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Novel Approach to Bacterial Identification That Uses the Autobac System

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A new system for the rapid identification of gram-negative bacilli on the Autobac system is described. This system utilizes growth inhibition profiles to a panel of differentially inhibitory chemical agents. These profiles are analyzed with a two-stage quadratic discriminant analysis to arrive at the organism identification. The system identifies 30 different groups of gram-negative bacilli, including the most clinically significant *Enterobacteriaceae* and glucose nonfermenters. A total of 3,726 strains, distributed among the 30 groups, was tested. The Autobac system agreed with the conventional biochemical identification 88.4% of the time. When the individual group results were weighted to reflect clinical frequency, the result was a 93.1% agreement.

The original Autobac system (7) was designed to provide rapid (3 to 5 h) qualitative susceptibility test results. Subsequent to its introduction, its capabilities have been expanded to include 5-h quantitative minimal inhibitory concentration determinations (6) and urine screening (5). This paper details a new system which incorporates the use of differentially inhibitory chemical compounds and a complex computerized algorithm to identify gram-negative bacilli.

The idea of using inhibitory compounds, such as antimicrobial agents, to predict the identity of bacterial strains has been suggested numerous times. Gilardi (4) used susceptibility profiles to assist in the identification of nonfermenting gram-negative bacteria. Susceptibility profiles were also used by Sutter and Finegold (10) to assist in the identification of gram-negative anaerobic bacteria. The use of statistical models to evaluate antimicrobial susceptibility test results for the purpose of bacterial identification has also been investigated. Friedman and MacLowry (3) proposed the use of a Bayesian model. A linear discriminant analysis was utilized by Darland to identify nine species of *Enterobacteriaceae* (2). Sielaff and co-workers (9) proposed a system based on the quadratic discriminant function. This work utilized only common clinically prescribed antimicrobial agents. Selective changes in antimicrobial resistance patterns pose a potential problem for any system that relies solely on clinically prescribed antimicrobial agents. This problem was partially addressed by Buck and co-workers (1) when they substituted several differentially inhibitory chemical agents for some of the clinical antimicrobial agents. These nontherapeutic chemical agents are subjected to less selective pressure and thus a more stable system would be expected.

This report describes an expansion and refinement of this system, now feasible for routine use in the clinical microbiology laboratory.

MATERIALS AND METHODS

Autobac identification system. The Autobac identification system consists of five main components: light-scattering photometer, incubator-shaker, data terminal, disk dispenser, and 19-chamber cuvette (Fig. 1).

To perform an identification with the Autobac system, the gram-negative bacilli must first be isolated from the patient specimen on both a sheep blood agar plate and a MacConkey agar plate. Three observations are made on the MacConkey agar plate: whether growth occurred; and if growth occurred, whether lactose was fermented; and whether the bile salts in the medium were precipitated. Lactose fermentation is indicated by a pink to red coloration of the colonies. Bile precipitation is indicated by a haze in the media surrounding the colonies when viewed by transmitted light. One observation, presence or absence of swarming growth, is made from the blood agar plate, and two rapid biochemical tests, a spot indole and a spot oxidase, are performed with a colony from that plate. The spot oxidase test is the standard test, using a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride. The spot indole test uses the method of Vracko and Sherris (11).

To prepare an Autobac cuvette, the 18 different antimicrobial disks (Table 1) are dispensed into the cuvette from the disk dispenser. A standardized inoculum is prepared by picking bacteria from well-isolated colonies on the blood agar plate. This material is dispersed in ca. 5 to 6 ml of Autobac saline in the special inoculum standardization tube. The bacterial

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FIG. 1. The Autobac identification system components: light-scattering photometer, incubator-shaker, and data terminal.

concentration is standardized by placing the tube in the standardization port in the photometer and observing the deflection of the needle in the standardization meter. The concentration can be adjusted by adding either more bacteria or more saline. When a properly standardized inoculum is achieved, a 3-ml sample is removed and added to a 26.5-ml tube of Autobac low-thymidine Eugonic broth. After mixing, the tube is attached to the cuvette, and the standard rotation sequence delivers 1.5 ml to each of the 19 chambers. The cuvette is then placed on one of the trays of the incubator-shaker and incubated for 3 h, after which the cuvette is placed in the photometer. The data terminal will request both an accession number for the specimen and an isolate number (to distinguish between isolates when more than one is obtained from a single specimen). The terminal will then request the results of the six primary plate observations/tests. Next, the photometer reads the cuvette and computes a light-scatter index (LSI) value for each chamber. These values are utilized in a two-stage quadratic discriminant analysis to arrive at an identification. The results are printed out on a separate report form (Fig. 2). The two most probable identifications are indicated along with their respective relative probabilities.

QDA. The quadratic discriminant analysis (QDA) is a multivariate statistical technique. It requires a learning sample or data base which consists of a sample of strains from each of the bacterial groups to be included in the identification system. The information required for each strain in the data base includes the true identity, as determined by a reference identification procedure; the data from the observations made from the ancillary observations and tests from the colonies on primary plating media; and the Autobac LSI values for each of the inhibitory chemical agents used in the system. Unlike the Autobac susceptibility systems, the Autobac identification system does not truncate the LSI values at 0.00 and 1.00. Instead, values less than 0.00 and greater than 1.00 are assumed to provide information useful for differentiation. Each strain can be visualized as residing in *n*-dimensional space. Each dimension represents a different inhibitory agent, and its scale is the scale of LSI values. Figure 3 shows a two-dimensional representation of this. Strain number 8 of group A has an LSI of 0.03 for agent I and an LSI of 0.76 for agent J. This procedure can be expanded to any number of dimensions by adding additional inhibitory agents, and the strain can be located in the higher-dimensional space in the same fashion. Every strain has its own profile of LSI values and hence its own unique position in *n*-dimensional space (although it is possible for more than one strain to have the same profile).

Implicit in these techniques is the assumption that bacteria which are of the same type will tend to have similar values for each of the variables. This will cause them to form clusters in *n*-space (Fig. 3). If the variables are appropriate for differentiation (assuming that the groups are in fact differentiable), then there will be a minimum amount of overlap between adjacent clusters.

TABLE 1. Panel of agents to be used in the Autobac identification system

Agent	Disk mass (μg)
Acriflavine	30
Brilliant green	3.
Cobalt chloride	375
Cycloserine	78
Cycloserine	240
3,5-Dibromosalicylic acid	750
Dodecylamine hydrochloride	18.7
Floxuridine	36
Malachite green	3
Methylene blue	255
Omadine disulfide	5.5
Sodium azide	75
Thallous acetate	150
Carbenicillin	40
Cephalothin	13.5
Colistin	13
Kanamycin	5.4
Novobiocin	48

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AUTOBAC MTS

ID TEST      ACCN.NO. 1234      ISO.NO. 1

GMA+ MLA- BIL- SWM- SIN- OXI-

CHMBR  1    2    3    4    5    6    7    8    9    10   11   12   13   14   15   16   17   18
LSI    0.62 1.13 -.02 0.60 0.22 0.84 0.04 0.26 0.53 0.18 0.89 0.61 0.49 0.68 1.45 0.98 1.31 0.39
G.I.=0.97 (2.43)

GRAM NEGATIVE

      ID            PROB
A.CALCOACETICUS     0.93
Y.PSEUDOTUBERCU     0.04

DATE: 5/14/80  TIME: 11:35    BY:
PT.NAME/ID:
COMMENT:
```

FIG. 2. Autobac identification test report form.

The quadratic discriminant function is based on the multivariate normal probability model. Figure 4 is an example of bivariate (two-dimensional) normal probability distributions. Each distribution is represented as a series of concentric equiprobability ellipses. The probability level associated with each ellipse is the probability that a member of that group could fall at least that far from the center of the distribution. Therefore, the farther from the center, the lower the probability of belonging to that group. In Fig. 4, the unknown falls on the 0.05 probability ellipse for group A and on the 0.10 probability ellipse for group B. As a result, the unknown is more likely to belong to group B than to group A and should be assigned to group B.

The following is the formula for the quadratic discriminant function:

$$f(x)_i = p_i\,(2\pi)^{-\frac{NV}{2}}\,|S_i|^{-\frac{1}{2}}\,e^{-\frac{q_i}{2}}$$

where p_i is the prior probability of group i, NV is the number of variables, and $|S_i|$ is the determinant of the covariance matrix for group i:

$$q_i = (X - \bar{x}_i)'\, S_i^{-1}\, (X - \bar{x}_i)$$

where X is the vector of LSI values for the unknown organism, $\bar{x}_i$ is the mean vector for the i-th group, $'$ means the matrix transpose, S_i^{-1} is the inverse of the covariance matrix for the i-th group.

The equation without the p_i is the probability density function for the multivariate normal model. The elements of the covariance matrix are computed by the following formula:

$$S_{x_i x_j} = \frac{n\sum_{k=1}^{n} x_{ik}\,x_{jk} - \sum_{k=1}^{n} x_{ik} \sum_{k=1}^{n} x_{jk}}{n^2 - n}$$

where $S_{x_i x_j}$ is the covariance between variables x_i and x_j (for $i = j$, the formula reduces to that of the variance of variable x_i); and n is the number of observations of both variables x_i and x_j.

The mean vector for each group is computed. The elements of these mean vectors are computed by the following formula:

$$\bar{x}_{ik} = \sum_{k=1}^{n} x_{ijk}/n_k$$

where $\bar{x}_{ik}$ is the LSI mean for the i-th variable in the k-th group, x_{ijk} is the LSI value for the i-th variable for

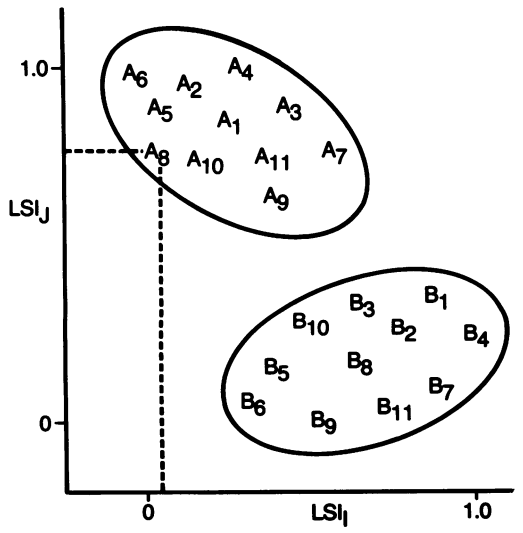

FIG. 3. Bivariate representation of the strains of two bacterial groups, A and B, challenged with two antimicrobial agents, I and J.

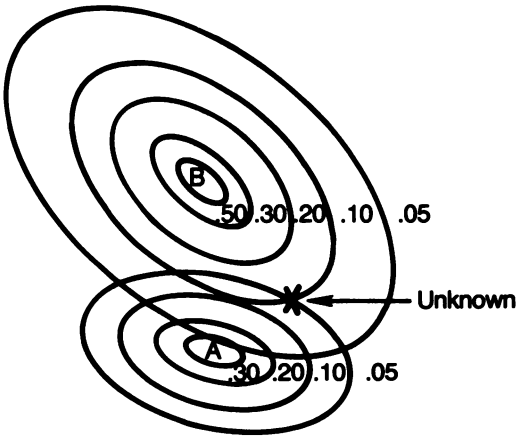

FIG. 4. Equiprobability levels for two bivariate normal probability models. Unknown falls on the 5% probability level for A and the 10% level for B. Therefore the unknown is assigned to B.

TABLE 2. Autobac identification compound panels for the first-stage QDA

Panel	Disk mass (μg)
Oxidase positive	
Cobalt chloride	375
Cycloserine	78
Cycloserine	240
3,5-Dibromosalicylic acid	750
Dodecylamine hydrochloride	18.7
Floxuridine	36
Omadine disulfide	5.5
Carbenicillin	40
Cephalothin	13.5
Colistin	13
Kanamycin	5.4
Novobiocin	48
Oxidase negative	
Acriflavine	30
Brilliant green	3
Cycloserine	78
Cycloserine	240
3,5-Dibromosalicylic acid	750
Methylene blue	255
Omadine disulfide	5.5
Thallous acetate	150
Carbenicillin	40
Cephalothin	13.5
Kanamycin	5.4
Novobiocin	48

the j-th strain in the k-th group, and n_k is the number of strains in the k-th group.

The prior probability is a factor in which various kinds of information can be introduced. This is usually previously known information, but is not limited to that. The relative cost (in medical terms) of misidentifying a species would be a good example, but is difficult to quantify. In the present case, the primary plate observations/tests are information known before the Autobac test and are included in the prior probability factor p_i. The p_i is obtained by combining the primary plating data (observations on the blood and MacConkey agar plates and the spot oxidase and spot indole tests), using a Bayes statistical approach. The following is the formula used to calculate p_i:

$$p_i = \prod_{j=1}^{NT} r_{ij}$$

where p_i is the prior probability in group i, r_{ij} is the probability of the observed result for the j-th test for

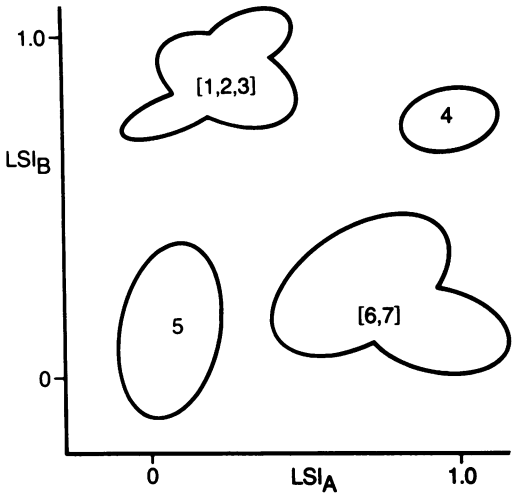

FIG. 6. Interpretation of the first-stage QDA results in Fig. 5.

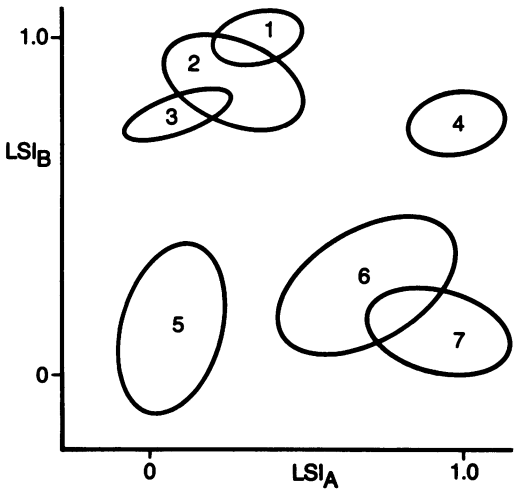

FIG. 5. Bivariate representation of seven bacterial groups after the first-stage QDA, showing relative isolation of some groups and overlap of others, using LSI data for antimicrobial agents A and B.

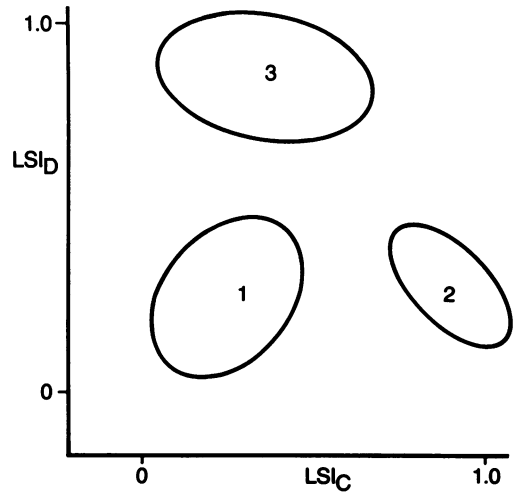

FIG. 7. Bivariate representation of the second-stage QDA of the first-stage supergroup 1, 2, 3, using LSI data for antimicrobial agents C and D.

TABLE 3. Overlap of groups requiring second-stage QDA

Identification by Autobac	Other possible true identification	Panel[a]	
Shigella sp.	*E. coli*	Cobalt chloride	(375)
		Cycloserine	(78)
		Floxuridine	(36)
		Malachite green	(3)
P. vulgaris	*P. mirabilis*	Acriflavine	(30)
		Brilliant green	(3)
		Omadine disulfide	(5.5)
Providencia sp.	*P. mirabilis*	Brilliant green	(3)
	P. vulgaris	Cobalt chloride	(375)
	Morganella morganii	Cycloserine	(240)
	Serratia sp.	Methylene blue	(255)
		Omadine disulfide	(5.5)
C. freundii	*E. coli*	Cycloserine	(240)
	E. cloacae	Dodecylamine hydrochloride	(18.75)
	E. agglomerans	Malachite green	(3)
		Sodium azide	(75)
		Colistin	(13)
E. aerogenes	*K. pneumoniae*	Cycloserine	(78)
	E. cloacae	Malachite green	(3)
		Omadine disulfide	(5.5)
		Kanamycin	(5.4)
		Novobiocin	(48)
E. agglomerans	*E. coli*	Brilliant green	(3)
	K. pneumoniae	Dodecylamine hydrochloride	(18.75)
	C. freundii	Floxuridine	(36)
		Sodium azide	(75)
		Novobiocin	(48)
Pseudomonas sp.	*P. aeruginosa*	Brilliant green	(3)
		Cobalt chloride	(375)
		Malachite green	(3)
		Methylene blue	(255)
P. putida/fluorescens	*P. aeruginosa*	Brilliant green	(3)
		3,5-Dibromosalicylic acid	(750)
		Methylene blue	(255)
		Omadine disulfide	(5.5)
Alcaligenes sp.	*Pseudomonas* sp.	Cobalt chloride	(375)
		Dodecylamine hydrochloride	(18.75)
		Floxuridine	(36)
		Cephalothin	(13.5)

[a] Disk mass in micrograms per milliliter is in parentheses.

the *i*-th group, and NT is the number of primary plate results.

When the quadratic discriminant function has been computed for all groups, the group with the greatest value is selected as the specific identification for the unknown organism.

For the identification system being discussed here, the data on each strain (18 LSIs plus six primary isolation plate observations and spot biochemical tests) are analyzed by a two-stage QDA, which is a modification of the procedure discussed above. Based upon the oxidase test result, a subset of 12 LSIs is selected from the panel of 18 LSIs generated for that strain. The oxidase-positive and oxidase-negative panels are shown in Table 2. The six primary plate observations/spot tests are used to compute the prior probability. A 12-dimensional QDA is then run using the appropriate oxidase panel of LSIs. If the strain is identified by this first stage as *Shigella* species, *Proteus vulgaris*, *Providencia* species, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Citrobacter freundii*, *Pseudomonas putida/fluorescens*, *Pseudomonas* species, or *Alcaligenes* species, a second-stage QDA is performed by the computer. For each of the bacteri-

TABLE 4. No. of strains tested for inclusion in data base

Taxonomic group	No. of strains run
Acinetobacter calcoaceticus	197
Aeromonas sp.	123
Alcaligenes sp.	108
Citrobacter diversus	94
Citrobacter freundii	146
Edwardsiella tarda	93
Enterobacter aerogenes	49
Enterobacter agglomerans	100
Enterobacter cloacae	107
Escherichia coli	156
Flavobacterium sp.	182
Hafnia alvei	49
Klebsiella pneumoniae	203
Klebsiella sp. (other than above)	101
Moraxella sp.	68
Morganella morganii	116
Proteus mirabilis	162
Proteus vulgaris	106
Providencia sp. (including *P. rettgeri*)	222
Pseudomonas aeruginosa	180
Pseudomonas cepacia	102
Pseudomonas maltophilia	111
Pseudomonas putida/fluorescens	173
Pseudomonas stutzeri	99
Pseudomonas sp. (other than above)	105
Salmonella sp. (including Arizona)	209
Serratia sp.	111
Shigella sp.	98
Yersinia enterocolitica	109
Yersinia pseudotuberculosis	47
Total	3,726

al groups mentioned, a specific panel of LSIs is selected to differentiate that group from other groups which significantly overlap it in the original 12-dimensional QDA. Figures 5 through 7 are a graphical representation of this procedure in two dimensions. In Fig. 5, the ellipses represent equiprobability ellipses, at a given probability level, for seven bacterial groups. Groups 1, 2, and 3 overlap as do groups 6 and 7. If the identity resulting from the first stage QDA were either group 4 or group 5, no further analysis would be necessary, as neither of these groups overlaps with any other group. Therefore, the identity from the first stage would be reported. If the identity resulting from the first-stage QDA was group 2, for example, a second-stage QDA would be performed since there is a significant amount of overlap with groups 1 and 3. The result of the first stage would be interpreted as in Fig. 6, where groups 1, 2, and 3 are treated as a single group. The second stage would then focus only on differentiating group 2 from groups 1 and 3. This is done by selecting only those antimicrobial agents which aid in that differentiation, as seen in Fig. 7. Table 3 lists, for each of the nine groups above requiring the second-stage QDA, the groups with which it has a significant overlap and the antimicrobial agents used to differentiate them.

RESULTS

To test the feasibility of the system described above, a large data base was collected. This data base included strains from 30 different gram-negative bacterial groups, both *Enterobacteriaceae* and glucose nonfermenters. Most of the clinically significant organisms are identified to the species level, with the remainder being identified to the genus level. Table 4 is a listing of the strains in the data base. To assess the accuracy of the Autobac identification system, these strains were identified by both the Autobac method and by standard biochemical test procedures. The level of agreement between the Autobac method and the standard reference method can be seen in Table 5. The column on the right lists the percent agreement for each individual bacterial group. At the bottom, the unweighted average is simply the total number of strains for which both methods agree divided by the total number of strains tested. Most of the strains were identified in 3 h. Only a few slow-growing strains required additional incubation (up to 5 h).

There also appears, at the bottom of Table 5, a weighted average. This weighted average is determined by weighting the individual percent agreements by the percent incidence found in column 2. The incidence data was obtained from a 1975–76 Bacteriological Report on Projected Incidences for all >100 Bed Acute Care Hospitals in the United States (Professional Market Research, Inc.). Since the number of strains in each group in the data base does not even approach a clinical distribution, the weighted average was computed to give an idea of the overall level of accuracy the typical laboratory could expect from the system.

As the table shows, 60% of the groups had greater than 90% agreement. Even more significant is the fact that the average level of agreement for the first four groups, *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis*, and *Pseudomonas aeruginosa*, which comprise more than 75% of the gram-negative work load, was greater than 95%.

To test the stability of the Autobac identification system, four bacterial strains with multiple on-scale LSI values were selected for repeated testing. The strains were an *E. coli*, a *P. mirabilis*, a *P. aeruginosa*, and an *Alcaligenes odorans*. Table 6 shows the results of approximately nine months of repeated testing. After 560 tests, the *E. coli* was misidentified only once, being identified as a *C. freundii*. The *P. mirabilis* was never misidentified in 560 tests. The *P. aeruginosa* was misidentified 11 times out of 552, with 10 of the 11 being identified as *P. putida/fluorescens* and 1 being identified as *Pseudomonas* species. Finally, the *A. odorans* was misidentified 16 times out of 560, with 14 of the 16 being

TABLE 5. Agreement between the Autobac and the reference method after two-stage QDA identification

Organism	% Incidence	No. correct/ no. run	% Agreement (unweighted)
E. coli	41.00	147/156	94.2
K. pneumoniae	13.70	188/203	92.6
P. mirabilis	12.60	160/162	98.8
P. aeruginosa	9.90	173/180	96.1
E. cloacae	5.90	90/107	84.1
Hafnia alvei	2.20	40/49	81.6
Serratia sp.	1.90	107/111	96.4
P. vulgaris	1.40	96/106	90.6
M. morganii	1.00	112/116	96.6
P. maltophilia	1.00	103/111	92.8
P. cepacia	1.00	83/102	81.4
P. stutzeri	1.00	91/99	91.9
P. putida/fluorescens	1.00	138/173	79.8
C. freundii	0.75	102/146	69.9
C. diversus	0.75	91/94	96.8
Klebsiella sp.	0.72	82/101	81.2
E. aerogenes	0.43	45/49	91.8
Providencia sp.	0.35	181/222	81.5
Pseudomonas sp.	0.25	85/105	81.0
A. calcoaceticus	0.25	187/197	94.9
Salmonella/Arizona sp.	3.00	179/209	85.6
Shigella sp.		93/98	94.9
E. agglomerans		62/100	62.0
Edwardsiella sp.		91/93	97.8
Y. enterocolitica		99/109	90.8
Y. pseudotuberculosis		45/47	95.7
Alcaligenes sp.		71/108	65.7
Aeromonas sp.		116/123	94.3
Flavobacterium sp.		178/182	97.8
Moraxella sp.		58/68	85.3
Avg		93.1 (Weighted)	88.4 (Unweighted)

identified as *Flavobacterium* species and 2 as *Aeromonas* species.

DISCUSSION

The Autobac gram-negative bacterial identification system represents a radical departure from other currently available bacterial identification systems. Although no other commercial system utilizes growth inhibition profiles as data for identification, several investigators, as noted in the introduction, have shown that antimicrobial susceptibility profiles are valuable for bacterial identification (1–4, 9, 10). In addition, over the years, many chemical agents (brilliant green, crystal violet, sodium chloride, bile salts, etc.) have been added to differential media to inhibit the growth of certain bacterial species, while allowing other species to grow. Therefore, the use of growth inhibition as a determinant in bacterial identification has a firm foundation in the literature.

The present study has shown that the Autobac identification system is both accurate and reliable. It is very much a rapid system, and the automated instrument interpretation of the test results removes the subjectivity of the test result interpretation so characteristic of the traditional biochemical test procedures. The quantitative LSI values of the Autobac system allow the extraction of more information from a single test than the dichotomous results obtained from conventional biochemical tests. The multivariate statistical analysis of the Autobac test data utilizes information on correlations between tests which the traditional univariate branching schemes cannot do. We therefore feel that the Autobac gram-negative bacterial identification system readily lends itself to routine use in the clinical microbiology laboratory.

TABLE 6. Stability study on the Autobac identification system

Strain	No. correct/ no. tests	% Correct
E. coli (511108)	559/560	99.8
P. mirabilis (571101)	560/560	100.0
P. aeruginosa (521205)	541/552	98.0
A. odorans (641518)	554/560	97.1

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