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# 4-h Identification of *Pseudomonas aeruginosa* with 9-Chloro-9-(4-Diethylaminophenyl)-10-Phenylacridan

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A total of 361 gram-negative bacilli were evaluated for their ability to grow in the presence of 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan. The minimum time required for the production of visual turbidity in brain heart infusion broth was determined to be 4 h in shake cultures at 35°C. The minimal inhibitory concentration (MIC) of 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan in brain heart infusion broth was determined for 174 isolates. The MICs for all 40 *Pseudomonas aeruginosa* isolates tested were >50 μg/ml. The MICs for the other 53 pseudomonads tested were ≤5 μg/ml. Among 81 other gram-negative bacilli tested, the MICs for 4 were 15 μg/ml, the MICs for 15 were 10 μg/ml, the MICs for 21 were 5 μg/ml, and the MICs for 41 were ≤1 μg/ml. Based on these data, 361 gram-negative bacilli were inoculated into brain heart infusion broth containing 15 μg of 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan per ml and incubated on a shaker for 4 h. The only bacteria that produced visual turbidity were identified as *P. aeruginosa* (170 of 170 isolates).

*Pseudomonas aeruginosa* is the most frequently isolated nonfermentative bacterium in clinical microbiology laboratories (5, 7). Although cetrimide is commonly used as a selective agent to assist in the isolation and identification of *P. aeruginosa*, several studies (1, 3, 4) have shown that it is not specific for *P. aeruginosa* and that some strains are even inhibited. A new antimicrobial agent, 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan (C-390), was incorporated into pseudomonas agar F and several other media in a study with stock cultures and was found to be both selective and differential for *P. aeruginosa* (4). Robin and Janda (Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C164, p. 298) compared the isolation rate of *P. aeruginosa* with selective media from 102 sputum and stool specimens to that with media containing C-390. The isolation rate of *P. aeruginosa* (12 of 102 specimens) was similar on all media, but contaminating growth from sputum specimens was reduced on C-390 medium. In addition, pseudomonads other than *P. aeruginosa* failed to grow on C-390 medium.

The present evaluation has expanded the number of species of bacteria studied and has defined the optimum concentration of C-390 for incorporation into a broth to allow the identification of *P. aeruginosa* within 4 h.

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## MATERIALS AND METHODS

**Media.** Brain heart infusion broth (BHIB; Difco Laboratories, Detroit, Mich.) was prepared according to the instructions of the manufacturer. Trypase soy agar with 5% sheep blood (BA) and tryptic soy broth with 20% glycerol were obtained from Scott Laboratories, Fiskeville, R.I.

**Bacteria.** A total of 361 gram-negative bacilli were studied (Table 1). There were 170 *P. aeruginosa* isolates, 101 other pseudomonads, and 90 gram-negative bacteria representing seven genera and seven Centers for Disease Control groups.

Sixty-one of the *P. aeruginosa* isolates were recent clinical isolates and were identified with the Oxi/Ferm system (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Nutley, N.J.) and conventional biochemicals (2) in our laboratory. The remaining bacteria (300 isolates) were stock cultures that had been identified by the Houston City Health Department reference laboratory with conventional biochemicals (8).

Recent clinical isolates were subcultured on BA and maintained at 4°C. Stock cultures were maintained at −70°C in tryptic soy broth with 20% glycerol.

**Antimicrobial agent.** The antimicrobial agent C-390 (Norwich-Eaton Pharmaceuticals, Norwich, N.Y.) was supplied by the manufacturer as a powder. A 100-mg amount of C-390 was dissolved in 1 liter of BHIB (C-390 BHIB) and autoclaved at 121°C for 15 min. C-390 BHIB was then diluted with sterile BHIB to obtain final concentrations ranging from 1 to 50 μg/ml, and 1.0 ml was dispensed into sterile, capped tubes (12 by 75 mm).

**Inoculum.** Bacterial isolates were subcultured at least twice on BA at 35°C. After overnight incubation, a small portion of one colony was picked with an

TABLE 1. Distribution of isolates

| Bacteria | No. of isolates |
|---|---|
| *Pseudomonas aeruginosa* | 170 |
| *P. acidovorans* | 5 |
| *P. alcaligenes* | 3 |
| *P. cepacia* | 10 |
| *P. denitrificans* | 4 |
| *P. diminuta* | 10 |
| *P. fluorescens* | 5 |
| *P. mallei* | 1 |
| *P. maltophilia* | 23 |
| *P. paucimobilis* | 4 |
| *P. pickettii* | 1 |
| *P. pseudoalcaligenes* | 7 |
| *P. putida* | 6 |
| *P. putrefaciens* | 4 |
| *P. stutzeri* | 14 |
| *P. testosteroni* | 3 |
| *P. vesicularis* | 1 |
| *Acinetobacter* spp. | 16 |
| *Aeromonas* spp. | 3 |
| *Alcaligenes* spp. | 12 |
| Centers for Disease Control groups | 10 |
| *Flavobacterium* spp. | 8 |
| *Enterobacter* spp. | 15 |
| *Escherichia coli* | 12 |
| *Klebsiella pneumoniae* | 14 |

inoculating needle and ground against the inside of the tube above the liquid. The tube was then tilted so the inoculum mixed with the broth. After inoculation, the medium had a barely detectable degree of turbidity when examined against an indirect light source.

**Incubation.** Broth media were incubated aerobically at 35°C for 4 h on a gyratory shaker (model R-2; New Brunswick Scientific Co., New Brunswick, N.J.) at 250 rpm.

**Interpretation.** Growth in broth media was examined visually against an indirect light source. A moderate to heavy degree of turbidity was considered a positive reaction.

**MICs of C-390.** MICs were determined for C-390 by broth dilution (6) in BHIB.

## RESULTS

**Determination of minimum incubation time.** The minimum incubation time was determined by inoculating 71 isolates into BHIB. One milliliter of BHIB was inoculated with a small portion of one colony and incubated aerobically at 35°C on the shaker. The numbers of bacterial isolates which produced visual turbidity at 2, 3, and 4 h were 18 of 71 (25%), 60 of 71 (83.3%), and 71 of 71 (100%), respectively (Table 2).

**MIC testing of C-390.** Table 3 shows the results of MIC tests for C-390 with 174 bacterial isolates. The MIC for *P. aeruginosa* (40 of 40 isolates) was >50 μg/ml. Among the other *Pseudomonas* spp. tested, the MIC for 10 isolates was 5 μg/ml and that for 43 isolates was ≤1 μg/ml. Among the remaining 81 gram-negative bacteria tested, the MIC for two *Aeromonas* spp. and two *Enterobacter* spp. was 15 μg/ml, the MIC for 15 isolates was 10 μg/ml, the MIC for 21 isolates was 5 μg/ml, and the MIC for 41 isolates was ≤1 μg/ml.

C-390 BHIB at 15 μg/ml was selected for further testing since only *P. aeruginosa* grew at this concentration. A total of 361 bacterial isolates (Table 1) were tested in C-390 BHIB and BHIB control tubes by emulsifying a portion of one colony in the broth. All isolates tested demonstrated moderate to heavy turbidity in BHIB control tubes when incubated for 4 h at 35°C on the gyratory shaker. *P. aeruginosa* isolates (170 of 170) gave a positive result in C-390 BHIB when incubated for 4 h, whereas all of the other bacterial isolates tested were negative.

## DISCUSSION

Marold et al. (4) reported that the addition of C-390 to agar media enhanced their selective and differential properties for *P. aeruginosa* when incubated overnight at 35°C. Our results demonstrated that the addition of C-390 to BHIB allowed isolates of *P. aeruginosa* to be differentiated from other gram-negative bacilli within 4 h. BHIB was used as the base medium for this evaluation and yielded excellent growth of *P. aeruginosa* within 4 h (Table 2). Some strains of *P. aeruginosa* were positive as early as 2 h in C-390 BHIB, and many strains were positive within 3 h. We also performed a limited series of tests with tryptic soy broth as the base medium for C-390 and found that results were equivalent to those obtained with BHIB.

C-390 medium might be incorporated into commercial systems that identify nonfermentative, gram-negative bacilli since this single agent will identify *P. aeruginosa*. This would allow the manufacturers of commercial systems to delete biochemicals that would otherwise be required

TABLE 2. Time required to produce visual turbidity

| Bacteria | No. tested | No. of isolates showing visual turbidity in BHIB at: | | |
|---|---|---|---|---|
| | | 2 h | 3 h | 4 h |
| *Acinetobacter* spp. | 16 | 5 | 16 | 16 |
| *Aeromonas hydrophila* | 2 | 0 | 2 | 2 |
| *Alcaligenes* spp. | 5 | 0 | 4 | 5 |
| Centers for Disease Control groups | 10 | 1 | 8 | 10 |
| *Flavobacterium* spp. | 8 | 2 | 5 | 8 |
| *Pseudomonas aeruginosa* | 15 | 6 | 14 | 15 |
| *Pseudomonas* spp. other than *P. aeruginosa* | 15 | 4 | 11 | 15 |

TABLE 3. MICs of C-390

| Bacteria | No. tested | No. of strains at the following MIC (μg/ml): | | | | |
|---|---|---|---|---|---|---|
| | | ≤1 | 5 | 10 | 15 | >50 |
| *Pseudomonas aeruginosa* | 40 | | | | | 40 |
| *P. acidovorans* | 3 | 3 | | | | |
| *P. alcaligenes* | 3 | 3 | | | | |
| *P. cepacia* | 5 | 5 | | | | |
| *P. denitrificans* | 2 | 2 | | | | |
| *P. diminuta* | 5 | 5 | | | | |
| *P. fluorescens* | 5 | 3 | 2 | | | |
| *P. mallei* | 1 | 1 | | | | |
| *P. maltophilia* | 5 | 3 | 2 | | | |
| *P. paucimobilis* | 2 | 2 | | | | |
| *P. pickettii* | 1 | 1 | | | | |
| *P. pseudoalcaligenes* | 6 | 4 | 2 | | | |
| *P. putida* | 5 | 4 | 1 | | | |
| *P. stutzeri* | 6 | 3 | 3 | | | |
| *P. testosteroni* | 3 | 3 | | | | |
| *P. vesicularis* | 1 | 1 | | | | |
| *Acinetobacter* spp. | 16 | 8 | 8 | | | |
| *Aeromonas* spp. | 3 | | | 1 | 2 | |
| *Alcaligenes* spp. | 12 | 7 | 3 | 2 | | |
| Centers for Disease Control groups | 10 | 6 | 4 | | | |
| *Flavobacterium* spp. | 8 | 7 | 1 | | | |
| *Enterobacter* spp. | 10 | | 2 | 6 | 2 | |
| *Escherichia coli* | 12 | 12 | | | | |
| *Klebsiella pneumoniae* | 10 | 1 | 3 | 6 | | |

for identification of *P. aeruginosa* and would allow them to strengthen their data base by the addition of biochemicals for identification of other bacteria.

Although the MIC for 40 of 40 *P. aeruginosa* strains tested was >50 μg/ml, a lower C-390 concentration (15 μg/ml) was selected for use in routine procedures for several reasons. *P. aeruginosa* (170 of 170 isolates) was the only bacterium among the 361 isolates tested capable of growth in 15 μg of C-390 per ml. C-390 produced a blue color when added to broth media, and at concentrations higher than 15 μg/ml, the intensity of color interfered with ease of interpretation of turbidity. In addition, this concentration required less C-390 and was therefore more economical.

C-390 is a water-soluble, autoclavable compound which does not interfere with the oxidase reaction or pigment production of *P. aeruginosa* (4). We found that dilutions of C-390 in BHIB are stable for at least 4 weeks when stored in the dark at 4°C. Though the mechanism of action for C-390 remains to be elucidated, several observations were noted during our study. Isolates that were negative in C-390 BHIB at 4 h (Table 3) also remained negative after stationary incubation overnight. A subculture of these negative isolates on BA at 4 or 24 h, even at a C-390 concentration of 40 μg/ml, resulted in growth, indicating that C-390 was not bactericidal. The medium produces equivalent results with overnight incubation in stationary culture. The only purpose of the shaker is to shorten the incubation time. No difference in differential activity was found when C-390 BHIB was inoculated with isolates that were grown on BA for 4, 24, or 48 h. In addition, equivalent results were obtained when colonies were inoculated into C-390 BHIB from MacConkey agar.

In summary, the inoculation of 361 gram-negative bacilli into BHIB containing 15 μg of C-390 per ml resulted in growth of only *P. aeruginosa* within 4 h.

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