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Automated, Rapid Identification of Bacteria by Pattern Analysis of Growth Inhibition Profiles Obtained with Autobac 1

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A scheme for identifying bacteria has been devised which utilizes the inhibition patterns obtained by Autobac 1 with routine and unusual antimicrobial agents and with other differentially inhibitory chemical compounds. Over 600 compounds were initially identified from the literature, and over 125 of these were selected for further testing on the basis of antibacterial activity most conducive to the instrument-generated differential scheme. Numerical growth index information derived by light scatter comparisons from the instrument were analyzed by computer, utilizing the quadratic discriminant function statistical technique. In comparison with conventional methods, accuracy for the 10 bacterial genera studied was 95% or greater. Results indicate a potential for both bacterial identification and antimicrobial agent susceptibility testing in the clinical laboratory within 3 to 5 h when using this automated approach.

Considering the impressive recent advances in computer technology and the pressure for computerization of clinical laboratories in general, it seems inevitable that there will be an increasing prominence of computer systems in the clinical microbiology laboratory.

One of the many possible tasks for such a system is the identification of bacteria, and schemes for this based on conventional biochemical tests have been available for some time (2, 5, 6, 8, 10, 14, 17). A more recent approach taken by several authors is the identification of bacteria through computer analysis of susceptibility patterns (4, 7, 16).

Friedman and MacLowry (7) examined broth dilution susceptibility data for 31 species of bacteria, including both gram-positive and gram-negative organisms, with a computer program based on a probability formula called Baye's theorem. Using 11 antimicrobial agents, they found approximately 86% agreement with conventional biochemical tests.

Darland (4) studied the possibility of identifying eight species of gram-negative bacteria by using the zone sizes obtained with a standard disk diffusion technique for 12 antimicrobial agents. A computer program based on discriminant analysis was used to analyze the data, and it was found that the computer identification was correct in 82% of the cases.

Sielaff et al. (16) applied another variation of this latter statistical technique, called the quadratic discriminant function, to the identification of nine groups of gram-negative bacteria, using susceptibility data derived through the use of a recently marketed semiautomated device for susceptibility testing (Autobac 1, Pfizer, Inc.). A large number of antimicrobial agents were tested to select the most effective; when 18 were used, 97.3% of the organisms were correctly identified, and when 14 were used the accuracy was 95.6%.

This latter study obtained an overall identification accuracy that could be considered acceptable for routine use and produced a truly rapid (3 to 5 h) and accurate, semiautomated identification system.

The work discussed herein was designed to overcome remaining problems from the study by Sielaff et al. (16) described above. First, a large number of different taxonomic groups needed to be included in the system to make it more practical. Second, the problem of plasmid-mediated resistance to routinely used antimicrobial agents is encountered. Any system based entirely on susceptibility patterns obtained with antimicrobial agents used commonly for therapeutic treatment is subject to future variations in susceptibility patterns due to the emergence of R-factors. Therefore, we have investigated the ability of the system to identify a large number of groups and examined the possibility of incorporating antimicrobial compounds that are not used in human or

veterinary medicine, or else are not used routinely as therapeutic agents and, thus, are relatively invulnerable to changes in susceptibility patterns.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study were fresh clinical isolates obtained from the Clinical Microbiology Laboratory, University of Utah Hospital, supplemented where necessary with stock cultures maintained at the University of Utah. Fourteen different taxonomic groups of organisms (10 genera) were used, with 24 or 25 strains per group, making a total of 346 cultures. Organisms were identified according to standard methods (11). The 14 groups were: *Escherichia coli, Klebsiella pneumoniae, Enterobacter* sp., *Citrobacter freundii, Citrobacter diversus, Serratia* sp., *Proteus mirabilis, Proteus vulgaris, Proteus morganii, Proteus rettgeri, Salmonella* sp., *Shigella* sp., *Pseudomonas aeruginosa,* and *Acinetobacter calcoaceticus* var. *anitratus* (*Herellea*). All cultures were maintained on slants of tryptose blood agar base (Difco) at 4°C. All tests with antimicrobial agents were done with overnight cultures plated on Columbia blood agar base (Difco) with 5% sheep blood and incubated at 37°C.

Antimicrobial agents. Over 600 miscellaneous chemical compounds were identified and reviewed in an extensive literature search. These are compounds that reportedly possess antimicrobial properties (including dyes, disinfectants, uncommon antibiotics, other organic compounds, and inorganic salts). Over 125 of them were tested by performing minimal inhibitory concentration studies against the bacterial strains described above (unpublished data). Four of these compounds, brilliant green, chlorhexidine diacetate, D-cycloserine, and 2′,3′,4′-trihydroxyacetophenone (gallacetophenone), were selected for further investigation on the basis of their capacity to differentiate bacterial species. Data from previous experiments (16) were analyzed by computer to select the combination of eight commonly used therapeutic agents that resulted in maximum identification accuracy. The ones selected were: carbenicillin, cephalothin, doxycycline, methenamine mandelate, nitrofurantoin, polymyxin B, streptomycin, and tetracycline. Thus, a set of 12 antimicrobial agents was created, which exactly filled the number of chambers in an Autobac cuvette.

Antimicrobial agents commonly used for susceptibility testing were elution disks obtained from Pfizer, Inc., at concentrations used for Autobac 1 (18). Brilliant green was obtained from K and K Laboratories, Irvine, Calif.; chlorhexidine was supplied by Ayerst Laboratories, Montreal, Canada; D-cycloserine was supplied by Eli Lilly and Company, Indianapolis, Ind.; and gallacetophenone was purchased from Eastman Organic Chemicals, Rochester, N.Y. Disks containing these latter compounds were made by hand. A stock solution of appropriate concentration was made, and 10 μl of this solution was placed on each disk with a repeating syringe dispenser (Hamilton Co., Reno, Nev.).

Susceptibility profiles. All susceptibility profiles were generated using Autobac 1 (Pfizer Diagnostics) (12, 13, 18). This system consists of several specialized components, the most prominent being a photometer that measures fixed-angle light scatter. Disks containing desired antimicrobial agents are loaded into a special 13-chambered cuvette, one disk per chamber. The first chamber serves as an uninhibited growth control and receives no disk. The organism to be tested is standardized in saline solution with the photometer unit and then diluted into broth and inoculated into the cuvette. The cuvette is incubated on a specially designed incubator-shaker (220 rpm) for 3 to 5 h at 36°C. To read the results, each cuvette is placed on the carriage in the photometer unit, where each cuvette chamber is automatically scanned and compared with the control. Any significant difference in growth indicates susceptibility to the agent and is represented by a value called a light-scattering index (LSI) value, ranging from 0.00, which represents total resistance, to 1.00, which represents susceptibility, and calculated in increments of 0.01. The procedure and theory have been given in greater detail elsewhere (13).

Statistical technique and computer program. Computer identification was accomplished through the use of a Fortran program, which utilized the quadratic discriminant function, a statistical technique based on the multivariate normal distribution. In this approach it is assumed that the LSI values for each taxonomic group follow a normal distribution with each antimicrobial agent (16). Although this assumption is not strictly accurate, the procedure is robust enough to be accurate in spite of this.

When using N number of variables (antimicrobial agents), each taxonomic group can be regarded as a cluster of closely related points in N-space. The center of a particular group's cluster is located in N-space by its vector of mean values for the variables being used. The cluster's size, shape, and orientation is determined by the group's covariance matrix. In practice, the population mean vectors and covariance matrixes are unknown and are, therefore, estimated using a sample of organisms of known identity from each taxonomic group. To identify an unknown organism, the discriminant function is computed for each group, using the vector of LSI values obtained for the unknown organism and the mean vector and covariance matrix for each group in turn. The unknown is assigned to the group with the largest computed value for the discriminant function. This procedure has been discussed in greater detail in a previous publication (16), and more information is available elsewhere (1, 3, 9).

This multivariate technique offers distinct advantages over the traditional dichotomous tree-branching procedure. The branching procedure requires that at each node (or test) a decision be made based solely on that test. This means that, in many cases, an organism that is atypical for even one test is excluded from correct identification. The discriminant function approach, on the other hand, does not require decisions to be made based on the results of

single tests. Rather, it views the profile of test results as a whole and makes but a single decision based on the totality of the test results.

RESULTS

Table 1 represents LSI data generated using the 12 selected compounds. Each bacterial group has a characteristic average LSI value with each of the 12 compounds, thus creating distinct patterns. This data base was analyzed with the quadratic discriminant function computer program, and the results represented in Table 2 were obtained. The data base was used to calculate the values marking the position of each taxonomic group. Using these values, the organisms in the data base were presented again to the computer for identification. The overall agreement with conventional biochemical tests was 97.1% (Table 2). The computer identification was 100% correct in most of the groups, and in all but two groups, *E. coli* and *C. freundii*, the identification accuracy was greater than 90%. All four of the misidentified strains of *E. coli* were identified as *Shigella* by the computer. Of the three *C. freundii* cultures misidentified, two were identified as *E. coli* and one was identified as *C. diversus*.

DISCUSSION

This work represents an attempt to reduce the number of antimicrobial agents used in the identification profile and increase the number of different organisms considered, while still maintaining the high identification accuracy achievable with this statistical technique. The results point out a problem in distinguishing *E. coli* from *Shigella*. Work is now underway to correct this problem. If this problem were eliminated, the overall accuracy would be even greater, i.e., around 98%. The problem of separating *Citrobacter*, *Enterobacter*, and *E. coli* was not unexpected, since it was encountered in previous work (16).

This was the initial approach to investigating the use of diverse antimicrobial compounds for identifying bacteria. A limited number of these compounds were combined with some of the routinely used therapeutic agents previously found useful for discrimination between groups of bacteria (16), and the results demonstrate the feasibility of using such compounds. Brilliant green and gallacetophenone are readily available from commercial sources but are not used in human or veterinary medicine. Cycloserine has activity against a variety of bacteria but, due to its toxicity, is limited to use as a secondary agent against tuberculosis (15). Chlorhexidine is used in some countries as an antiseptic (15). A panel of compounds similar to these could probably be used as a standard set without undue concern over increasing resistance. However, it would still be necessary to monitor the inhibition patterns by comparison with conventional biochemical tests and update the data base if necessary. We are presently investigating other compounds to find a set that will adequately identify a further expanded set of gram-negative organisms. We are also investigating a similar scheme with gram-positive bacteria.

This concept is significantly different from using the normally generated susceptibility data for identification. It would be necessary to add another "identification" cuvette(s) containing a set of standard compounds, and the data from these compounds could either be used alone or in conjunction with the susceptibility data for identification. With the addition of a computer interface (now under investigation), the data can be transferred automatically to

TABLE 1. *Average LSI values given by 14 groups for compounds in final set*

Antimicrobial agent	Avg LSI value													
	E. coli	*Klebsiella*	*C. freundii*	*C. diversus*	*Enterobacter*	*Serratia*	*P. mirabilis*	*P. vulgaris*	*P. morganii*	*P. rettgeri*	*A. calcoaceticus*	*P. aeruginosa*	*Shigella*	*Salmonella*
Brilliant green	0.80	0.24	0.43	0.39	0.26	0.82	0.71	0.75	0.88	0.81	1.00	0.33	0.85	0.21
Carbenicillin	0.84	0.31	0.74	0.33	0.82	0.75	0.95	0.82	0.96	0.79	0.98	0.50	0.88	0.94
Cephalothin	0.77	0.86	0.47	0.80	0.29	0.07	0.95	0.40	0.05	0.37	0.25	0.10	0.79	0.86
Chlorhexidine	0.97	0.69	0.75	0.64	0.37	0.83	0.66	0.63	0.73	0.69	0.50	0.62	1.00	0.77
Cycloserine	0.88	0.18	0.56	0.83	0.56	0.05	0.14	0.14	0.04	0.17	0.36	0.07	0.90	0.77
Doxycycline	0.60	0.51	0.50	0.80	0.48	0.43	0.11	0.36	0.43	0.16	0.98	0.19	0.68	0.37
Methenamine mandelate	0.12	0.31	0.33	0.41	0.26	0.40	0.76	0.70	0.81	0.73	0.42	0.17	0.40	0.59
Nitrofurantoin	0.92	0.59	0.78	0.85	0.52	0.49	0.26	0.42	0.39	0.31	0.16	0.10	0.99	0.99
Polymyxin	0.94	0.96	0.95	0.97	0.94	0.72	0.18	0.24	0.13	0.20	1.00	0.99	1.00	0.99
Streptomycin	0.69	0.74	0.77	0.89	0.89	0.77	0.85	0.85	0.81	0.70	0.86	0.56	0.68	0.71
Tetracycline	0.53	0.56	0.55	0.80	0.55	0.23	0.09	0.41	0.62	0.11	0.71	0.12	0.68	0.77
Gallacetophenone	0.31	0.23	0.29	0.31	0.24	0.60	0.53	0.59	0.83	0.57	0.66	0.36	0.44	0.25

TABLE 2. *Analysis of data base with the quadratic discriminant function computer program*[a]

Group name	No. correct/ total strains	Percent correct
E. coli	20/24	83.3
Klebsiella	25/25	100
Enterobacter	24/25	96
C. freundii	21/24	87.5
C. diversus	25/25	100
Serratia	24/24	100
P. mirabilis	25/25	100
P. vulgaris	23/25	92
P. morganii	25/25	100
P. rettgeri	25/25	100
Salmonella	25/25	100
Shigella	25/25	100
P. aeruginosa	24/24	100
A. calcoaceticus var. *anitratus* (*Herellea*)	25/25	100

[a] Total correct = 336/346; accuracy = 97.1%.

the computer for processing and storage, eliminating the need for data transcription, and thus this scheme seems to be a promising approach to a rapid and accurate, automated identification system.

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