included are the results of pyocin typing and immunotyping of most of these isolates.

MATERIALS AND METHODS

Selection and identification of strains. The strains of *P. aeruginosa* included in this study were isolated from clinical specimens submitted to the bacteriology laboratory of the Boston City Hospital. They were identified by, or under the supervision of, A. Kathleen Daly and Alice McDonald. Duplicate isolates from the same site in the same patient were not collected, and only one strain recovered from a patient was

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tested. ("Strains" and isolates are therefore used here interchangeably.) Strains were identified as *P. aeruginosa* by characteristic colonial morphology, pigment production, growth on selective agar (Cetrimide), citrate utilization, and failure to ferment lactose, sucrose, and mannitol.

Susceptibility tests. Quantitative tests for susceptibility to antimicrobial agents were done on Beef Heart Infusion Agar (Difco) by the inocula-replicating method of Steers et al. (21). Overnight broth cultures were tested in every instance with a 10^{-3} dilution of culture as inoculum; in addition, 31 randomly selected strains were also tested with undiluted cultures. The replicator delivers an inoculum of approximately 0.002 ml, containing approximately one to two million colony-forming units of the undiluted culture. The lowest concentration giving no visible growth after incubation for 20 to 24 hr at 37 C was taken as the minimum inhibiting concentration (MIC).

Antibiotics. Solutions of antibiotics were freshly prepared in sterile distilled water. To effect the solution of BLP-1654, it was necessary to use 5% $NaHCO_3$ and then to add HCl to achieve a final *p*H of 7.0 to 7.2. Samples of solutions containing 2,000 or 4,000 μg of each antibiotic per ml were stored at −20 C; each was thawed only once prior to use, and unused portions were discarded. Solutions of BLP-1654, AB-2288, and carbenicillin were freshly prepared for each set of tests and not stored. The antibiotics used and their suppliers were: sodium penicillin G, Pfizer; ampicillin trihydrate and BLP-1654, Bristol; disodium carbenicillin and AB-2288, Beecham; gentamicin sulfate, Schering; and polymyxin B sulfate, Burroughs-Wellcome.

Typing methods. Pyocin typing was performed by the method of Gillies and Govan (8) with eight indicator strains, kindly supplied by Jonas A. Shulman, Emory University School of Medicine.

Immunotyping of selected strains was performed in the laboratory of M. W. Fisher, Parke, Davis & Co., Detroit, Mich., by the method which he and his co-workers described (6).

RESULTS

Sources of the strains. The sources of the isolates are listed in Table 1. Although the lower respiratory tract accounted for 40% of the strains, the majority of strains from this source did not occur in pure culture and were usually not associated with pneumonia. Only 20% of the respiratory isolates grew out in pure culture or were associated with significant clinical infection of the respiratory tract. Infections of the urinary tract accounted for 23% of the isolates and postoperative wounds for another 11%. Blood cultures were positive in 10 of the 163 patients. Nearly 80% of the isolates were acquired in the hospital; only those strains recovered from skin and subcutaneous infections were predominantly community-acquired. Only one isolate from each patient was included.

TABLE 1. *Clinical sources of 163 isolates of Pseudomonas aeruginosa: Boston City Hospital—1970*

Source	No. of isolates	Per cent of isolates	Hospital-acquired strains	
			No.	Per cent[a]
Lower respiratory tract[b]	65	39.9	52	80
Urinary tract	38	23.3	30	79
Postoperative wound	18	11.4	18	100
Skin and subcutaneous tissue	13	8.0	3	23
Blood	10	6.1	8	80
Others[c]	19	11.7	15	79
All sources	163	100	126	77.3

[a] Per cent of strains from the same source.

[b] Only 20% of the strains were "significant pathogens"; the others were "colonizers."

[c] Six pleural fluids, three middle ear fluids, and one each from peritoneal fluid, subclavicular joint, renal carbuncle, bone, eye, liver biopsy, bile, decubitus ulcer, pharynx, and catheter tip.

Susceptibility to antibiotics. The upper panel of Fig. 1 shows the susceptibility of the strains of *P. aeruginosa* to polymyxin B, gentamicin, and five penicillins, when using a 10^{-3} dilution of culture as inoculum. Polymyxin B was the most active agent, the median MIC being 1.6 μg/ml; only two strains were not inhibited by 6.25 μg/ml. Gentamicin was nearly as active as polymyxin B, the MIC values ranging from 0.1 to 25 μg/ml (median, 3.1 μg/ml).

AB-2288 (also designated BRL-2288) was the most active of the five penicillins tested; the median of the 163 isolates tested was 12.5 μg/ml (range 0.1 to >400 μg/ml). It was generally four times more active than either carbenicillin and BLP-1654, both of which were about equally active against a 10^{-3} dilution of culture; only 3% of strains were resistant to 400 μg/ml or more of any of these three agents.

The lower panel of the figure shows the influence of inoculum size on the MIC of each antibiotic for 31 randomly selected strains tested in parallel. A twofold increase in the median MIC for polymyxin B and gentamicin was demonstrated. A more marked inoculum effect was noted with the penicillins; one-half of the strains were resistant to 400 μg/ml or more of carbenicillin and AB-2288, and 82% of the strains were resistant to similar concentrations of BLP-1654 when the undiluted inoculum was used. The results obtained with 31 isolates, using undiluted cultures with penicillin G and ampicillin, are not shown; these two penicillins were essentially in-

active (for any clinical application) against all of the isolates tested.

Pyocin types. One hundred and four strains were subjected to pyocin typing. Of these, 43 (41%) were classified into one or another of the specific pyocin types of Gillies and Govan (8); another 16 gave atypical reactions, and 45 strains did not inhibit the growth of any of the indicator strains. Types 5, 35, 1, 4, and 2 were the most frequent pyocin types isolated and comprised 58% of typable strains.

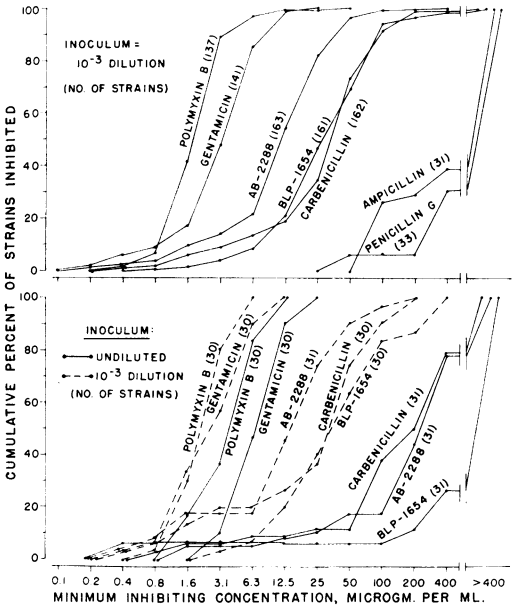

FIG. 1. *Susceptibility of Pseudomonas aeruginosa to polymyxin B, gentamicin, and five penicillins. Inocula-replicating method (20) with overnight cultures in Brain Heart Infusion Broth inoculated on Heart Infusion Agar.*

Immunotypes. Eighty-three strains were submitted for immunotyping; 10 of them were not typable, and the other 73 strains were separated into 7 different immunotypes (Table 2). Types 3 (22 strains), 4 (11 strains), 1, and 2 (10 strains each) were the most frequent. There was no relationship between the pyocin type and immunotype of individual strains; nontypable strains in one system were not necessarily nontypable in the other system. Nor was there any correlation between specific type and place of acquisition (community or hospital) of the organism. Immunotypes 2 and 4 appeared to be associated with a particular site of colonization, 9 of the 11 strains of type 2 and 8 of the type 4 strains being isolated from the respiratory tract. No other special predilection of specific immuno- or pyocin types for any particular site(s) could be discerned, nor was resistance to any of the antibiotics more frequent among strains of any one type.

DISCUSSION

P. aeruginosa is best known for the production of a green pigment, pyocyanin. It also produces another substance, pyocin, which inhibits the growth of some other strains of *P. aeruginosa* and is useful in the "typing" of isolates. In the most recent prevalence survey of nosocomial infections at Boston City Hospital, *P. aeruginosa* was cultured from 22% of all nosocomial infections but from only 3% of community-acquired infections (1); thus, it ranked next to *Klebsiella pneumoniae* as the most important nosocomial pathogen.

Significant progress has been made recently

TABLE 2. *Distribution of immunotypes of 83 strains of Pseudomonas aeruginosa isolated from various sites at Boston City Hospital—1970*

Type	Respiratory tract	Urine	Wound	Skin and/or subcutaneous tissue	Blood	Others	All sites	
							No.	Per cent
1	6	3	2			1	12	14
2	9	1	1				11	13
3	6	6	4	3	2	1	22	27
4	8	1			1		10	12
5	1					1	2	2
6	2		1			1	4	5
7	3	4			1		8	10
Mixed	2		1	1	1		4	5
NT[a]	2	2	2	2		2	10	12
Total	39 (47)[b]	17 (20)	11 (13)	6 (7)	4 (5)	6 (7)	83	(100)

[a] Not typable with available antisera.
[b] Values in parentheses indicate per cent.

in the treatment of severe pseudomonas infections. In 1954, Wright and co-workers (13) showed that the vast majority of isolates were not inhibited in vitro by neomycin, streptomycin, chloramphenicol, bacitracin, penicillin, tetracycline, and erythromycin. They were uniformly susceptible to polymyxin B in concentrations ranging from 1.6 to 12.5 μg/ml. In 1961, Postic and Finland (14) tested colistin, kanamycin, demethylchlortetracycline, oxytetracycline, and paromomycin, in addition to chloramphenicol, tetracycline, steptomycin, neomycin, and polymyxin, and demonstrated that only colistin and polymyxin B were effective agents and were about equally active. In 1965, Eickhoff and Finland (3) confirmed the quantitatively similar activity of colistin and polymyxin B. The present study shows that the strains recently isolated at the Boston City Hospital are susceptible to the same concentrations of polymyxin B, indicating that there has been no substantial change, in this respect, over the last 20 years. Nevertheless, because of its toxicity and the difficulty in achieving adequate levels in many tissues, the polymyxins are not suitable agents for the treatment of many patients with severe pseudomonas infections (12, 25).

The availability of gentamicin and carbenicillin has introduced a new dimension in the treatment of patients with pseudomonas infection. Both of these antibiotics have been shown to be active, separately and in combination, against *P. aeruginosa*. Gentamicin has been used at this hospital for more than 2 years, and only 2 of 141 strains recently isolated from clinical cases and tested in this study required more than 6.25 μg/ml for inhibition. When these data are compared with those reported from this hospital by Klein et al. in 1964 (11), no appreciable change in the susceptibility of *P. aeruginosa* to gentamicin over this 6-year period is noted. One reason for this lack of development of significant resistance may be that topical gentamicin is seldom or never used at this hospital. Workers in Atlanta have implicated the topical use of gentamicin as a cause of the occurrence and spread of gentamicin-resistant strains. (Jonas A. Shulman, *personal communication*).

Carbenicillin, AB-2288, and BLP-1654 differ from other penicillins in being active against *P. aeruginosa* and also against both indole-negative and -positive strains of *Proteus*. Structurally, AB-2288 resembles carbenicillin and BLP-1654 is related to ampicillin. Of the three new compounds, BLP-1654 was the least active in vitro against all species of *Proteus* (2), and it was quantitatively similar to carbenicillin in its activity against *P. aeruginosa* when tested with a dilute inoculum; however, it was essentially inactive against most strains when tested with an undiluted inoculum. AB-2288 was four times more active than carbenicillin when tested with a dilute inoculum, but these two penicillins were more nearly equal in activity when tested against undiluted cultures. Quantitatively the results obtained in this study with the diluted inoculum were similar to those reported by Smith and Finland (20) who noted a greater inoculum effect than was found in the present study. The median MIC of carbenicillin for *P. aeruginosa*, namely 50 μg/ml, is readily achieved in the serum when large intravenous doses are administered (20 to 40 g daily); more than 90% of strains were inhibited by 50 μg or less of carbenicillin per ml. In addition, Smith et al. (19) demonstrated that carbenicillin and gentamicin were synergistic in vitro against many strains of *P. aeruginosa*, and polymyxin and carbenicillin also acted synergistically against many other strains.

Because of the resistance of *P. aeruginosa* to most antibiotics, or the necessity to resort to toxic drugs, other means of treating these infections have been investigated. One therapeutic device makes use of a combination of a penicillinase inhibitor or a penicillinase-resistant penicillin such as methicillin or cloxacillin, and another penicillin which is sensitive to penicillinase such as ampicillin or benzylpenicillin (16, 17, 18). The penicillinase-resistant drug preferentially binds to the penicillinase elaborated by the organism, allowing the other penicillin to inhibit cell wall synthesis without itself being destroyed.

With the recognition that the majority of nosocomial infections are currently caused by gram-negative bacilli, it has become necessary, for epidemiological purposes, to utilize more precise methods of identifying and classifying these organisms. Bacteriocines and serological methods of typing have emerged as useful in the classification of strains of *P. aeurginosa*. Gillies and Govan (8) showed that pyocin typing is a valid and sensitive method of classifying *P. aeruginosa* for epidemiological purposes. They differentiated 37 specific pyocin types and have also been able to subclassify type 1, the most common type in their studies, into eight subtypes (9). Several "immunotypes" based on specific "protective" antigens have also been delineated (6) and used for epidemiological purposes (M. R. Moody and V. M. Young, *personal communication*) and also as a basis for the development of a heptavalent *Pseudomonas* vaccine (10).

In the present study, a number of pyocin and

immunotypes were recognized. No correlation could be discerned between the pyocin type and immunotype of individual strains. Among the strains typable by pyocin, no single type predominated; the strains were distributed more or less evenly among 14 recognized types and three patterns of inhibition not previously given a specific number by Gillies and Govan (8). However, many of the strains tested were not inhibited by any of the eight indicator strains employed. In addition, pyocin typing, as employed here, was not always reliable and reproducible. The pyocin produced by a large proportion of these strains did not inhibit growth of any of the indicator strains. This may have been due in some instances to poor elaboration of pyocin by some of the strains, but it may also have been due to occasional fluctuations in the temperature or time of incubation of the test cultures (8, 9).

A larger percentage of the present strains that were tested could be classified by immunotyping than by pyocin typing (88% versus 57%). Immunotype 3 accounted for 27% of the typable strains and was composed of strains of a variety of pyocin types, which, in turn, were both hospital-acquired and community-acquired and were evenly distributed among various anatomic sites of infection.

The data suggest that, in this hospital, immunotypes 2 and 4 were more frequently associated with a respiratory site than with other anatomic foci; no other significant differences among infections in various sites were identified. However, Moody and co-workers (*personal communication*) found that within their hospital certain immunotypes had a specific predilection for various sites of infection. Thus, blood stream invasion was most frequent with immunotypes 2 and 7; these two types were not frequently associated with infections of the respiratory or the urinary tract, whereas types 3 and 6 were significantly associated with upper respiratory tract infections but not with postoperative wound infections or bacteremia.

Phage typing was not carried out in the present study, although an earlier one by Postic and Finland (15) suggested the feasibility and possible usefulness of this method for epidemiological purposes.

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