Pseudomonas cepacia Strains Isolated from Water Reservoirs of Unheated Nebulizers

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Pseudomonas cepacia strains were isolated from the water reservoirs of unheated nebulizers in a hospital setting. The isolates were characterized by morphology, biochemical tests, and antibiotic susceptibility patterns. An interesting feature of these organisms was their capacity for sustained multiplication in either doubly deionized or doubly distilled water as well as 5% dextrose or 0.9% saline. They could not multiply in doubly deionized and then doubly distilled water or in any of several parenteral nutrition solutions studied. Isolation of *Pseudomonas cepacia* from the water reservoirs of unheated nebulizers suggests that this equipment may serve as a source of respiratory tract exposure to the organism.

Pseudomonas cepacia (multivorans, EO-1), previously considered only as a plant pathogen, has been isolated from hospital water supplies, physiological saline, and disinfectant solutions (1, 3, 10, 12) and has been demonstrated to be an agent of nosocomial infection (3, 10, 12, 16, 18). Strains of the organism isolated from commercial distilled water and from reservoirs of mist therapy units have been characterized by Carson et al. (4). The present study describes the morphological, biochemical, and drug susceptibility characteristics of strains of *P. cepacia* isolated from water reservoirs of unheated nebulizers.

Grieble et al. (8) previously reported an epidemic of *P. aeruginosa* infections which was traced to improperly maintained nebulizers. Although no such epidemic has been reported for *P. cepacia* infections, the increasing frequency of reports of resistant *P. cepacia* infections in seriously ill patients (13, 15, 16, 17, 18) suggests the possibility of future outbreaks in the hospital setting. Isolation of *P. cepacia* from the water reservoir of unheated nebulizers suggests that this equipment may serve as a source of respiratory tract exposure to the organism.

MATERIALS AND METHODS

Isolation technique. Samples were collected aseptically from 70 different water reservoirs of unheated nebulizers (Puritan-Bennet Corp., Kansas City, Mo.) from various sections of a 1,400-bed hospital including intensive care units and open wards. All units were sampled by a microbiologist (S. M.

G.) after the nebulizers had been in use for at least 6 h. Since the units were not dated there was no way to establish how long they were actually in service. Each water reservoir was sampled once. A 1.0-ml aliquot was plated onto agar plates (nutrient agar, 1.5%; Difco). These were incubated aerobically in a stationary position at 37 C. All glassware used was acid washed (10% HCl), rinsed three times in deionized water, and autoclaved before use.

Biochemical characterization. Pigmentation was tested on Flo and Tech agars (BBL). Motility was examined in hanging drop preparations from deionized water and nutrient broth cultures incubated at 37 C for 24 h. Oxidase activity was tested by streaking colonies from 18-h nutrient agar plates onto cytochrome oxidase test papers (PathoTec). Nitrate respiration was tested by the method of N. R. Smith, R. E. Gordon and F. E. Clark (*in* V. B. D. Skerman (ed.), *Abstracts of Microbiological Methods*, 1969). Intracellular reserves of polyhydroxybutyrate were detected by the method described by Stanier et al. (19) utilizing Sudan black stain. Oxidative fermentation tests (5) were performed by the method of Hugh and Leifson (11) employing OF (oxidative fermentation) basal medium (BBL). GasPak (BBL) jars were used to test anaerobic growth.

Antibiotic susceptibility testing. In vitro antibiotic susceptibility was determined by the single disk method of Bauer et al. (2) employing standard antibiotic-impregnated disks (BBL).

Electron microscopy. Log-phase cultures of bacteria, grown in nutrient broth or in deionized water, were centrifuged ($8,000 \times g$ for 10 min) into a concentrated suspension. One drop of this suspension was placed on Formvar- and carbon-coated grids which were then processed by two methods. For critical-point drying, the grids were fixed in 3% glutaraldehyde in Millonig buffer (pH 7.3) for 1 h. They were then dehydrated in successively higher

concentrations of ethanol, ethanol and amylacetate, and finally in pure amylacetate. The amylacetate was replaced by CO₂ utilizing a Denton DCP-1 critical-point drying apparatus (Denton Vacuum Inc., Cherry Hill, N.J.), and the specimens were coated with carbon and platinum in a Denton DB502 high-vacuum evaporator. For negative staining, the grid was floated on the surface of 0.5% potassium phosphotungstate containing a trace of bovine serum albumin for 15 s and air dried.

Both preparations were examined on an RCA EMU-3H electron microscope.

Growth studies. An inoculum of approximately 10⁴ organisms, consistent with previous studies (6), was used for all growth studies. Flasks were incubated aerobically in stationary positions. For 4 C (± 0.3 C) incubation a Foster refrigeration unit was used. The temperature was monitored by a thermometer placed on the same shelf as the flasks to be tested. The thermometer was examined at the time of entry and removal of flasks at 0, 24, and 48 h. For 22 C incubation, ambient room temperature was used. This varied from 21.2 to 23.5 C. These flasks were examined at 1-h intervals for the first 8 h and at 24 and 48 h. For 37 C incubation, a National incubator (water jacketed; Scientific Products, Evanston, Ill.) was used. The temperature was 37.2 ± 0.4 C. Samples were taken at the same intervals as 22 C samples. For 46 C incubation, a Precision Scientific incubator (Scientific Products) was used. The temperature was 46.3 ± 0.2 C. Samples were taken only at 0, 24, and 48 h.

Growth media used. Intravenous solutions employed as media in this study were 5% and 50% dextrose, 0.9% saline (Abbott Laboratories), and several intravenous nutrition solutions. The nutrition solutions included a casein hydrolysate (5% Amigen, Travenol Laboratories), a fibrin hydrolysate (5% Aminosol, Abbott Laboratories), and two crystalline amino acid solutions. The formulation of these solutions and the experimental design were as previously reported (6). Deionized water (Continental Water Co., Chicago, Ill.) and nutrient broth (Difco) were used for comparison.

RESULTS

Water samples taken from the water reservoir of 70 randomly selected unheated nebulizer units yielded three *P. cepacia* isolates.

The titers of these three isolates averaged over 10⁷ colony-forming units per ml. There was no turbidity or any flocculent material present in the reservoirs that would have indicated contamination.

These three *P. cepacia* strains were compared to a strain of *P. cepacia* previously isolated from commercial distilled water. This fourth strain was obtained from the Center for Disease Control (CDC) Environmental Microbiology Section, Phoenix, Ariz. All strains possessed the same characteristics for the traits tested (Table 1). All strains were gram-nega-

TABLE 1. Characteristics of four *P. cepacia* strains described in this study

Test	Response
Pigment	—
Motility	+
Oxidase	+
Gelatinase	+
Gas in nitrate broth	—
Polyhydroxybutyrate	+
Growth at:	
4 C	—
46 C	—
Acids from:	
Glucose	+
Lactose	+
Fructose	—
Maltose	+
Mannitol	+
Xylose	+
Anaerobic growth	—

tive short rods, unpigmented, motile by direct observation when grown in nutrient broth (but not when grown in deionized water), oxidase and gelatinase positive, and could not produce nitrite or gas when grown in nitrate broth. Polyhydroxybutyrate could be demonstrated in all strains. They did not grow in deionized water or nutrient broth at either 4 or 46 C. They all produced acid oxidatively within 24 h from glucose, lactose, maltose, mannitol, and xylose, but not from fructose. None of the strains could grow in prereduced nutrient agar when incubated anaerobically. All strains could grow exponentially within 24 h in deionized or distilled water but not in water that was first doubly deionized and then doubly distilled in acid-washed glassware.

The antibiotic disk susceptibility patterns for the four strains revealed slight differences. All strains were resistant to erythromycin (15 μ g), penicillin (10 U), lincomycin (2 μ g), methicillin (5 μ g), novobiocin (5 μ g), ampicillin (10 μ g), streptomycin (10 μ g), colistin (10 μ g), polymyxin B (300 U), carbenicillin (100 μ g), and gentamicin (10 μ g), and susceptible to nalidixic acid (30 μ g) and sulfamethizole (1 mg). Our three isolates were also susceptible to tetracycline or chloramphenicol (30 μ g each), and two strains were susceptible to kanamycin, cephalothin, or cephaloridine (30 μ g each). In contrast, the CDC strain was resistant to tetracycline, chloramphenicol, kanamycin, cephalothin, or cephaloridine.

Electron microscopy showed no discernible differences in the size or shape of bacteria when grown in deionized water or in nutrient broth. Cells of all strains possessed up to two

monopolar flagella and pili (Fig. 1). These structures were more prevalent in cells grown in nutrient broth.

All strains could grow in deionized water, physiological saline, or 5% dextrose, but could not multiply in 10 or 50% dextrose or in 3% saline or in any of the several commercially available and investigational intravenous nutrition solutions studied (Fig. 2). This was true when cultures were incubated either at 22 or 37 C.

DISCUSSION

The water reservoirs of unheated nebulizers can be a source of nosocomial infection (8). *P. cepacia*, an organism shown to be a potentially lethal pathogen (12, 16), was found to exist in high concentrations in 3 of 70 units sampled. Our findings confirm that casual

observation cannot judge contamination of these units by this organism, since *P. cepacia* causes no turbidity at concentrations as high as 10^7 colony-forming units/ml (4).

These isolates have been identified as *P. cepacia* based on the following characteristics. They were all unpigmented, oxidase-positive, gram-negative rods that were resistant to polymyxin B and produced no gas from nitrate broth. All other characteristics agree closely with the schemes for identification proposed by Gilardi (7) and Carson et al. (4), with the exception of acid production from fructose, which was negative to very weak in all of our isolates and in the CDC reference strain but was reported as positive in 74 of 74 strains by G. L. Gilardi (presented at the 75th Annual Meeting of the American Society for Microbiology, New York, N.Y., 1975).

FIG. 1. (a) *P. cepacia* (Hines strain 1) grown in deionized water, negatively stained with phosphotungstic acid. Filare scarce. Note length of flagella. (b) *P. cepacia* (CDC, Phoenix) grown in deionized water, negatively stained with phosphotungstic acid. Peritrichous piliation is more common. (c) *P. cepacia* (Hines strain 2) grown in nutrient broth, negatively stained with phosphotungstic acid. Pili and flagella are found on nearly all cells. (d) *P. cepacia* (Hines strain 3) grown in nutrient broth, critical point dried, and shadowed with carbon and platinum. Note arrangement and length of pili.

FIG. 2. Multiplication of *P. cepacia* which were isolated from deionized water in intravenous solutions at 32 C.

No discernible difference in electron microscope morphology was found when the organisms were grown in water or in nutrient broth. Our findings agree with those of Carson et al. (4) that cells grown in water appear to be nonmotile, but they do possess flagella as evinced by electron microscopy (Fig. 1). Why they appear nonmotile cannot be explained at present.

Although these organisms can grow in a wide variety of intravenous solutions, our data (Fig. 2) show that they do not multiply in intravenous feeding (hyperalimentation) solutions under the usual conditions.

This study suggests that deionized or distilled water in the reservoirs of unheated nebulizers can be a growth medium for *P. cepacia*. This finding has interesting implications. Since *P. cepacia* are resistant to chlorhexidine and a variety of quaternary ammonium compounds (1, 12, 16), it is evident that simply disinfecting the units and changing the water probably would not eliminate these organisms unless a disinfectant which has been proven to be effective against *P. cepacia* is used. In addition, our data confirm the unusual drug susceptibility pattern of *P. cepacia* as opposed to that of *P. aeruginosa*, *P. fluorescens*,

and most other pseudomonads. Our isolates and the CDC strain were resistant to both carbenicillin and gentamicin. This is consistent with several recent reports (4, 9, 14, 17, 18) describing clinical infections of *P. cepacia*. This organism has been reported to be sensitive in vitro and in vivo to sulfamethoxazole and trimethoprim (15), and our data confirm in vitro sensitivity to sulfa derivatives. We did not investigate trimethoprim.

In conclusion, *P. cepacia* could be isolated from the water reservoirs in unheated nebulizers in a hospital environment. *P. cepacia* is likely to be found as a contaminant of these units since the organism can grow—not merely exist, but grow—in water with the slightest trace of nutrients.

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