Time- and Media-Saving Testing and Identification of Microorganisms by Multipoint Inoculation on Undivided Agar Plates

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Motility and various biochemical activities of isolates of bacteria and yeasts were tested on undivided agar plates by using a simple, manually operated multipoint inoculation apparatus that allowed the analysis of 25 isolates per 9-cm-diameter petri plate. Fermentation of all 17 carbohydrates tested as well as 13 other biochemical activities commonly used for identification of bacteria were readily demonstrated by the multipoint inoculation plate method, and the results agreed very well with those of conventional tube tests. In addition to speedy inoculation and low cost of materials, the multipoint inoculation plate method offers several other advantages when compared with conventional tube tests or with some of the manufactured test kits currently available for recognizing members of the family *Enterobacteriaceae*.

In recent years, there has been a rapidly growing interest among clinical microbiologists in meeting the steady increases in specimens to be analyzed and in costs of labor and materials by introducing various time- and media-saving procedures, such as combining several tests in one tube, microscale methods, and attempts to automate laboratory testing of clinical isolates of microorganisms. Furthermore, a number of manufactured multitest kits designed for identification of bacteria belonging to the family *Enterobacteriaceae* on the basis of their biochemical activities have become commercially available. Whereas several such systems still are being evaluated, the improved API and Enterotube identification systems appear to be sufficiently reliable in comparisons with conventional tube tests and are presently accepted and widely used (e.g., in Sweden). However, these systems also have disadvantages, such as lack of flexibility, contributing to a fairly high cost of materials per isolate identified. In addition, the API system requires a considerable amount of work for manual inoculation of its 20 separate wells, and in the flexible Minitek system manual addition of bacteria and also media, reagents, and sometimes oil to the chosen number of wells is necessary.

Since the first multipoint inoculation apparatus was introduced by S. D. Garrett in 1946, several devices for microbiological work based on the multipoint principle have been described (see review in reference 7). Their main clinical use is currently in phage and bacteriocin typing

of bacterial strains, and in several clinical laboratories multipoint inoculators also enable large-scale routine antibiotic susceptibility testing of microorganisms by using the agar dilution method instead of the more variable disk diffusion method (14, 15). Such inoculators have also been used for a long time in microbial genetics for work with phages or antibiotics as well as for testing of auxotrophy, carbohydrate utilization, or other genetic markers of bacterial mutants, recombinants, transductants, etc. (i.e., in simple situations where defined media can be used and either growth or no growth is to be recorded).

Multipoint inoculators have also been suggested for demonstration of metabolic activities of clinical isolates of bacteria. The organisms to be analyzed are inoculated into the compartments of divided petri dishes (13) or Teflon plates (7). From these master plates the bacterial strains are transferred to multicompartiment plates containing test media by using a multipoint inoculator. These methods had not been developed or much used until recently, when multipoint testing with microtiter trays was again adopted for evaluation (6, 12).

We wish to report our positive experience from large-scale testing of clinical isolates of bacteria and yeasts on undivided agar plates, using a simple multipoint inoculation apparatus. On each regular 9-cm-diameter plate, one or two activities of 25 isolates have been tested, thus making expensive multicompartiment trays and elaborate media-dispensing routines unnecessary. The system is entirely based on generally

accepted tests. When necessary, however, the conventional test substrate or the inoculation procedure has been modified to fit the agar plate condition. In comparison with classical tube testing, this system allows considerable reductions in costs of media and reagents, as well as in labor with glassware service, media preparation, and inoculation, with little loss of reliability. Since the system offers flexibility and several other advantages in addition, it constitutes, in our opinion, an attractive alternative to the manufactured identification kits currently available. This test method, described and evaluated below, is hereafter referred to as the multipoint inoculation plate (MIP) method.

MATERIALS AND METHODS

Microorganisms. The strains of bacteria and

yeasts tested were aerobic or facultative organisms isolated from clinical specimens received at the Bacteriological Laboratory at the Regional Hospital in Umeå, Sweden.

Replication equipment. The replication equipment was communicated by G. Bertani. The pin replicator (replicator A, Fig. 1A) was made by a local workshop and consists of a square acrylic plastic (Plexiglas) handle (70 by 70 by 15 mm) to which 25 stainless steel pins (40 by 3 mm) have been attached 13 mm apart. The tips of the pins must be level.

For testing of H_2S production and motility, we have developed a second replicator (replicator B, Fig. 1B), equipped with injection needles (50 by 0.8 mm) instead of steel pins and by which the microorganisms to be tested are inoculated deeply into the agar.

The commercially available autoclavable nylon microculture container (ELESA, Casella Pascoli, I-20100, Milan, Italy) measures 60 by 60 by 12 mm and is divided into 25 cubical compartments (volume, about

FIG. 1. Multipoint inoculation equipment. (A) Steel pin replicator; (B) needle replicator; (C) autoclavable microculture container; (D) disposable container.

1 cm³ each), one for each replicator pin (Fig. 1C). During autoclaving and subsequent handling, the container was stored in a glass petri dish whose lid did not touch the top of the culture container. Water condensing under the lid might otherwise have caused cross-contamination between the compartments of the container when in use. While this study was being finished, a thin-walled and inexpensive disposable plastic microculture container of similar size (Kemi-intressen, Sundbyberg, Sweden) became available in Sweden (Fig. 1D). This container can also be used for MIP testing.

The distance between the plated culture samples obtained with this equipment is an appropriate compromise between media economy and speedy inoculation on one hand and the risk of interference between too-large neighbor zones of reaction on the other.

Use of equipment. Before use, about 0.5 ml of sterile saline per compartment was dispensed into the culture container. When identifying gram-negative bacteria, tryptophan broth (see below) was used instead of saline. Parts of a colony of each strain to be tested were then transferred to individual compartments by using autoclaved toothpicks or a platinum loop, and a record of strain numbers and corresponding container and compartment (numbered 1 to 25) numbers was kept. (It is advisable to leave a compartment, e.g., number 25 [the lower-right corner], uninoculated to avoid later confusion concerning the identity of the spots of growth on the test plates.) The pins of the replicator were dipped into ethanol, flamed, and allowed to cool for a few seconds before the tool was ready for rapid inoculation of the test plates desired with strains present in the container. To avoid later flowing and mixing of the culture drops applied, we found it advisable to dry the plates (1 h at 37°C with their lids off) before use.

The strain number, results of the tests chosen, and final strain diagnosis were recorded on an appropriate data sheet (25 strains per sheet). Each batch of test medium was controlled by including standard strains in the set to be tested to ensure that the negative as well as the expected weakly and strongly positive reactions were demonstrated. If tests requiring anaerobic incubation were used (amino acid decarboxylases or oxidation/fermentation of glucose), a strain of *Pseudomonas aeruginosa* was included to check that satisfactory anaerobic conditions were achieved.

General cultivation conditions. Unless otherwise stated, the tests were incubated in air at 37°C and read 18 to 24 h after inoculation. When needed, anaerobic conditions were obtained by using GasPak jars or a glove box containing H₂ (10%), CO₂ (5%), and N₂ (85%).

By using dried test plates (see above) with a high concentration of agar (Oxoid agar no. 1, 3% [wt/vol]) problems due to swarming by *Proteus* were avoided. Thus, except for the motility test medium (see below), all commercially available test agars used had to be further solidified.

The media were dispensed into 9-cm-diameter plastic petri dishes. The results of all tests were easier to read if plates with extra-thick agar were used (e.g., 40 to 50 ml per plate), since the risk of too-wide reaction zones was then minimized and the optical contrast

between positive reactions and the negative background was enhanced.

Tests. For convenience, the review by Cowan and Steel (2) is used as a general reference for test media instead of the original references. When Cowan and Steel include alternative methods, the particular method here used is stated.

(i) **Esculin hydrolysis.** Blackening in and around the growth spots on regular esculin agar (2) indicated hydrolysis.

(ii) **Carbohydrate utilization.** Proteose peptone no. 3 (Difco) with carbohydrate (D-arabinose, D-cellobiose, dulcitol, D-galactose, D-glucose, inositol, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-ribose, D-sorbitol, D-sucrose, D-trehalose, or D-xylose [10 g/liter]) and bromothymol blue (50 mg/liter) added was used for staphylococci as well as for gram-negative bacteria to test carbohydrate utilization. Although not necessary, a doubled concentration of bromothymol blue facilitated reading of the tests. If anaerobic incubation was performed to facilitate differentiation between oxidation and very weak fermentation, NaNO₃ (425 mg/liter) had to be included in the test medium (see Results). For yeasts, the basal medium described by Martin and Schneidau (11) but with bromocresol purple as a pH indicator (33 mg/liter) was used. Utilization of a carbohydrate was demonstrated by heavy growth and a change in color of the pH indicator in and around the growth spot. Fermentation usually produced a sizable halo of indicator color change around each spot (Fig. 2).

(iii) **Citrate utilization.** Utilization of citrate as a carbon and energy source was indicated by visible growth and a shift in pH towards alkalinity (dark-blue halo) on Simmond citrate agar (Oxoid).

(iv) **Decarboxylases (arginine, lysine, and ornithine).** Moeller decarboxylase broth base (Baltimore Biological Laboratory [BBL]) was supplemented with the appropriate amino acid (10 g/liter) plus NaNO₃ (425 mg/liter), adjusted to pH 5.8 to 6.0, and solidified as described above. After inoculation, the plates were incubated anaerobically. The results were interpreted as described in Results.

(v) **Deoxyribonuclease.** Deoxyribonuclease agar (Oxoid) was used for gram-negative bacteria as well as for staphylococci, and after incubation the plates were flooded with toluidine blue (1 g/liter) or with hydrochloric acid (1 M) to obtain purple halos against a dark-blue background or zones of clearing, respectively, around deoxyribonuclease-positive spots of growth.

(vi) **Gelatin liquefaction.** Gelatin agar (2) was inoculated and developed by flooding it with the acid mercuric chloride solution of Frazier (2) after incubation. Distinct halos of clearing indicated hydrolysis of gelatin.

(vii) **Hippurate hydrolysis.** Hippurate hydrolysis by gram-negative bacteria was clearly demonstrated by growth and pink color on hippurate agar (2).

(viii) **Hydrogen sulfide production.** To test for hydrogen sulfide production, regular triple sugar iron (TSI) agar (2) must be used, e.g., that of Oxoid employed here (see Results). Extra-thick agar gave more distinct positive reactions. After inoculation with replicator A, replicator B was punched through the inoc-

ula and halfway through the agar layer. H₂S production was indicated by blackening in the depth of the agar, best observed from the bottom side of the plate (Fig. 3).

(ix) **Indole production.** To test for indole production, the broth and plates used were based on tryptone (Difco, 10 g/liter), and indole was detected by using Kovacs reagent (2).

(x) **Motility.** The motility test medium of Difco containing 0.5% agar could be used, but supplementation with beef extract (Difco, 3 g/liter) promoted growth and simplified reading of the results. Alternatively, 2,3,5-triphenyltetrazolium chloride could be added (0.05 g/liter) to improve visualization of bacterial growth. If strains of *Proteus* were present among the bacteria to be tested, their swarming had to be prevented by including chloral hydrate (1 g/liter [3]) in the test medium (see Results). The plates were inoculated using replicator B, which was punched halfway through the agar layer. Incubation for 40 h at 22°C was necessary for demonstration of motility in some strains in MIP as well as in tube testing.

(xi) **ONPG test.** For the *o*-nitrophenyl- β -D-galactopyranoside (ONPG) test, solidified ONPG broth (2) was used, and a yellow halo around a bacterial inoculum, due to the *o*-nitrophenol released, indicated a positive reaction.

(xii) **Phenylalanine deaminase.** To test for phenylalanine deaminase, after incubation phenylalanine agar (Oxoid) plates were flooded with ferric chloride (100 mg/ml), and the presence of phenylpyruvic acid resulted in rapid development of green color in and around the growth spots.

(xiii) **Urease.** Bright-red halos were produced by urease-positive strains growing on urea agar base (Oxoid) plus the amount of urea recommended. This MIP test had to be incubated at 22°C (see Results).

Chemicals. Chloral hydrate was kindly supplied by Ferrosan AB, Malmö, Sweden. ONPG was from E. Merck A.G., Darmstadt, West Germany, and 2,3,5-triphenyltetrazolium chloride was from BDH Chemicals Ltd., Poole, England.

RESULTS

MIP tests not requiring evaluation. Several conventional tests are normally performed on agar plates. Therefore, the corresponding MIP tests were carried out mainly to make sure that the growth spots created clearly visible zones of reaction and, above all, that the latter did not become too wide and interfere with reading of the adjoining tests. With the following tests, the results were so clear-cut that a closer evaluation was regarded as superfluous: esculin hydrolysis, deoxyribonuclease, gelatin liquefaction, and hippurate hydrolysis.

Evaluation of MIP tests. In the following,

TABLE 1. Comparison between conventional testing and MIP testing of biochemical and physiological activities of gram-negative bacteria

Activity	No. of tests compared	Conventional test ^a	Agreement (%)
Esculin hydrolysis		AP	EO ^b
Carbohydrate ^c utilization:			
Glucose	350	B	100
Lactose	350	B	100
Mannitol	350	B	100
Citrate utilization	350	AT	100
Decarboxylases:			
Arginine	100	B	100
Lysine	100	B	100
Ornithine	100	B	100
Deoxyribonuclease		AP	EO
Gelatin liquefaction		AP	EO
Hippurate hydrolysis		AP	EO
Hydrogen sulfide production	350	AT	98
Indole production	350	B	95-100 ^d
Motility in:			
Regular medium	350	AT	100 ^d
Regular medium + chloral hydrate	200	AT	100 ^d
ONPG (β -galactosidase)	350	B	98
Phenylalanine deaminase	350	B	100
Urease	350	B	100

^a Media: B, broth; AT, agar in test tube; AP, agar in petri dish (see also text).

^b EO, Evaluation omitted (see text).

^c For carbohydrates other than those listed (see text), evaluation was regarded as superfluous.

^d See text.

the application of a number of tests to MIP performance is described. The comparisons of MIP results with those of the corresponding conventional tests are summarized in Table 1 and refer to analyses of strains of gram-negative bacteria isolated from urine, wound, and chest infections and most often belonging to the family *Enterobacteriaceae*.

Carbohydrate utilization. The results of MIP fermentations of mannitol, lactose, and glucose by enteric bacteria were compared with those of tube tests, and 100% agreement was obtained (Table 1). A mannitol and a lactose plate are shown in Fig. 2. Since fermentation of all 17 carbohydrates tested (see Materials and Methods) appeared to be demonstrated equally well with the MIP method, further careful evaluations were omitted. It thus appears that fermentation testing of almost any carbohydrate can be applied to this method.

To avoid confusion between very slow fermentation and the weak acid reaction resulting from

FIG. 2. Carbohydrate fermentation plates inoculated with strains of enteric bacteria and incubated overnight. (Top) Lactose; (bottom) mannitol.

FIG. 3. TSI plate inoculated with strains of enteric bacteria and incubated overnight, viewed from the bottom side.

oxidation of a carbohydrate by certain obligate aerobic bacteria (e.g., pseudomonads), the fermentation plates could be incubated anaerobically. By using aerobically as well as anaerobically incubated plates, the MIP glucose test thus became equivalent to the classical oxidation/fermentation (OF) test of Hugh and Leifson (2).

Mannitol fermentation by *Staphylococcus aureus* and the fermentations included in the classification system for other staphylococci recently described by Kloos and Schleifer (glucose, galactose, lactose, maltose, mannitol, rhamnose, ribose, and trehalose [8]), as well as a large number of carbohydrate utilization tests used for identification of yeasts (1, 9, 10), all appeared to be equally suitable for MIP performance. Occasional yeast strains, however, grew very slowly and needed more than 48 h of incubation. In such situations, the halos of acidity created by the strongly positive strains could become confluent and obscure the reaction of the slow strain. The latter then had to be retested without any organisms inoculated into the neighbor compartments.

Citrate utilization. In the citrate utilization test, bacterial growth was slow and false negative results could occur. However, this risk was not greater in MIP tests than in regular agar slope tests, since a comparison showed 100% agreement between the two methods (Table 1).

Decarboxylases (arginine, lysine, and ornithine). In the decarboxylase tests, differences in appearance between MIP and tube tests were found. Some of the acid metabolites from the small amounts of glucose fermented apparently evaporated from the plates (e.g., CO₂). Thus, growth of enteric bacteria resulted in a higher pH value on plates than in the conventional petrolatum-covered broth. Decarboxylase-negative strains therefore did not create yellow halos of acidity in MIP tests. On the other hand, positive strains created violet halos against an indifferently colored background. The results agreed very well with those of Moeller tube tests (Table 1), provided that all degrees of violet were scored as positive. Furthermore, after incubation the MIP plates should not be left in air for more than 30 min before reading, since all negative tests eventually became weakly pseudo-positive in air, possibly due to an accelerated evaporation of CO₂ and volatile organic acids.

Another important difference between tube and MIP decarboxylase testing was that NaNO₃ had to be added to the MIP medium (see Materials and Methods). Otherwise, growth was very poor on MIP plates incubated during particularly successful anaerobiosis. Presumably,

NaNO₃ served as an additional electron acceptor necessary in the Moeller medium during very strict anaerobic agar surface conditions.

Hydrogen sulfide production. Initially, we hoped to be able to combine the H₂S test with any fermentation test desired. However, this turned out to be very difficult. For an unknown reason, all positive reactions were completely suppressed by bromothymol blue, and the results with various sugar-phenol red agars incubated aerobically or anaerobically were often different from those with TSI agar. Thus, bacterial formation of H₂S appeared to be variable and easily influenced by the nutritional and atmospheric environment of the cells. Since results with TSI agar presently are the basis of taxonomy in this matter, the evaluation of MIP H₂S tests was finally performed with TSI agar (Fig. 3). The agreement with tube tests was then good (Table 1). The halos of acidity (if formed) were due to fermentation of lactose or sucrose, whereas utilization of the low concentration of glucose present did not result in any indicator change. Thus, the MIP TSI test differed from the tube test in that fermentation of glucose could be demonstrated in the bottom of a tube test but not in MIP plates.

Indole production. Since indole is volatile and also diffuses rapidly in agar, it could not be demonstrated in or around the MIP growth spots on a tryptone plate. This plate, however, contained bacteria which were induced for tryptophanase and suitable for further analysis of this enzyme activity. By using replicator A, heavy inocula of such bacteria were transferred from the overnight plate to a second tryptone plate with only 1% agar. The replicator pins were punched through the agar layer so that crypts containing bacteria were formed. The second plate was incubated for 0.5 to 1 h before flooding with reagent. This plate then had to be read immediately, and any pink color developed in the crypts was scored as positive. The agreement with tube tests was acceptable (95%) among 350 consecutive gram-negative isolates tested. Due to the many indole-positive strains (e.g., *Escherichia coli*) analyzed, the tendency towards false-negative MIP results was here emphasized, resulting in 3.5% doubtful and 1.5% false-negative MIP tests (Table 1).

Our attempts to make this MIP test simpler and more sensitive have so far been unsuccessful. At present, we therefore routinely use the broth test performed in the culture container. The bacteria are suspended in tryptone broth instead of in saline, and after inoculation of the test plates the container is also placed at 37°C. After incubation overnight, a drop of reagent is added to each of its microculture compartments

by using a pipette or a pipettor (i.e., semiautomatic pipette). This performance of course yields results which are identical with those of tube tests. The carry-over of tryptone broth to the test plates (about 1.5 μ l per spot) does not influence the outcome of the other MIP tests. Since the indole reagent is strongly acidic and may be harmful to the polymer of the container, the latter should be rinsed soon after use. Alternatively, a disposable container can be used (see Materials and Methods.)

Motility. The MIP motility test is essentially the same as the conventional test, and with nonswarming bacteria the two also yielded identical results (Table 1). However, because of the low concentration of agar in the motility medium, strains of *Proteus* present among the bacteria to be tested could swarm and invade all tests run on the same plate. The problem was circumvented by including 1 mg of chloral hydrate per ml in the medium. This concentration of chloral hydrate was optimal and effectively prevented swarming without inhibiting motility in all 34 strains of *Proteus* tested. Similarly, no negative effect of 1 mg of chloral hydrate per ml on motility was noted among 94 strains of other motile gram-negative bacteria studied. Thus, 100% agreement between tube and MIP testing of motility was obtained, despite the presence of chloral hydrate in the latter case (Table 1).

ONPG test. Since the ONPG test is capable of visualizing very low β -galactosidase activities, bacteria that utilized lactose rapidly gave a visible reaction after only a few hours of incubation of MIP plates, whereas slow strains required overnight growth. By that time, however, the reaction zones of the most active strains had diffused over the whole plate. The MIP ONPG test was therefore practical only for isolates that appeared negative on lactose fermentation plates. However, since the ONPG test is routinely used mostly for further analysis of such gram-negative bacteria (e.g., from fecal specimens), it is fortunate that the MIP ONPG test turned out to be useful in this particular situation.

We have been satisfied with the MIP ONPG test in the feces analysis laboratory ever since the following routine was adopted. Clones that appear lactose negative on brilliant green agar (Oxoid) or on deoxycholate agar (BBL) are transferred to the container for further testing. The lactose-negative character of the isolates is first rechecked on a regular lactose fermentation plate (see above) incubated overnight. Thus, sizable amounts of bacteria induced for β -galactosidase (if present) are obtained. Using replicator A, heavy inocula of such cells are then transferred to the ONPG plate, and the test is

read after 2 to 4 h at 37°C. The results agree well with those of overnight ONPG broth tests (Table 1).

Phenylalanine deaminase activity and pigment production. After incubation, phenylalanine plates were first inspected for halos of color due to bacterial pigments, as this whitish plate nicely visualized diffusible pigments produced by *Pseudomonas aeruginosa* (blue-green or yellow), *P. fluorescens* (yellow), *P. maltophilia* (brown), etc. This plate was also suitable for detection of fluorescent pigments by using UV light. The plates were then flooded with reagent, and phenylpyruvic acid formed was indicated by additional distinctly green reactions. The halos never became too large, and the agreement with the Henriksen tube test (2) was 100% (Table 1).

Urease. After overnight incubation at 37°C, strains of *Proteus* usually showed strong reactions on MIP urea plates, and the bright-red halos produced were large and tended to obscure the results of the neighbor strains. The urea plates were therefore incubated at 22°C. At this temperature, however, bacteria with weaker urease activities (strains of *Klebsiella pneumoniae*, *Enterobacter*, etc.) gave rather small and less intensely colored halos. Therefore, all such discrete reactions had to be scored as positive. If this is kept in mind, the agreement with the urea broth test of Stuart et al. (2) is complete (Table 1). The urease test was the only MIP test which could not be incubated for more than 24 h. This was due to the confluence of strong reactions mentioned.

MIP method in routine use. After the above evaluation of MIP tests had been completed, additional studies were performed to ascertain whether the MIP method was practical in the routine laboratory. Even if various MIP tests appeared reliable when evaluated individually, the method might have been invalidated by cross-contamination, loss of order of strains, or other technical problems when in practical use.

The limited set of tube tests previously used in this laboratory for first-stage identification of gram-negative bacteria in urine specimens (TSI, mannitol-Durham tube, indole, ONPG, and Voges-Proskauer) was used as a reference in a preliminary practicality trial of a set of MIP tests (citrate, glucose, lactose, mannitol, H₂S, indole, and phenylalanine deaminase). The following 222 strains of gram-negative bacteria were tested: 102 *E. coli*, 49 *Klebsiella-Enterobacter*, 5 *Citrobacter*, 32 *Proteus mirabilis*, 4 *P. vulgaris*, 15 *Pseudomonas*, and 15 *Salmonella*. The two methods disagreed on 4 strains, which were subject to an extended series of tests. It turned out that 2 H₂S-negative, indole-positive

strains of *Citrobacter* and 2 Voges-Proskauer-negative, indole-positive strains of *Klebsiella* were identified as *E. coli* by the tube set because it lacked a citrate utilization test. Most important, however, was the fact that no technical problems were encountered when several MIP tests were inoculated from the same culture container. The latter has also been further controlled with regard to contamination during handling. In our experience, occasional contamination occurs as a result of failure in isolating the original clone in a pure state. Therefore, the risk of contamination is no greater in MIP than in tube testing. Such problems are, however, often visible in MIP growth spots, in contrast to tube and kit tests.

Recently, the MIP method was introduced as routine in this laboratory, and microbiologists and technicians have rapidly become familiar with the method and accepted it. Furthermore, we have found that MIP test plates yield unchanged results after 1 month of storage in plastic bags at 4°C. This circumstance will simplify media preparation routines when the MIP method is adopted for regular use.

DISCUSSION

Any system introduced to simplify laboratory testing and identification of microorganisms must offer a sufficient degree of reliability in comparison with accepted reference methods. A number of steadily improving manufactured multitest kits are now available for recognition of members of the family *Enterobacteriaceae* on the basis of their biochemical activities. Many evaluations of such kits have been performed in recent years, and the API, Enterotube, and also Minitek and R/B tests have become increasingly accepted. Although they require less labor than the classical tube tests, each of these systems also has disadvantages (see introduction). In very small laboratories, kits are particularly useful, since they are almost independent of media preparation facilities and still enable a rather high degree of diagnostic sophistication and accuracy. On the other hand, the microtiter multipoint method (see introduction) might become useful in very large laboratories, although the elaborate routines for dispensing media are a problem and require automation.

In our opinion, the MIP method here presented and evaluated can also offer a sufficient degree of accuracy at each level of diagnostic sophistication, although independent evaluations in other laboratories are of course desirable. The method is practical in small as well as in large laboratories and is also economical. Table 2 shows an approximate comparison of costs of prepared media in conventional tubes,

TABLE 2. Approximate material cost comparison between different *Enterobacteriaceae* identification methods

Method	Relative material cost/test ^a
Conventional tube testing	100
Enterotube	26
API	14
MIP	3

^a For the Enterotube and API tests, the current prices in Sweden were used. The calculations for the conventional and MIP methods were based on the prices charged by the National Bacteriological Laboratory, Stockholm, Sweden (and by most other Swedish laboratories as well) for prepared media of the types used (contained in tubes and plastic petri dishes, respectively). The conventional tube and MIP methods allow various combinations of more than one test per unit. To simplify comparison, the degree of combination used in the Enterotube method (11 tests in 8 units) has here been applied also to the conventional tube and MIP methods. For each of the methods, the material cost per test is given relative to that per conventional tube test (defined as 100). The costs during later stages of testing (inoculation and reading) are not included in the table.

MIP plates, and two manufactured kits available in Sweden (Minitek is temporarily withdrawn from the Swedish market due to "technical problems," and no price can be obtained at present). According to our calculations, the material cost per MIP test is about 3% of that per tube test and apparently also less than the cost per Enterotube and API test. In addition, the cost per isolate identified can be further cut when using MIP, since the set of tests used can be reduced and adjusted according to the type of specimen and the particular level of diagnostic refinement desired. With a rigid system, such as API, 20 tests are spent on each isolate. Furthermore, the MIP method requires little time for handling since 25 inoculations are performed in 1 manipulation.

In addition to versatility, speedy inoculation, and low cost, MIP also offers other advantages here briefly pointed out.

(i) MIP enables inspection of colony-like growth spots, so that "colony" appearance, pigmentation, poor growth (risk of false-negative results), and sometimes contamination are observed.

(ii) Reading MIP is easy. No color comparison cards are necessary, as the results generally look like those of the conventional tests. Reading is further simplified because many positive and negative reactions on the same plate are compared against a background of uninoculated test medium. Thus, controls are automatically performed and any significant decline in medium

quality will be detected as a deviation from the ordinary visual impression.

(iii) The age, quality, and composition of media and reagents are known and can be changed.

(iv) No precaution against carry-over of test media has to be taken during inoculation (cf. microtiter).

(v) Antibiotic susceptibility testing by the agar dilution method can easily be added to MIP identification.

(vi) The MIP method can be used also for other tasks, such as large-scale testing of microbial growth requirements, response to temperatures, pH, atmospheric conditions, phages, bacteriocins, and toxic agents (tellurite, KCN, azide, bile salts, disinfectants, etc. and the agents used for resistogram typing [4, 5]).

(vii) The MIP method can be applied to many species of bacteria in addition to members of *Enterobacteriaceae*, as well as to yeasts.

(viii) If desired, the strains tested by the MIP method can be saved without spending much extra effort or space. By sealing one of the MIP plates, the strains can be stored at 4°C for several weeks to be further analyzed in nosocomial or other follow-ups. The MIP data sheets then serve as a temporary strain record.

(ix) In parallel with identification and antibiotic susceptibility testing of gram-negative bacteria, efficient screening for the presence of conjugative R plasmids in the strains can be performed by using the MIP equipment (L. G. Burman and R. Östensson, Plasmid, in press).

The drawbacks with the MIP system should also be pointed out. One is that certain tests have so far resisted application to MIP (e.g., malonate, methyl red, Voges-Proskauer, and gas formation). Secondly, MIP is impractical if several days of incubation of tests are required. Chloral hydrate must then be added to all plates, since 3% agar is not an absolute guarantee against late swarming of *Proteus*. Furthermore, strong positive reactions on the urea plate might become confluent.

However, we believe that the MIP method could become a valuable tool in microbiological

work and that further improvements and applications of the method are feasible.

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LITERATURE CITED

1. Ahearn, D. G. 1970. Systematics of yeasts of medical interest. Proc. Internat. Symposium on Mycoses 205:64-70.
2. Cowan, S. T., and K. J. Steel. 1976. Manual for the identification of medical bacteria. Cambridge University Press, London.
3. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1973. Microbiology, 2nd ed., p. 772. Harper & Row, Publishers, New York.
4. Elek, S. D., and L. Higney. 1970. Resistogram typing—a new epidemiological tool: application to *Escherichia coli*. J. Med. Microbiol. 3:103-110.
5. Elek, S. D., and C. Moryson. 1973. Resistotyping of *Staphylococcus aureus*. J. Med. Microbiol. 7:237-249.
6. Fung, D. Y. C., and P. A. Hartman. 1975. Miniaturized microbiological techniques for rapid characterization of bacteria, p. 347-370. In C.-G. Hedén and T. Illéni (ed.), New approaches to the identification of microorganisms. John Wiley & Sons, Inc., New York.
7. Hartman, P. A. 1968. Miniaturized microbiological methods. Academic Press, New York.
8. Kloos, W. E., and K. H. Schleifer. 1975. Isolation and characterization of staphylococci from human skin. II. Descriptions of four new species. Int. J. Syst. Bacteriol. 25:62-70.
9. Lennette, E. H., E. H. Spaulding, and J. P. Truant (ed.). 1974. Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
10. Lodder, J. (ed.). 1970. The yeasts. A taxonomic study, 2nd ed. North-Holland Publishing Co., Amsterdam.
11. Martin, M. V., and J. D. Schneidau. 1970. A simple and reliable assimilation test for the identification of *Candida* species. Am. J. Clin. Pathol. 53:875-879.
12. Newsom, S. W. B. 1975. Easy, economic typing of enterobacteria, p. 435-443. In C.-G. Hedén and T. Illéni (ed.), New approaches to the identification of microorganisms. John Wiley & Sons, Inc., New York.
13. Sneath, P. H. A., and M. Stevens. 1967. A divided petri dish for use with multipoint inoculators. J. Appl. Bacteriol. 30:495-497.
14. Steers, E., E. L. Foltz, and B. S. Graves. 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Washington, D.C.) 9:307-311.
15. Washington, J. A., II. 1969. Antimicrobial susceptibility of *Enterobacteriaceae* and nonfermenting gram-negative bacilli. Mayo Clin. Proc. 44:811-824.