Nursery Outbreak of *Pseudomonas aeruginosa*: Epidemiological Conclusions from Five Different Typing Methods

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In April 1971, nine cases of *Pseudomonas aeruginosa* septicemia occurred in a high-risk nursery. The epidemiology of the outbreak was studied by pyocin production, pyocin sensitivity, serological typing, antibiotic susceptibility, and phenotypic properties such as colonial morphology, pigment, and hemolysis. Ten isolates of *P. aeruginosa* were recovered from 9 newborns and from 13 environmental sources. Twenty-one of the 23 isolates had identical pyocin production patterns against 60 different indicator strains and were of the same serotype. These 21 isolates were designated as the "outbreak strain"; the other 2 isolates had no epidemiological significance. The results of pyocin sensitivity, antibiotic susceptibility tests, and phenotypic properties were dissimilar. They would yield incorrect epidemiological conclusions if used alone. The outbreak strain dissociated in vitro and these phenotypic changes accounted for the variable results by the latter three typing methods. Although the precise mode of introduction of the organism into the nursery could not be determined in retrospect, the epidemiological data strongly suggested that one infant contracted a *P. aeruginosa* infection, and this strain spread throughout the nursery by means of contaminated resuscitation equipment.

Exposure to *Pseudomonas aeruginosa* constitutes a serious danger to newborn infants during their stay in a hospital nursery. Unless the utmost care is taken, *P. aeruginosa* will survive and often multiply in such diverse nursery environments as resuscitators, humidifiers, incubators, milk formulas, breast pumps, washbasins, water faucets, sinks, sponges, drains, detergent and disinfectant solutions, and personnel (see references 1, 4–7, 11, 17, 19, 20, 21, and 24). Whitby and Rampling (23) recently cultured *P. aeruginosa* from 84 of 154 such hospital environmental samples.

Healthy children exposed to *P. aeruginosa* normally resist serious infection, but respiratory or intestinal colonization may occur without any symptoms to warn of this potentially dangerous event (5–7). Premature infants are more likely to acquire serious infections (15) which can often result in nursery outbreaks of septicemia, meningitis, diarrhea, and pneumonia (1, 11, 19). The particular type of infection usually depends upon the condition of the children, site of entry, number of organisms introduced, and the virulence of the infecting strain.

Currently, epidemiologists use several methods to compare or "fingerprint" isolates: bacteriocin production (10, 12, 13, 25), bacteriocin sensitivity (10, 16), bacteriophage susceptibility (22), antibiotic susceptibility, agglutination in antisera to somatic antigens (14), or simply gross phenotypic properties. Combinations of these have been used successfully, but no standardized method presently exists.

In this article, we describe an outbreak in which we used five different epidemiological typing methods for comparing all the isolates.

**MATERIALS AND METHODS**

**Media.** Medium 81 contained 28.5 g of Trypticase soy broth without glucose (BBL), 10 g of KNO₃, and 1,000 ml of distilled water. This medium was dispensed into 16- by 125-mm screw-capped tubes (10 ml per tube) and autoclaved. All other media were
from commercial sources and were prepared according to the manufacturers' instructions.

Identification of \( P. \) aeruginosa. The isolates studied were identified by colonial morphology, oxidase reaction, oxidative utilization of glucose, oxidation of gluconate to 2-ketogluconate, growth on Pseudosel agar (BBL), pyocyanin production on Pseudomonas agar P (Difco), and fluorescein production of Pseudomonas agar F (Difco).

**Antibiotic susceptibility pattern determinations.** Antibiotic susceptibility was determined by the method of Bauer et al. (2). Minimal inhibitory concentrations were determined by serial dilution of sulfadiazine in Mueller-Hinton agar (BBL).

Typing by pyocin sensitivity. The pyocin sensitivity of each isolate was determined by a slight modification (7) of the method described by Farmer and Herman (10). Pyocin lysates were prepared from standard pyocin-producing strains A through X which were grown in Medium 81 for 24 h and treated with chloroform. The 24 pyocin lysates were then added to lawns of outbreak isolates previously described (8, 10).

Typing by pyocin production. Pyocin lysates were prepared from each of the outbreak isolates as described above, and the pyocin production pattern was determined with the following pyocin indicators: 27 of Farmer and Herman (10), 8 of Gillies and Gowan (12), 5 of Gowan and Gillies (13), 12 of Zabransky and Day (25), and 8 of L. W. Griffith, V.A. center, Wilmington, Del. The lysates (containing pyocin or bacteriophages, or both) from each outbreak isolate were also tested against each other in all possible combinations. If these strains are identical, none should produce a pyocin that would inhibit any other (18). Pyocin reactions were recorded according to the code described by Farmer (9) and shown in Table 1.

**Serological typing.** The O antigens of each isolate were determined by William T. Martin, Center for Disease Control, Atlanta, Ga. We retyped several isolates with the procedure and antisera kindly supplied by W. T. Martin. Each isolate was grown on a mechanical shaker at 37 C for 24 h in a flask containing 30 ml of Brain Heart Infusion broth (BBL), heated at 121 C for 30 min, and centrifuged for 30 min at 5,000 × g. The supernatant fluid was discarded and the cells were suspended in 5 ml of saline. Each isolate was typed by slide agglutination with antisera to the 13 O antigens of Habs (14).

**Pigment production.** Each isolate, grown in Medium 81 for 24 h at 37 C, was inoculated onto a surface (2 by 2 cm) of Trypticase soy agar (TSA;BBL) in a sterile REPLI dish (Dyos Plastics, 242 Tolworth Rise South, Surbiton, Surrey, England). The dish was then incubated at 37 C and pigment production was recorded every 24 h. Each isolate was inoculated in a 4-mm long streak on Mueller-Hinton agar, and fluorescein production was determined after 24 h. The formation of autoplaques (3) was also noted on these plates.

**Hemolysis of sheep blood agar (SBA).** Each isolate was inoculated in a 2-mm circle onto TSA with 5% defibrinated sheep blood and incubated at

| Table 1. Code for reporting pyocin production patterns* |
|--------------------------------------------------------|
| 3 Pyocin reactions                                | Representation |
| + + +                                                 | 1               |
| + + -                                                 | 2               |
| + - +                                                 | 3               |
| - + +                                                 | 4               |
| + - -                                                 | 5               |
| - + -                                                 | 6               |
| - - +                                                 | 7               |
| - - -                                                 | 8               |

* A strain with a pyocin production pattern + + + − + − + − − − would be coded “348” (+ + + = 3, − + − + = 4, − − − = 8). If the total number of pyocin reactions was not evenly divisible by 3, a second (+ + = A, + = B, − = C, − = D) and third alphanumeric (+ = E, − = F) were used to code the remaining reactions.

37 C. Zones of hemolysis and the number of different distinct bands were measured and noted every 24 h.

**Dissociation among the isolates.** We made the working hypotheses that 21 of the isolates were of the same strain in a genetic and epidemiological sense and that the differences in phenotypic properties were due to dissociation (26) which had occurred. Each of the isolates, picked from single isolated colonies, was treated to enhance dissociation in vitro and then put into environments which simulated the conditions of the outbreak. These experiments consisted of storing the isolates in the following sources: TSA slants at 37 C and room temperature; Trypticase soy broth at 37 C and room temperature; sterile distilled water at room temperature; distilled water with rubber or plastic suction tubing; and sterile blood of various red cell and Rh types. After several weeks, a loopful from each source was streaked onto TSA and the plate was incubated at 37 C. The colonies were examined with a dissecting microscope and any colonies different from the wild-type were tested for susceptibility to sulfadiazine and sulfathiazole, pigment and phage production, hemolysis of sheep blood, and formation of autoplaques.

**RESULTS**

Two cases of \( P. \) aeruginosa sepsis occurred on 21 and 22 February 1971. In addition to the blood specimens, endotracheal aspirate material from the two cases grew \( P. \) aeruginosa. Infection with \( P. \) aeruginosa was uncommon in the high-risk nursery studied. From 28 March 1971 through 16 April 1971, seven additional cases of \( P. \) aeruginosa sepsis occurred. Endotracheal aspirate material from these cases also yielded \( P. \) aeruginosa.

Eight of the nine infants suffered from both weight and gestational age prematurity and asphyxia requiring endotracheal intubation. All the infants were nursed in isolettes with oxygen and high humidity.
Tracheal cultures from eight infants yielded *P. aeruginosa*. Thus, the respiratory tract appeared to be the likely portal of entry. On 23 April 1971, cultures were taken from nine other infants occupying the same nursery and from various environmental sources (Table 2).

The environmental culture survey showed that equipment used for resuscitation was contaminated with *P. aeruginosa*; thus, control measures instituted on 26 April were directed to this probable source. The wall suction apparatus was discontinued and mucous trap suction by mouth was instituted. Oxygen humidifiers were scrubbed immediately after use and autoclaved, and plastic suction tubings were discarded after one use. The one O-Syl solution that yielded *P. aeruginosa* was later shown not to be associated with the outbreak. To date, no additional cases of pseudomonas infection have been observed in this nursery.

By 1 May 1971, 23 isolates were obtained from the following sources: 10 blood cultures (9 infants), 5 suction bottles, 6 suction bottle tubings, 1 oxygen bottle tubing, and 1 O-Syl solution.

**Pyocin production patterns.** Isolates 1 through 21 had identical pyocin production patterns. Each of the 60 pyocin reactions of the 21 isolates were identical even to size and shape of the zone of inhibition. This striking identity suggested that all 21 isolates were the same strain (called "the outbreak strain") which had spread through the nursery. In addition, none of the 21 isolates produced a pyocin against any of the others. However, isolate 23 from the O-Syl solution and isolate 22 from a suction bottle tubing had different pyocin production patterns (Table 3).

**O antigens.** Isolates 1 through 21 strongly agglutinated in antisera to O antigens 7 and 8 of the Habs schema; thus, serologically they were also identical. The two isolates with different pyocin production patterns also possessed different O antigens: O-Syl isolate 23 was O 2, 5, and suction bottle tubing isolate 22 was O 6 (Table 3).

**Phenotypic differences among the 21 isolates of the outbreak strain.** The absolute identity of the 21 isolates in their pyocin production patterns and serotypes convinced us that the outbreak was due to a single strain of *P. aeruginosa* which had spread throughout the nursery. Preliminary antibiotic susceptibility showed that all strains were resistant by disk to tetracycline, cephalothin, chloramphenicol, kanamycin and ampicillin; and susceptible to gentamicin, polymyxin B, and carbenicillin. However, 5 outbreak strains (isolates 7, 8, 12, 15 and 17) were resistant to >1,000 μg/ml of sulfadiazine whereas the remaining 16 were susceptible to this agent. This difference in antibiotic susceptibility prompted us to look for additional phenotypic differences. We defined four phenotypic groups based on differences such as antibiotic susceptibility, pigment and phage production, hemolysis of sheep blood, and formation of autodigestion. These differences were reproducible and are summarized in Table 4.

**Pyocin-sensitivity patterns.** Pyocin-sensitivity patterns of isolates 22 and 23 were quite different from the remaining 21 isolates. The pyocin-sensitivity patterns of 15 of the remaining 21 isolates were identical; however, all 5 isolates of phenotype D were more sensitive to pyocins than the remaining ones. The pyocin-sensitivity patterns of one representative of these four groups are shown in Fig. 1.

**Comparison of typing methods.** The epidemiological conclusions obtained from each method, independent of the others, are summarized in Table 5. Pyocin production and serological typing each yielded correct results, but each of the other three methods would have resulted in incorrect epidemiological conclusions if they were used alone.

**Dissociation of phenotype D isolates into phenotype B.** When old TSA slants of phenotype D (all 5 isolates) were streaked on TSA, two colony types were usually observed, the wild type and a mutant which was easily distinguishable. The mutant colony was picked, purified, and then tested for the properties listed in Table 4. The mutant was identical to phenotype B in all properties tested. To rule out any possible chance of contamination, three of us repeated this experi-
ment independently and obtained the same results, namely, dissociation of phenotype D into phenotype B. Thus, dissociation in the infants and environment probably resulted in the differences in phenotype. This explains why incorrect epidemiological results were obtained with three of the typing methods. We are currently trying to show dissociation of the other phenotypes.

### DISCUSSION

The initial two cases of *P. aeruginosa* sepsis occurred on 21 and 22 February 1971. The first case was a mature female who developed bilateral pneumonia 2 days after admission to the nursery. She required endotracheal intubation. The source of the *P. aeruginosa* infection in this infant can only be postulated. Prior to this case, there had been no cases of *P. aeruginosa*
TABLE 5. Different epidemiological conclusions obtained from the five typing methods

| Typing method              | Epidemiological conclusion: No. strains involved in outbreak |
|----------------------------|-------------------------------------------------------------|
| Pyocin production         | Single strain*                                              |
| Serological typing        | Single strain                                               |
| Pyocin sensitivity        | Two different strains                                        |
| Antibiotic susceptibility | Four different strains                                       |
| Phenotypic properties     | Four different strains                                       |

*Results do not include isolates 22 and 23 which were different and not related to the outbreak.

infection during the preceding 6 months. Thus, it would appear unlikely that the organisms were introduced from the nursery environment, although this possibility cannot be ruled out. Contamination either from the mother, during passage through the birth canal, or from personel in the nursery appears to be more probable (6, 1).

The third case of pseudomonas sepsis occurred on 28 March 1971 and involved an infant born outside of the institution studied. The infant developed pneumonia and required endotracheal intubation and frequent tracheal suctioning.

Although several weeks had elapsed since the initial case, it appears likely that resuscitation equipment was the source of the outbreak strain in this and subsequent cases. About one-fourth of the samples of equipment yielded the outbreak strain, which lends credence to the hypothesis that this equipment was the common source of the outbreak strain. Although no direct evidence exists to prove that resuscitation equipment acted as the mode of transmission in the cases presented, the elimination of infections following the institution of control measures directed to this probable source is highly suggestive. Prior to the institu-
tion of control measures on 26 April 1971, nine infants, eight of whom died, developed P. aeruginosa sepsis.

Five different techniques were used to study the epidemiology of the outbreak: pyocin production, pyocin sensitivity, serological agglutination of somatic antigens, antibiotic susceptibilities, and phenotypic properties. Only two of these "fingerprinting techniques" gave results consistent with the epidemiological evidence. Pyocin production and serological typing indicated that 21 of the 23 isolates were of the same strain and that this was a single-strain outbreak. If pyocin sensitivity had been the only epidemiological marker used, the explanation would have been different. Results from pyocin sensitivity indicated that there were two different outbreak strains in the environment and in the patients. Since phenotype D isolates and phenotype B isolates are actually of the same strain, incorrect epidemiological conclusions would have resulted. If the outbreak isolates had been compared only by antibiotic susceptibilities or gross phenotypic properties, the epidemiological conclusions would have been even less accurate.

Pyocin production and serological typing have proved to be stable epidemiological markers when properly controlled (see references 1, 10, 12, 13, and 25). Both methods have been thoroughly tested in epidemiological studies. Pyocin sensitivity was described in 1965 by Osmond (16), but has only recently been used to compare strains in epidemiology (10). The molecular basis of pyocin sensitivity is similar to that for bacteriophage susceptibility, but unfortunately, colonial types of the same strain which have dissociated often have different phage susceptibilities (26). This has limited the use of phage susceptibility as an epidemiological marker. In this study dissociants of the outbreak strain had different pyocin sensitivity patterns which would have led to incorrect conclusions had pyocin sensitivity been used alone. Although pyocin sensitivity is a convenient marker for routine typing, its lack of stability may limit its usefulness. We have also observed changes in pyocin sensitivity in another outbreak we are typing.

It is tempting to compare strains by antibiotic susceptibility, colony type, pigment production, hemolysis, and other simple markers because this can be done with little effort. However, these markers must not be used to discount the possibility of an outbreak or cross-infection caused by a single strain of P. aeruginosa, because they are not stable genetic markers. P. aeruginosa strains quickly dissociate both in vivo and in vitro and these dissociants are usually quite different from the wild-type strain (26). A stable marker such as pyocin production or serological typing should be used to investigate and define the epidemiology of a possible outbreak.

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