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Rapid Flow Cytometric Bacterial Detection and Determination of Susceptibility to Amikacin in Body Fluids and Exudates

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A flow cytometry-based method for rapid and quantitative detection of bacteria in various clinical specimens and for rapid determination of antibiotic effect is described. Achieving such a measurement with high sensitivity required discrimination between bacteria and other particles which were often present in clinical samples in high concentrations. This discrimination was facilitated by detecting the bacterial characteristic light scatter and fluorescence signals following staining, e.g., with the fluorescent nucleic acid-binding dye ethidium bromide, as well as by measuring bacterial proliferation during short time intervals. Antibiotic susceptibility was measured by observing the inhibition of such proliferation. The method was applied to 43 clinical specimens from various sources, such as wound exudates, bile, serous cavity fluids, and bronchial lavage. Bacterial detection, achieved in less than 2 h, agreed with results of conventional methods with a sensitivity of 74% and a specificity of 88%. Susceptibility to amikacin was detected in 1 h in 92% of 13 positive specimens.

Bacteriological analysis of clinical specimens, which involves bacterial detection, identification, and determination of antibiotic susceptibility, is currently a lengthy procedure. Consequently, empiric antibiotic therapy is often initiated prior to the arrival of results from the microbiology laboratory. Such therapy is not necessarily optimal with regard to efficacy, toxicity, and specificity, as well as cost and selection pressure towards resistant hospital strains. In some cases, therapy is completely superfluous, the biological agent being nonbacterial. For these reasons extensive efforts are being directed towards the development of rapid methods for bacteriological analysis (11, 22). Indeed, the potential clinical impact of such methods has been demonstrated (5). Attempts to automate bacteriological analysis have yielded considerable progress. Nevertheless, most methods still require at least 5 to 6 h and suffer from additional drawbacks, including lack of sensitivity and restricted applicability (3, 7, 9, 23). Several reports have suggested methods for bacterial detection based on flow cytometry (FCM), a technology which allows extremely rapid measurements of multiple optical parameters at the single-cell level (20). Sahar et al. described the rapid identification of *Streptococcus pyogenes* in the presence of saliva by immunofluorescence staining (22). Van Dilla et al. (25) used double labeling of DNA with AT- and GC-specific strains for differentiation among several bacterial strains. Detection and quantitation of *Bacteroides gingivalis* were performed in bacterial mixtures (14). The detection of extremely low concentrations of *Escherichia coli* in blood by DNA stains was described previously (18).

In this work we describe a flow cytometry-based method for the rapid and quantitative detection of bacteria in other clinical fluid specimens and for the determination of their susceptibility to antibiotics within 2.5 h. The method was applied to 43 clinical specimens from various body fluids and exudates. Bacterial detection was in good agreement (84%) with the results of conventional analysis. Susceptibility to amikacin was detected in 92% of 13 cases.

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MATERIALS AND METHODS

Clinical specimens. A total of 46 clinical specimens, kindly provided by C. Block of the Sheba Medical Center Clinical Microbiology Laboratory, were either analyzed on the day of arrival at the laboratory or stored at 4°C and examined on the following day. In most cases a sample of the original fluid specimen was obtained. In 14 cases specimens were supplied on a swab in semisolid medium (Transwab; Medical Wire and Equipment Co., Corsham, Wiltshire, United Kingdom). The specimens included fluids from serous cavities, wound exudates, bile, and bronchial lavage. Organisms in these specimens, identified according to standard methods (16) by the Sheba Medical Center Clinical Microbiology Laboratory, included *Enterobacter* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *P. stutzeri*, *Staphylococcus aureus*, *S. epidermidis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Acinetobacter* spp.

Laboratory organisms and growth conditions. Bacterial strains used as model systems included *E. coli* 346, *S. pyogenes* group A type M5 (both kindly provided by I. Ofek), *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *P. mirabilis* ATCC 29906.

The bacteria were maintained on nutrient agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.), except for *S. pyogenes*, which was maintained on blood agar (Hylabs, Rehovoth, Israel); all bacteria were stored at 4°C. Prior to each experiment with model bacterial suspensions, the bacteria were transferred to tryptic soy broth (TSB; Biolife, Jerusalem, Israel) (supplemented with 10% fetal calf serum in the case of *S. pyogenes*) and the cultures were incubated at 37°C with shaking (100 strokes per min) until the mid-logarithmic phase. Each bacterial suspension was diluted as needed with 50 mM phosphate-buffered saline (PBS) (pH 7.3).

Microscopy. Samples were observed by phase-contrast or epifluorescence microscopy with a 40× objective. Bacterial concentrations were determined with a Petroff-Hausser counting chamber (Thomas Scientific, Philadelphia, Pa.).

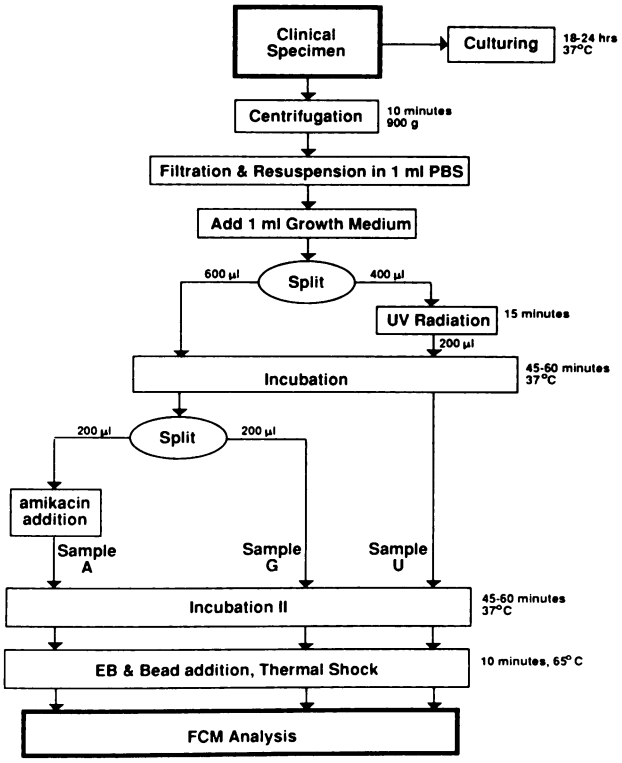

FIG. 1. Flow chart of specimen preparation and measurement procedure. For UV radiation, up to 1 ml of sample contained in a small uncovered sterile aluminium foil cup was placed 10 cm from a UV lamp (Philips model 413 P/40X30W) under a sterile hood and exposed to radiation for 15 min. For EB addition, EB was added at a final concentration of 25 μg/ml.

Specimen pretreatment. (i) Recovery of specimens from swabs. Swab transport media were suspended in 8 ml of PBS and shaken vigorously in a vortex mixer (Scientific Industries Inc., Bohemia, N.Y.), and the test tubes were placed on ice for approximately 10 min until the agar was deposited at the bottom. The supernatant was collected and used for further procedures.

(ii) Specimen centrifugation. Each specimen was diluted 1:1 with PBS (except for swab forms) and centrifuged (model RC5C centrifuge; Sorvall Instruments, Wilmington, Del.) for 10 min at 900 × *g*.

(iii) Elimination of antimicrobial agents from specimens. A filtration unit which allowed transfer of liquid from both sides of a 25-mm-diameter sterile disposable polysulfone filter (Gelman Sciences, Inc., Ann Arbor, Mich.) was used. Samples (1 ml) were inserted from the top, and vacuum was applied. PBS (5 ml) was injected through the bottom at a pressure of 2 atm (1 atm = 101.29 kPa) to resuspend particulate matter from the filter. Vacuum was again applied, and 1 ml of PBS was used to resuspend particulate deposits from the filter. This suspension was removed for further procedures.

Summary of the procedure applied to clinical specimens. The procedure is summarized in Fig. 1. Samples (10 μl) of each specimen were inoculated onto TSB-glucose agar plates and incubated at 37°C for 18 to 24 h to determine the bacterial content of each specimen. Additional 10-μl samples were inoculated into 1 ml of TSB and into 1 ml of TSB supplemented with 100 μg of amikacin (Amikin; Bristol Laboratories, Syracuse, N.Y.). The turbidity of these samples after incubation for 18 to 24 h at 37°C served to define bacterial susceptibility to amikacin. Approximately 1 ml of the original fluid specimen or the specimen recovered from a swab was used for the FCM procedure (Fig. 1).

FCM. Fluorescence and forward light scatter signals for each particle were measured on a model 50H flow cytometer (Ortho Diagnostic Systems, Westwood, Mass.) with 200 mW of laser power at a wavelength of 488 nm. Fluorescent light was collected perpendicularly to the excitation beam through a 600-nm high-pass filter. All beam splitters were removed, and a photomultiplier voltage setting of 2.0 to 2.5 was used with the signal detected in the logarithm peak mode. The forward light scatter signal was detected in the logarithm peak mode at a photomultiplier voltage setting of approximately 2.0. The graphic presentations were copied directly from the computer screen with a Tektronix 4632 hard-copy unit at constant screen- and copy-intensity levels. Fluorescent polystyrene spheres (stock suspension of 10^8 beads per ml, as determined with the Petroff-Hausser counting chamber) with a diameter of 1.55 μm (Polysciences, Warrington, Pa.) were added to each sample to a final concentration of 10^6/ml. These spheres served to quantitate the bacterial concentration in the sample by counting the number of particles with fluorescence and light scatter signals in the ranges found to be typical of bacteria (see region 1 in Fig. 2A) after the sphere count has reached a predetermined level (usually 2,000). In addition, these spheres served to monitor the optical alignment of the instrument during the experiment.

Datum analysis. Bacterial growth was evaluated by the growth index (GI), defined as

$$GI = 100 \times (N_G - N_U)/N_U \quad (1)$$

where N_G and N_U were the concentrations of bacteria in samples *G* and *U*, respectively (as defined in Fig. 1 and below). Similarly, the effect of an antibiotic on bacterial growth was evaluated by the antibiotic index (AI), defined as

$$AI = 100 \times (N_G - N_A)/N_A \quad (2)$$

where N_A was the concentration of bacteria in sample *A* (as defined in Fig. 1 and below). Only AI values greater than 50% were considered to indicate inhibition. This limit was set by examination of the statistical variation in GI values measured for the negative samples.

RESULTS

Figure 2A presents the FCM distribution obtained from a suspension of *E. coli* in PBS after staining with ethidium bromide (EB). Each bacterium in the measured sample is depicted as a dot in the graph according to the fluorescence and narrow-angle forward light scatter signals it produced. These signals reflected the amount of bacterial nucleic acid and the bacterial size, respectively. Bacteria as a whole formed a well-defined distribution, owing to their relatively homogeneous nucleic acid content and size. However, some strains were found to exhibit poor permeability to EB. In an attempt to circumvent this problem and to achieve intense homogeneous staining of bacteria, we tested the following treatments: (i) addition of detergents (such as Triton X-100 or cetyltrimethylammonium bromide) to the bacterial suspension in the presence of EB; (ii) use of alternative dyes, such as acridine orange (Sigma Chemical Co., St. Louis, Mo.) and Hoechst 33342 (Calbiochem, Lucerne, Switzerland), which are known to penetrate membranes of living cells; (iii) exposure of the bacterial suspension to a thermal shock in the presence of EB.

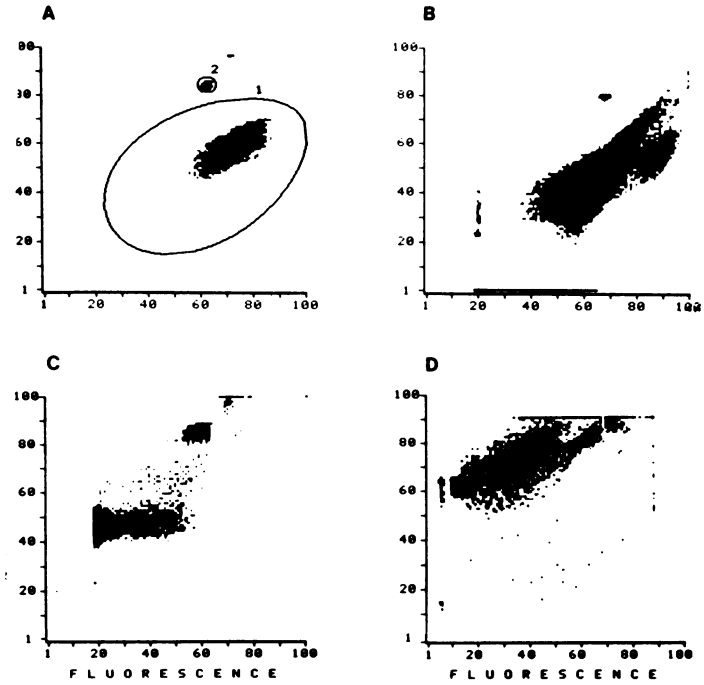

FIG. 2. FCM distributions depicting forward light scatter versus fluorescence of each particle in EB-stained samples. Synthetic fluorescent latex particles (region 2 in panel A) were added to each sample to serve as internal standards. (A) *E. coli* suspended in PBS (region 1). (B) Wound exudate sample containing a high concentration (2×10^7 per ml) of bacteria relatively free of background noise. (C) Wound exudate sample containing bacteria staining more weakly than those in panel B and overlapping a typical distribution of background noise particles. (D) Sterile peritoneal fluid sample showing a high concentration of background noise particles.

Treatments (i) and (ii) applied to several model bacterial strains were found to be unsatisfactory. Cetyltrimethylammonium bromide facilitated EB permeability, but an intensely stained precipitate which entrapped bacteria suspended in clinical samples or in growth media was formed. Treatment (ii) yielded poor, unstable, and nonhomogeneous staining of several bacterial strains. Treatment (iii) was found to give the best results, yielding intense staining in all cells of all strains tested. The thermal shock treatment was found to be optimal at 65°C for 10 min, conditions which also terminated bacterial growth without causing cell lysis in all bacterial strains tested. The EB staining procedure was applied to clinical specimens, and FCM measurements were taken. Figure 2B shows the results obtained from a wound exudate specimen found to contain *P. aeruginosa*. This was a typical example of FCM distribution of bacteria in a clinical sample, and it showed a well-defined bacterial signal which resembled that of bacteria suspended in PBS. In comparing the bacterial distributions presented in Fig. 2B and C, the latter of which was obtained from a wound exudate specimen containing *E. coli*, *P. mirabilis*, and *P. aeruginosa*, it was apparent that bacterial optical properties varied considerably from specimen to specimen. Indeed, bacterial strain and physiological state, as well as the nature of the clinical specimen, were all found to influence signals of bacteria in the FCM measurements. That the measured signals were from the bacteria in the sample was confirmed by the following observations. First, quantitative inoculation of a bacterial suspension into a clinical specimen which was found to be sterile by plating yielded an FCM signal similar to the signal obtained from bacteria in PBS. This signal was absent in measurements taken on a sample of the original specimen. Second, in positive specimens, quantitative measurements taken on samples supplemented with growth media and incubated at 37°C showed an increase with time in the counts of particles having optical signals appropriate to bacteria.

When measuring clinical specimens, we encountered problems of background noise caused by particles other than bacteria. Such particles were found to be present in all clinical samples, including sterile specimens from healthy individuals. Intact blood cells (especially erythrocytes) contaminated many specimens and interfered with the measurements, owing to their high concentration, which obscured the presence of any other particles. This problem was resolved by a light centrifugation which differentially deposited blood cells, leaving bacteria in the supernatant. Optimal conditions were $900 \times g$ for 10 min and yielded 45 to 75% of the bacteria and $<10^6$ blood cells per ml (from whole blood) in the supernatant. The blood cells remaining in the supernatant after this treatment did not interfere with the bacterial measurement, as they produced optical signals very different from those of bacteria. However, this treatment did not eliminate all noise caused by nonbacterial particles, as some were found to have sedimentation properties and optical signals overlapping those of bacteria. An extreme case of such noise is presented in Fig. 2D, which shows the distribution obtained from a sterile peritoneal fluid specimen; this distribution overlaps the bacterial distribution shown in Fig. 2B. These noise particles varied considerably between specimens, both in their concentration and in their optical properties. Although the fluorescence intensity and the concentration of noise particles were usually lower than those characteristically measured for bacteria in positive specimens (Fig. 2B), some specimens contained intensely stained noise particles (Fig. 2D) and others contained poorly stained bacteria (Fig. 2C). Thus, it appeared impossible to

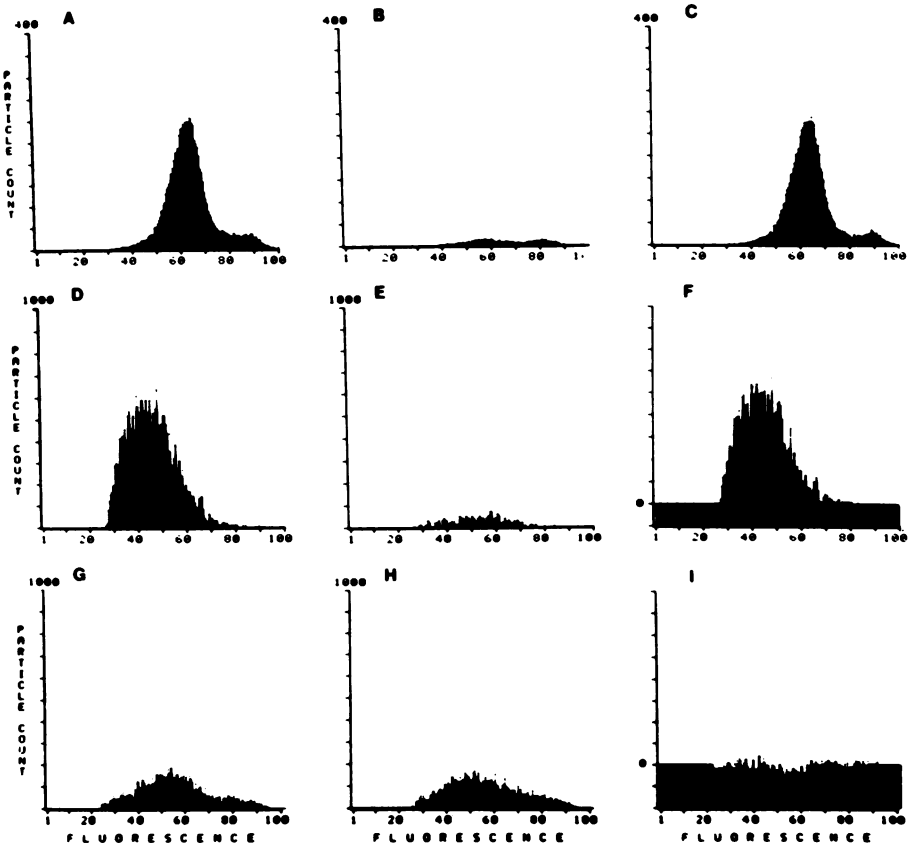

FIG. 3. Detection of bacteria by measurement of proliferation. Three specimens are shown: a wound exudate (same as in Fig. 2B) in panels A to C; a wound exudate (same as in Fig. 2C) in panels D to F; and peritoneal fluid (same as in Fig. 2D) in panels G to I. (A, D, and G) Sample G—samples allowed to grow for 90 to 120 min. (B, E, and H) Sample U—samples in which growth was arrested prior to the 2-h incubations. (C, F, and I) Channel-by-channel subtraction of sample U from sample G. The distributions in panels F and I were shifted upwards by a constant to allow depiction of negative counts obtained in the subtractions.

confidently distinguish noise particles from bacteria with a single FCM measurement of a clinical specimen.

Specific FCM detection and measurement of bacteria in clinical specimens were achieved by a dynamic approach, differentiating bacteria from noise on the basis of their ability to proliferate. Two samples of a specimen were compared: one which was provided with favorable growth conditions and one in which bacterial growth was terminated. UV radiation was used to arrest bacterial growth for the duration of the experiment. In all strains tested, at least 90% of the population was found to be arrested within 10 min of the UV treatment. In some strains, there was considerable lysis (up to 60%) of bacteria during radiation, as assessed by microscopic counts (with a Petroff-Hausser counting chamber). Figure 3A shows a histogram of the fluorescence distribution obtained from the specimen shown in Fig. 2B. The FCM measurement was taken on a sample which was supplemented with growth media and incubated at 37°C for 2 h. Figure 3B shows measurements obtained from a similar sample of the same specimen in which bacterial growth was terminated prior to incubation by exposure to UV radiation. Figure 3C shows an algebraic channel-by-channel subtraction of panel B from panel A, representing the net result of bacterial growth. A similar dynamic analysis of the specimen shown in Fig. 2C is presented in Fig. 3D, E, and F. Although the FCM distribution obtained from this specimen overlapped the distribution of typical sterile specimens, here, too, a signal corresponding to proliferating bacteria was clearly observed by the difference between panels D and E shown in panel F. When such measurements were taken on a sterile specimen (Fig. 2D), the distribution of noise particles in the nonradiated sample (Fig. 3G) remained unchanged when compared with that in the UV-radiated sample (Fig. 3H), resulting in a difference (Fig. 3I) which was close to zero.

A total of 46 clinical specimens were obtained for FCM analysis. Three (6%) were discarded because they contained extensive precipitations in which bacteria were entrapped. The remaining 43 were each divided to yield two samples for FCM analysis: sample G was incubated for 90 to 120 min under favorable growth conditions, and sample U was UV radiated before incubation. The GI quantified and normalized the difference between samples U and G and was evaluated for each specimen (Fig. 4). Specimens were classified as positive or negative according to the results of bacteriological cultures performed in parallel. In all cases but one these culture results were found to agree with those obtained in the Sheba Medical Center Clinical Bacteriology Laboratory. Of 19 positive samples, 14 (74%) were correctly determined by our method (GI, >50%). Of 24 negative samples, 22 (87%) were correctly determined (GI, <50%). In the specimens yielding true-positive results, the minimal bacterial concentration after incubation was 3×10^4/ml. The maximal noise particle concentration in the specimens cor-

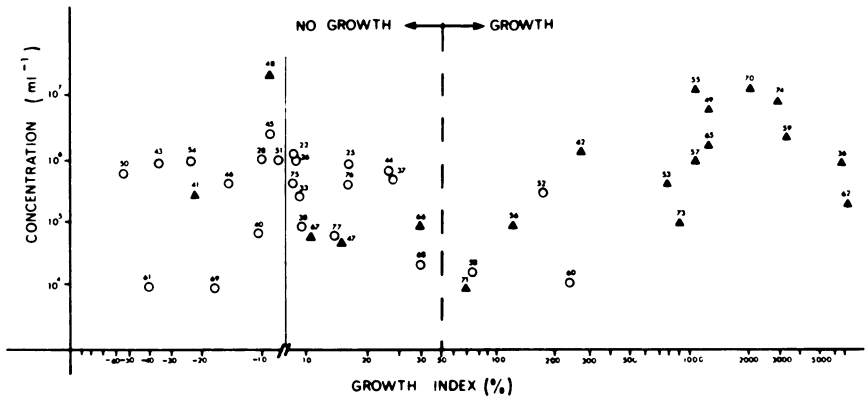

FIG. 4. GI (defined by equation 1) distribution versus total particle concentration evaluated for 19 positive samples (▲) and 24 negative samples (○) (determined by culturing). A GI of 50% was defined as the cutoff point above which a sample was considered to exhibit positive growth. Each point is identified by a sample number.

rectly determined to be negative was 10^6/ml. The seven (16%) specimens yielding false results in our measurements are summarized in Table 1.

The effect of amikacin was tested in 13 of the positive specimens. An AI which assessed the difference between samples G and A was evaluated for each specimen (Fig. 5). The average AI was 394%, and 12 of 13 specimens had an AI of greater than 50%; 1 specimen was borderline. TSB cultures of all of these specimens were susceptible to amikacin at the concentration tested.

DISCUSSION

The results show that a dynamic FCM analysis allows bacterial detection as well as identification of sterile specimens within less than 2 h in a variety of clinical specimens. The potential of this method for the rapid determination of antibiotic susceptibility was demonstrated by detecting the effect of amikacin on the specimens determined to be positive.

The majority of specimens arriving at clinical microbiology laboratories are fluids, rendering them amenable to FCM analysis. Our study included fluids from serous cavities, wound exudates, bile, and bronchial lavage. Although many of these specimens were heavily contaminated with blood, blood specimens as such were excluded at this stage of the study because of the small proportion of positives and because blood specimens often contained extremely low bacterial concentrations. Specimens which tended to exhibit normal flora, such as sputum from the upper respiratory tract and urine, were also excluded from this study.

Intense homogeneous staining of all bacterial strains of interest is needed to achieve a well-defined FCM signal. Several studies (8, 15) reported that bacterial staining by acridine orange after fixation was a sensitive method comparable to the traditional Gram stain. Although acridine orange and Hoechst 33342 were reported capable of penetrating viable eucaryotic cells (20), this was not the case for some bacterial strains. Enhancing bacterial permeability by use of a detergent resulted in undesirable side effects in the fluid of the specimens and in growth media. EB staining combined with a short thermal shock yielded satisfactory bacterial fluorescence in all clinical specimens tested. Most blood cells were eliminated by centrifugation, and the remaining cells were easily distinguishable from bacteria. Nevertheless, other particles, probably lysed cells and tissue debris, were found to bind EB to a certain extent, producing a noise signal closely resembling that of bacteria and obscuring the presence of low concentrations of bacteria in the specimen. For this reason, a dynamic approach was adopted to detect bacterial proliferation by comparing a sample of the specimen in which bacteria were allowed to grow with a sample in which growth was terminated. UV radiation was chosen for this purpose since, compared with chemical treatment or heat, it was less likely to cause changes in the noise particles or the fluid of the specimen. This was important, since any detectable difference between growing and sustained samples must only indicate the presence of bacteria. Bacterial cells were found to undergo partial lysis (10 to 60%) because of the UV radiation. This lysis increased the measurement sensitivity, since the difference between the radiated and nonradiated samples was increased. Basing bacterial detection on bacterial growth presented the problem of antimicrobial activity which might have resulted from antimicrobial therapy the patient had received or from natural antimicrobial activity in serum. Elimination of fluid

TABLE 1. Summary of specimens yielding false bacterial detection results

Sample no.[a]	Status[b]	Source	Specimen form	Concn
41	FN	Pleural fluid	Fluid in test tube	
48	FN	Surgical wound	Fluid in test tube	10^4 CFU/ml[c,d]
47	FN	Peritoneal fluid	Swab	2×10^3 CFU/ml[c]
66	FN	Surgical wound	Swab	10^4 CFU/ml[c]
67	FN	Bile	Swab	10^4 CFU/ml[c]
58	FP	Peritoneal fluid	Swab	2×10^4 Particles per ml[e]
60	FP	Pleural fluid	Fluid in test tube	10^4 Particles per ml[e]

[a] As in Fig. 4.
[b] FN, False-negative; FP, false-positive.
[c] Determined by viable counts on TSB-glucose agar plates.
[d] This specimen contained 10^7 (nonviable) bacteria, as determined by microscopy and by FCM.
[e] Determined by FCM.

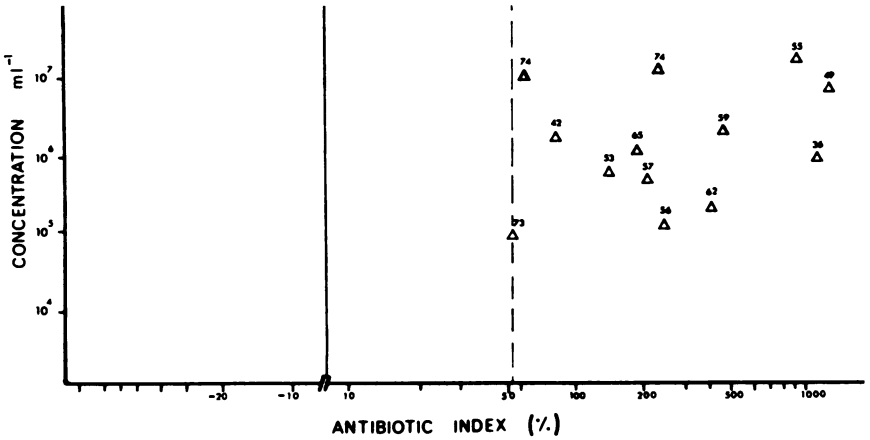

FIG. 5. AI (defined by equation 2) distribution versus total particle count calculated for 13 of the true-positive samples shown in Fig. 4. An AI of 50% was defined as the cutoff point above which a sample was considered to exhibit antibiotic susceptibility. Points to the left of the dotted line indicate resistance, and those to the right indicate susceptibility.

from the specimen by use of a filtration-resuspension system resolved this problem but at the expense of some loss of bacteria (40 to 60%).

The specificity of bacterial detection was 88% (14 true-positives of 16 positive results). The two false-positive results seemed to be due to the fact that these specimens contained a low concentration (~10^4/ml) of particles and the fact that a fixed volume (0.5 μl) of the sample was measured. As a result, less than 20 particles were measured in these cases (as compared with the usual hundreds to thousands), with very significant statistical variations. Thus, the difference between samples G and U in these cases does not reflect a real difference between samples G and U. Such problems could easily be avoided by setting a minimum number of particles to be collected, thus measuring a larger volume of the specimen when the particle concentration is low.

The sensitivity of bacterial detection was 74% (14 true-positives and 5 false-negatives). Three of the false-negative results (Table 1) were produced by specimens in the form of swabs transported in semisolid medium designed for easy plating, but recovery of the material from the swabs involved considerable dilution. Viable counts of these recovered suspensions were less than 10^4 CFU/ml. These false-negative results may perhaps be avoided by use of a fluid transport medium. One of the false-negative results was due to a large number of nonviable bacteria obscuring the presence of a small proportion of viable bacteria. This problem might have resulted from antibiotic therapy received by the patient.

Bacterial growth determined by the GI was based solely on the difference between total particle counts in samples G and U. Additional information may, however, be gained by observing the pattern of particle distribution obtained in the FCM measurement. While bacteria were usually distributed in a relatively narrow peak, noise tended to be distributed in a broader, more random fashion. A definition of GI based on both particle count and distribution pattern differences might improve the sensitivity of our method.

The duration of the FCM test for bacterial detection and susceptibility determination was in the order of 2.5 h, considerably less than other rapid methods that are in routine use in clinical laboratories. The sensitivity of the FCM measurement was the result of the detection and analysis of bacteria on a single-cell level, allowing the detection of small changes in bacterial concentration as well as the measurement of optical properties of each cell reflecting bacterial size, shape, and composition. These properties were affected by both bacterial growth and antibiotic susceptibility. We demonstrated this by analyzing the effect on the positive clinical specimens of amikacin at a concentration known to inhibit most pathogens. The resulting AI values showed that growth inhibition could be measured after 1 h of amikacin exposure. Under these conditions one of the specimens was borderline (AI = 50%).

Several investigators have reported attempts to shorten susceptibility testing time. They include early readings of results of classical methods (4, 13, 17), direct inoculation of specimens in susceptibility tests (4, 6, 7, 12, 19, 21), and automation (11, 24). Some drawbacks of these approaches affect our method in a similar fashion. It has been observed (2, 10) that MICs determined after short incubation periods differ from those obtained after incubation for 15 to 18 h. Mixed infections are undoubtedly a problem in direct-inoculation susceptibility testing. Several studies have revealed major discrepancies between susceptibility test results obtained by direct inoculation and those obtained after culturing each bacterial strain and testing it separately (1, 3, 7). Although the clinical significance of both short-incubation MICs and mixed-culture direct susceptibility test MICs has been questioned, others have emphasized both the need for and the feasibility of developing such methods (1, 3, 7, 11). Methods such as the one described here may allow the rapid initiation of effective treatment against the major infecting organism and monitoring of the patient's condition at short intervals with adjustment of the treatment if other strains multiply.

Some drawbacks of the rapid methods previously described are avoided in the FCM procedure. Direct-inoculation studies suffer from the problem of insufficient growth (1), which the FCM method overcomes, owing to its high sensitivity. An additional reported problem has been the lack of standardization of the inoculum, which impairs the interpretation of results. This problem does not apply to the FCM test, since each specimen produces its own control sample (sample U). The "mass increase effect" (3) which produced false resistance results when measuring turbidity because of bacterial filamentation in fact increased the sensitivity of the

FCM results, since filamentation caused detectable optical changes in the bacteria. An additional advantage of the FCM method may be its potential for the identification of specific pathogens via immunofluorescence labeling of specific bacterial antigens (21).

Despite its potential to perform clinical bacteriological analysis rapidly and automatically, the ability of an FCM-based method such as the one presented here to become routinely used in clinical laboratories is still limited. The instrumentation involved is sophisticated and costly and requires highly trained personnel for reliable operation. The development of dedicated instrumentation suitable for the clinical laboratory setting is needed. Some progress in this direction has already been made (23).

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