Evaluation of an Immunofluorescent-Antibody Test for Rapid Identification of *Pseudomonas aeruginosa* in Blood Cultures

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An immunofluorescent-antibody test was developed for rapid detection of *Pseudomonas aeruginosa* in blood cultures. The test uses a murine monoclonal antibody specific for all strains of *P. aeruginosa*. In initial tests, bright uniform immunofluorescence signals were seen when each of the 17 international serotypes, as well as 14 additional isolates of *P. aeruginosa*, were examined. No immunofluorescent staining was observed when 37 other gram-negative and 15 gram-positive species were studied. In a clinical study, the assay was applied to broth smears of 86 gram-negative bacilli isolated from 74 bacteremic patients and 28 additional clinical isolates of *Pseudomonas* sp. and other oxidase-positive gram-negative bacilli recovered from various other body sites. Smears were made directly from blood cultures which were positive for gram-negative bacilli by Gram staining. Eleven (15%) of 74 patients with gram-negative bacteremia had a positive test for *P. aeruginosa*. Including the results of these 11 isolates recovered in a prospective study and an additional 10 isolates from a retrospective study, we obtained a sensitivity and specificity of 100% (21 positive specimens and 103 negative specimens, respectively). These preliminary results suggest that this is a useful reagent for rapid presumptive identification of *P. aeruginosa* in blood cultures. With the immunofluorescent-antibody test, *P. aeruginosa* could be identified within 1 h of Gram stain evidence of gram-negative bacteremia.

Pseudomonas aeruginosa is an important cause of nosocomial bacteremia and pneumonia, especially in immunocompromised patients. In patients receiving organ transplants, this organism was the most common cause of bacteremia among the aerobic gram-negative bacilli and was responsible for 50 (28%) of 180 episodes (4). Case fatality rates ranged from 30 to 50%, despite the use of antibiotics (2, 18; V. T. Andriole, editorial, J. Lab. Clin. Med. 94:196-199), and have consistently remained higher than the mortality associated with bacteremia caused by other gram-negative bacilli (23). Nonetheless, early institution of appropriate antibiotics improves the outcome of *P. aeruginosa* bacteremia, even in neutropenic patients (3, 23). A review of 410 infections in cancer patients showed that cure rates were reduced from 74 to 46% in patients in whom there was a 1- to 2-day delay in the use of appropriate antibiotics (3). Therefore, there is an obvious need for laboratory techniques which permit more rapid identification of *P. aeruginosa*.

The *P. aeruginosa* immunofluorescent-antibody (IFA) test (Genetic Systems Corp., Seattle, Wash.) was evaluated for use in the clinical laboratory as a rapid presumptive identification method for *P. aeruginosa* isolated from blood cultures. Production of mouse monoclonal antibodies to porin protein F has been reported by others (9, 17). Previous work has also shown that certain epitopes on this protein may be present on all *P. aeruginosa* strains but not other gram-negative species. Antibodies to these epitopes are candidates for an antibody-based diagnostic assay (9, 11). To be useful in a clinical setting, however, the assay must be quick, easy to interpret, and adaptable to current technology. We describe an assay that uses an antibody to an epitope on porin protein F and accurately detects *P. aeruginosa* within 1 h after a Gram stain identifies gram-negative rods in a blood culture.

MATERIALS AND METHODS

Monoclonal antibody to porin protein F was cloned after fusion of a murine myeloma line with spleen cells from an immunized mouse by standard methods (16, 19). Whole boiled bacterial cells and isolated outer membranes (10) spiked with purified porin protein (22) were used as immunogens. Throughout the cloning procedure, culture supernatants were assayed for antibodies by standard enzyme-linked immunosorbent assays (6) with purified porin as the solid-phase antigen. Ascitic fluid was produced (19), and the monoclonal antibody was purified by protein A chromatography (7). Purified protein F was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Hancock and Nikaïdo (13) and subsequently immunoblotted by the method of Towbin et al. (20). Samples were examined with and without 2-mercaptoethanol in the same buffer. For direct immunofluorescence assays, the monoclonal antibody was conjugated to fluorescein isothiocyanate by procedures previously described (8). Examination of the specificity and cross-reactivity of the antibody was by direct immunofluorescence assays. These were performed with bacteria which were removed from the appropriate agar medium and suspended in phosphate-buffered saline. A drop was then placed on a 30-well microscope slide and allowed to air dry. The slides were stored at -70°C until used.

When blood specimens were examined, they were cultured by using three different systems. Specimens from Fred Hutchinson Cancer Research Center were cultured by two different methods: (i) direct inoculation into dextrose phosphate broth/GC agar biphasic bottles (PML Microbiologicals, Tualatin, Oreg.) and a peptone broth bottle (B-D Vacutainer Systems, Rutherford, N.J.) and (ii) processing with a 10-ml Isolator tube (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) with subsequent inoculation of the sediment to a combination of tryptic soy agar supplemented

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with 5% sheep blood, chocolate agar with enrichment, and candida bromcresol green agar plates (PML). The third system, used by the Laboratory of Pathology of Seattle, Inc. (Swedish Hospital Medical Center), Seattle, Wash., cultured specimens in three different media, BACTEC aerobic, anaerobic, and aerobic-resin broths (NR6A, NR7A, and NR16A, respectively; Johnston Laboratories, Inc., Towson, Md.).

Slides from positive blood cultures were prepared for staining in one of two ways. (i) Liquid medium specimens (5 μ l) were mixed with 1 drop of water in a 15-mm-diameter well of a slide, or (ii) suspensions of bacteria from agar plates were diluted in normal saline to a 0.5 McFarland standard, pipetted onto a well, and immediately aspirated, leaving a film of bacteria. Slides prepared by either method were air dried at 35°C and fixed in 95% ethanol for 10 min.

In the indirect method of staining, 1 drop (approximately 25 μ l) of prestaining reagent (10) was applied to the slide to expose the antibody-reactive sites. After 10 min, the prestain reagent was washed off with distilled water for 1 min and air dried. One drop of unlabeled monoclonal antibody reagent was then added to each well. After 15 min, the slides were again washed and air dried. A drop of anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Tago, Inc., Burlingame, Calif.) was then applied, incubated for 15 min, and washed and dried as described above. Finally, a drop of Evans blue counterstain was added for 2 to 3 min, followed by another distilled-water wash.

The multistep direct method differed from the indirect method by use of a monoclonal antibody conjugated directly to FITC, which eliminated the need for the second-step antibody. Incubation time was 15 min for this antibody reagent. Otherwise, the staining procedures were as described above.

The single-step direct staining method combined the prestain reagent, FITC-labeled monoclonal antibody, and Evans blue counterstain into a single reagent. One drop of this combined reagent was added to each well, incubated for 15 min, and washed and air dried as described above. After the final rinsing and drying steps in each method, cover slips were applied to the slide by mounting with equal parts of glycerol-phosphate-buffered saline and examined by fluorescence microscopy with FITC filters. Positive reactions were bright apple green and were graded with regard to increasing intensity as 1+ to 4+.

Control strains of *P. aeruginosa* and *Escherichia coli* were stained with each group of slides. Additionally, 28 isolates of *Pseudomonas* species and other oxidase-positive gram-negative bacilli obtained from clinical specimens were stained by IFA to test for possible cross-reactions. These organisms included the following: two *P. cepacia*, five *P. fluorescens*, four *P. maltophilia*, two *P. paucimobilis*, two *P. putida*, two *Alcaligenes xylosoxidans* subsp. *denitrificans*, two *Achromobacter* group V-D, and single isolates of *P. pseudoalcaligenes*, *P. vesicularis*, *Aeromonas hydrophila*, *Aeromonas* species, *Alcaligenes faecalis*, *Bordetella bronchiseptica*, Centers for Disease Control group II-B, *Moraxella nonliquefaciens*, and *Moraxella osloensis*. Specimen slides from these organisms were prepared in the same manner as those from agar plates. Control slides were fixed and stored at -70°C.

Ten *P. aeruginosa* strains were used to evaluate short-term antigen stability. These strains were recovered from patients who were bacteremic before the study period and had been frozen in tryptic soy-bovine serum albumin and stored at -70°C. The strains were thawed, subcultured onto

tryptic soy agar-5% sheep blood plates, and incubated at 35°C for 24 h. On the next day, organisms from the plates were inoculated into dextrose phosphate/GC agar bottles. After 18 h, multiple slides were prepared as previously described for liquid medium isolates, fixed, and stained at intervals of 1, 8, and 24 h by the multistep and single-step direct methods.

Additional control slides of *P. aeruginosa* prepared as described above were stored at -70°C to examine the effect of long-term storage on staining intensity. At 30 and 110 days, 10 representative slides were removed from storage. Five were fixed for 10 min in 95% ethanol and examined by the multistep direct method. The staining intensity was compared with the originally recorded reading and with the other five slides which were not refixed in ethanol.

RESULTS

Selection of the monoclonal antibody. Most of the anti-porin monoclonal antibodies were eliminated from consideration for use in the diagnostic assay because of lack of significant binding, cross-reactivity with other species, or inability to obtain highly FITC-conjugated preparations that retained activity. One antibody, designated 1G1, was selected to be used in the test. This antibody yielded a bright, uniform immunofluorescence signal specific for *P. aeruginosa* when examined in an indirect-immunofluorescence assay format. Subsequent conjugation of the antibody with FITC resulted in the same binding characteristics without the need for a second antibody step. The binding characteristics of the antibody selected to be used in the diagnostic assay are shown in the following lists of the results of preclinical studies with the IFA test. (An asterisk denotes that the clinical isolate was obtained from either local hospitals or the Centers for Disease Control. Identities were confirmed by standard laboratory methods.) (i) The *P. aeruginosa* strains examined (all tested positive) were 17 IATS serotype strains (ATCC 33348 to 33364 and 27853) and 13 other clinical isolates*. (ii) The other gram-negative rods (all tested negative) were *P. alcaligenes**, *P. aureofaciens**, *P. acidovorans* ATCC 15668, *P. cepacia* ATCC 25416, *P. diminuta* ATCC 11568, *P. fluorescens**, *P. maltophilia**, *P. mendocina**, *P. paucimobilis* ATCC 29837, *P. pertucinogena**, *P. pickettii**, *P. putida* ATCC 12633, *P. putrefaciens**, *P. pseudoalcaligenes**, *P. stutzeri**, *P. testosteroni**, *P. vesicularis**, *Acinetobacter calcoaceticus**, *Bacteroides fragilis**, *Bacteriodes oralis* ATCC 33269, *Branhamella catarrhalis**, *Campylobacter sputorum* ATCC 33491, *Cardiobacterium hominis**, *E. coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Fusobacterium nucleatum* ATCC 25586, *Haemophilus influenzae**, *Haemophilus parainfluenzae**, *Klebsiella pneumoniae**, *Legionella pneumophila**, *Leptotrichia buccalis* ATCC 14201, *Moraxella lacunata* ATCC 17967, *Neisseria meningitidis**, *Neisseria sicca**, *Proteus mirabilis**, and *Treponema denticola**. (iii) The gram-positive organisms (all tested negative) were *Actinomyces israelii* ATCC 10049, *Bifidobacterium dentium**, *Corynebacterium ulcerans**, *Eubacterium limosum* ATCC 8486, *Lactobacillus cateniforme* ATCC 25536, *Micrococcus luteus**, *Peptococcus asaccharolyticus**, *Peptostreptococcus anaerobius**, *Rothia dentocariosa* ATCC 17931, *Staphylococcus aureus* ATCC 10832, *Staphylococcus epidermidis**, *Streptococcus faecalis* ATCC 12959, *Streptococcus pyogenes* ATCC 12972, *Streptococcus pneumoniae**, and *Streptococcus sanguis* ATCC 10556. Confirmation that 1G1 bound to porin protein F was obtained by immunoblot

analysis. Purified protein F was subjected to sodium dodecyl sulfate gel electrophoresis and transferred to nitrocellulose. Subsequent analysis revealed that one major band, at an apparent molecular weight of 39,000, was bound by the antibody. Interestingly, the monoclonal antibody was able to bind to the reduced and nonreduced forms of the protein (Fig. 1). This is similar to one of the monoclonal antibodies reported by Hancock and Mutharia (11). The isotype of antibody 1G1 was determined to be an IgG2b.

Clinical evaluation of the IFA test. Of 74 patients 11 (15%) with gram-negative bacteremia had a positive test in the prospective study. Including instances of polymicrobial bacteremia, 86 different isolates of gram-negative bacilli were recovered (Table 1). A total of 124 blood cultures were obtained from the 74 patients. Table 2 shows the number of blood cultures tested by the three different methods. The percent recovery of *P. aeruginosa* was higher by the indirect method: 24% versus 4% for the multistep direct method and 15% for the single-step direct method. However, no one test method was judged to be superior to another, since the methods were not performed concurrently and the numbers of *P. aeruginosa* correctly identified by the IFA test correlated completely with the isolation and identification of *P. aeruginosa* by conventional methods.

All 21 *P. aeruginosa* isolates from the 11 patients exhibited 4+ fluorescence staining from all of the blood culture systems tested, including aerobic and anaerobic media, compared with control strains of *P. aeruginosa*, which consistently stained as 3+, and *E. coli*, which were always negative. No false-positive reactions occurred among the 20 other genera and species recovered from blood cultures. Autofluorescence, a dull olive green or yellow color, was observed in one specimen containing both *E. coli* and *Providencia stuartii* and in another specimen yielding only *E. coli*. However, the autofluorescence did not pose a significant interpretive problem, since both the intensity and color of the reactions could be easily distinguished from the specific antibody staining. No positive reactions were seen among the 28 isolates of *Pseudomonas* species and other oxidase-positive, gram-negative bacilli.

FIG. 1. Immunoblot analysis of purified porin protein F with monoclonal antibody 1G1. Purified protein F was subjected to electrophoresis as described in the text. In lane 1, 2-mercaptoethanol was not present in the sample buffer, whereas it was included in lane 2. The numbers to the right indicate molecular weight.

TABLE 1. Organisms recovered from 86 isolations of gram-negative bacilli from 74 patients with bacteremia

Organism	No. of isolates (total no. recovered) ^a
<i>Pseudomonas aeruginosa</i>	11 (21)
<i>Pseudomonas maltophilia</i>	3 (5)
<i>Pseudomonas putida</i>	1 (1)
<i>Pseudomonas paucimobilis</i>	1 (1)
<i>Pseudomonas stutzeri</i>	1 (1)
<i>Escherichia coli</i>	20 (29)
<i>Enterobacter aerogenes</i>	2 (3)
<i>Enterobacter cloacae</i>	9 (10)
<i>Klebsiella pneumoniae</i>	10 (18)
<i>Serratia marcescens</i>	2 (5)
<i>Morganella morganii</i>	2 (2)
<i>Proteus mirabilis</i>	2 (2)
<i>Providencia stuartii</i>	1 (1)
<i>Acinetobacter calcoaceticus</i> var. <i>anitratus</i>	5 (9)
<i>Acinetobacter calcoaceticus</i> var. <i>lwoffii</i>	3 (4)
<i>Alcaligenes denitrificans</i>	1 (1)
<i>Moraxella osloensis</i>	1 (1)
<i>Haemophilus influenzae</i>	2 (3)
<i>Capnocytophaga</i> sp.	2 (2)
<i>Bacteroides fragilis</i>	4 (10)
<i>Fusobacterium nucleatum</i>	2 (2)
<i>Bacteroides oralis</i>	1 (3)

^a Includes multiple isolates from individual patients. All isolates were examined with the IFA test, and the only ones positive by the IFA test were the 11 *P. aeruginosa* isolates.

In a retrospective study, all 10 *P. aeruginosa* strains from our stock of previously collected isolates were positive in the IFA test. There was no decrease in staining intensity observed when staining was delayed for 1, 8, and 24 h from the time of fixation to the microscope slide. Maximum (4+) staining reactions were seen in smears prepared from 59 of 60 test wells, and the remaining reaction was 3+. No differences in staining intensity were seen in a comparison of the multistep and single-step direct methods. Neither long-term storage nor refixation in ethanol had a substantial effect on staining intensity.

DISCUSSION

Knowledge of the detection of *P. aeruginosa* might have important implications for antibiotic therapy, especially in immunocompromised patients. Newer beta-lactam antibiotics, such as ceftazidime, imipenem, and aztreonam, have been developed which have increased activity against gram-negative bacilli, including *P. aeruginosa*, compared with older drugs, and these new drugs have been used success-

TABLE 2. Patients and blood cultures examined by the IFA test

Test ^a	No. of patients	No. of blood cultures	No. of patients IFA positive/no. tested (%) ^b	No. of blood cultures IFA positive/no. tested (%)
Indirect test	33	62	8/33 (24)	17/62 (27)
Multistep direct test	28	44	1/28 (4)	1/44 (2.3)
Single-step direct test	13	18	2/13 (15)	3/124 (17)

^a See the text for descriptions of the tests.

^b Tests were evaluated sequentially as new modifications were developed. With each method, the number of patients with positive IFA tests corresponded exactly to results obtained by conventional isolation and identification.

fully to treat patients with gram-negative infections (1, 5, 14). However, it is still recommended that in immunocompromised patients with gram-negative bacteremia, especially those persons with *P. aeruginosa* bacteremia who are persistently neutropenic, combination therapy including the potentially toxic aminoglycoside antibiotics should be used (15, 24). Consideration could be given to discontinuing or not using the aminoglycoside antibiotic if it were known that the bacteremia was not caused by *P. aeruginosa*.

With conventional blood culture systems, 48 to 72 h may elapse from the time of venipuncture before a tentative identification of *Pseudomonas* sp. can be made, depending on the growth rate of the organism. Initial evidence of growth must be determined by visual inspection or radio-metric examination with the BACTEC system, followed by Gram staining to identify the presence of a gram-negative rod. However, tentative identification of *P. aeruginosa* requires examination of the results of routine subculture onto solid media and confirmation testing. Whereas it is not certain that detection of growth by the BACTEC method is superior to methods using visual inspection (21), the *Pseudomonas* IFA test appears to be a useful addition to either approach. With this test, a sample containing bacterial growth is taken directly from a positive blood culture, fixed to a microscope slide, and stained with the *Pseudomonas* IFA reagent, which allows immediate positive identification of *P. aeruginosa*. This method would shorten the time required for identification by at least 8 to 24 h.

Over the course of these experiments, a single-step direct test was developed which substantially reduced the time required to perform the IFA test from approximately 3 h for the indirect test to less than 1 h. This was accomplished without a change in the intensity of staining, which was identical by both methods. The single-step direct test was easy to perform and interpret. In addition, the clarity, intensity of staining, and absence of interfering nonspecific background fluorescence made it possible to perform rapid low-power scanning of a field to detect positive organisms. Control slides, used for quality control purposes, appeared to be stable and easily stored, since they retained the same degree of staining intensity for over 100 days.

Previous researchers have demonstrated the potential usefulness of anti-porin protein F monoclonal antibodies (12), and a diagnostic test using this monoclonal antibody has been reported (9). We improved upon these observations by constructing a test that uses a highly conjugated antibody with FITC, which eliminated the need for a second antibody step. In addition, the test can be used without boiling the samples and can be applied directly to smears of blood cultures.

We previously reported that a single isolate of *Alcaligenes denitrificans* reacted with the *Pseudomonas* IFA test (R. W. Schwartz, B. K. Ulness, D. J. Hamilton, R. P. Darveau, M. D. Cunningham, and G. W. Counts, Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 207, 1987). Subsequent to this, we learned that this organism, isolated from soil, was originally obtained from the American Type Culture Collection as *Pseudomonas* sp. ATCC 13867. The 86 gram-negative bacilli in blood cultures in our study included one isolate identified as *A. denitrificans*, which did not react with the IFA test.

In summary, we found the *Pseudomonas* IFA test to be highly sensitive and highly specific for detection of *P. aeruginosa* in blood cultures. With results available in less than 1 hour, it represents a valuable addition to the microbiology laboratory. Rapid detection of *P. aeruginosa* bacte-

remia will permit more accurate and targeted antimicrobial therapy. Further field testing of this new technique is clearly warranted.

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