Clinical Laboratory Evaluation of a System Approach to the Recognition of Nonfermentative or Oxidase-Producing Gram-Negative, Rod-Shaped Bacteria

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The Oxi/ferm tube, an eight-compartment system suitable for the recognition of nonfermentative, gram-negative, rod-shaped bacteria, was evaluated in conjunction with the oxidase test in a clinical microbiology laboratory setting. A comparison between this system and the 17-test routine examination was performed with 265 cultures representing 21 species. The results obtained with the Roche systems were supplemented with simple additional tests in accordance with Computer Identification Data provided by the manufacturer. The Oxi/ferm tube approach worked satisfactorily in establishing the identity of the bacteria isolated from clinical specimens augmented with some representatives from culture collections. Reactions within the Oxi/ferm system were reproducible and comparable to laboratory prepared and controlled substrates.

The "system" or "kit" approach has become established in many clinical laboratories as the routine method for diagnosing members of the *Enterobacteriaceae* (5). It is only proper that this modality should be extended to the recognition of other microorganisms, since every clinical microbiology laboratory would be capable of categorizing a particular group of protista whenever they are isolated as medically or epidemiologically significant inhabitants of pathological specimens. The system approach obviates the search for required media and reagents, has the proper measure of uniformity, stability, and reproducibility for interlaboratory comparison, and provides guidance to selected tests crucial in confirming the identity of the isolated microorganism. The Oxi/ferm tube (Roche Diagnostics, Nutley, N.J.) is a system approach to the recognition of obligately and facultatively aerobic, gram-negative, rod-shaped bacteria which are being encountered with increasing frequency in the hospital setting. The comparison of the Roche system with the tests routinely employed in this laboratory is the subject of this report.

MATERIALS AND METHODS

Bacteria. The majority of the organisms were isolated as previously described from clinical specimens and environmental cultures that had been obtained during epidemiological investigations (7). Approximately 19% of the 265 bacteria studied were supplied through the courtesy of P. B. Smith, Center for Disease Control, Atlanta, Ga.

Oxi/ferm tube. This device is an adaptation of the Roche Enterotube to the classification of oxidative and/or nonfermentative, gram-negative, rod-shaped bacteria. Like the Enterotube, the Oxi/ferm tube consists of an assembly of eight substrates, each in its separate compartment. All compartments are traversed by a channel that contains the inoculum rod. This rod, secured by a cap on each end, serves to transfer a comparatively heavy inoculum which was obtained from a single isolated colony growing on any of the media employed for the isolation of bacteria from clinical specimens. Inoculation is achieved by withdrawing the rod through each compartment. The rod is then discarded, except for one small remnant which has been appropriately scored and is left in place to insure proper atmospheric conditions for fermentation, decarboxylation, and detection of nitrogen gas. The manufacturer advocates the inoculation of a Trypticase soy agar or other nutrient agar plate prior to the reinsertion of the segment. This plate is incubated at 35°C overnight followed by incubation at room temperature. It constitutes a purity check on the culture, permits the determination of pigment production, and establishes a need for additional nutrient requirements. Besides the substrate for glucose fermentation, the tube includes tests for arginine dihydrolase (ADH), nitrogen gas (N₂) production from nitrate, H₂S and indole production, oxidative acid production from glucose and xylose (XYL), urease (U) elaboration, and citrate (CIT) utilization. An oxidase test should be performed on the colony used for inoculation. The Oxi/ferm tube is then incubated for 24 h, the reactions are determined by comparison with a color chart accompanying each package, and the assembly is reincubated for an additional 24 h when all additional changes are noted. The manufacturer has

provided a pad and numerical system which permits the laboratorian to determine a four-digit identification (ID) number. A manual also provided by the manufacturer identifies the organism for the corresponding ID number. If more than one bacterium generates this ID value, supplemental tests are suggested for the determination of the proper genus or species diagnosis.

Supplemental tests. A total of 32 additional tests are suggested for the identification of the 47 species and/or genera included in the manufacturer's manual. However, only a few of these are suggested to distinguish between bacteria with the same ID value. The supplemental tests are: alkalization of xylose in the corresponding compartment of the Oxi/ferm tube after a 48-h incubation as manifested by a change in the indicator from a neutral green color to blue; ampicillin susceptibility performed by the standard disk diffusion technique (1); growth under anaerobic conditions; brown pigment production or discoloration manifested on a Trypticase soy agar plate; susceptibility to carbenicillin established by the standard diffusion technique with a 50- μ g disk; growth on cetrимide agar (10); β -hemolysis; Gram stain reaction after overnight growth in broth (*Achromobacter xylosoxidans* presents a banded or mottled appearance); colistin susceptibility as determined by the standard diffusion modality using a 10- μ g disk; fluorescence determination performed with long-wave ultraviolet light on Trypticase soy agar or cetrимide agar after 24 and 48 h; fructose oxidation with the production of acid; gelatin hydrolysis; lysine decarboxylase; maltose oxidation with acid production; mannitol oxidation with acid production; motility as determined by microscopic inspection of a 4- to 6-h-old broth culture that had been incubated at 25°C; methyl red production; nalidixic acid susceptibility as determined by a standard diffusion technique with a 30- μ g disk; growth after a 18- to 24-h incubation in nutrient broth; odor; o-nitrophenyl- β -D-galactopyranoside hydrolysis; penicillin susceptibility as determined by a standard diffusion methodology with a 10-U disk; polymyxin B susceptibility as determined by a standard diffusion technique with a 300-U disk; pyocyanin production; wet, confluent ("runny") colonial growth on agar; growth characteristics at 25°C; rapid U production in less than 24 h; growth on Salmonella-Shigella agar; streptomycin susceptibility as determined by a standard diffusion technique with a 10- μ g disk; tetracycline susceptibility as determined by a standard diffusion technique with a 30- μ g disk; *Vibrio cholerae* polyvalent antiserum; growth at 42°C. Since susceptibility and other additional studies are performed routinely immediately after bacteria are isolated, a portion of these test results are available at the time of Oxi/ferm tube inspection. Actually, 21 of the supplemental 32 test results can be ready at the time the Oxi/ferm results become available.

Routine tests. Gram-negative, rod-shaped bacteria, not members of *Enterobacteriaceae*, are categorized in this laboratory by a battery of 14 tests which have been previously described (4). These examinations include: the oxidation-fermentation (O-F) test using glucose as a substrate; fluoresceine

and pyocyanin production; growth and acid production on 10% lactose slant; lysine decarboxylase; ADH; growth on cetrимide agar; action on litmus milk; gelatinase production; action on nitrate; deoxyribonuclease production; starch hydrolysis; and catalase and oxidase production. The oxidation of XYL, indole production, and CIT and U elaborations were added to the battery of tests for this comparison study. All media and substrates were prepared at the Long Island Jewish/Hillside Medical Center in accordance with the methods of Vera and Dumoff (11).

System challenge. Thirty-eight of the bacteria, obtained from the Center for Disease Control, were used to challenge the ability of the Oxi/ferm tube system in identifying the nonfermentative, gram-negative rods. Although these organisms had been previously analyzed by both approaches, they were submitted as coded specimens and examined with both the Oxi/ferm tube and the tests cited in the instruction manual under the heading, Computer Identification Data.

RESULTS

The findings with the Oxi/ferm tube are summarized in Table 1. The results described represent reactions obtained after a 48-h incubation, since this time was required by 126 bacteria for the manifestation of their activities. The expected reactions were elicited after 24-h incubation with 139 (52%) of the cultures examined. The battery of routine tests required the same time for the manifestation of microbial reactions. Table 1 demonstrates the variation in the reaction sequences of certain of the bacteria. Such variability has been reported in the literature (2, 3, 10). Thus, *A. xylosoxidans* reacted variably on substrates for ADH, N₂, and XYL. The two varieties of *Acinetobacter calcoaceticus* reacted variably with U and CIT, *Aeromonas hydrophila* with CIT, *Alcaligenes faecalis* with U and CIT, *Pseudomonas aeruginosa* with N₂ and U, *Pseudomonas alcaligenes* and *Pseudomonas pseudoalcaligenes* with CIT. The finding that one of six *A. hydrophila* produced acid on XYL was confirmed by standard tests. The failure of one strain of *Alcaligenes denitrificans* to produce N₂ was demonstrated repeatedly with the Oxi/ferm tube. Proper reactions were obtained with the routine test regimen. One strain of *Alcaligenes odorans* failed to utilize CIT in the Oxi/ferm tube but did so in the routine examination. The correct reaction was elicited on repeat examination. The failure of one representative of *P. aeruginosa* to produce ADH even on several repeated examinations in the Oxi/ferm tube could not be corroborated with the routine tests. On the other hand, the inability of *Pseudomonas putida* to produce acid oxidatively on XYL was confirmed with the routine tests. The feeble U pro-

TABLE 1. *Oxi/ferm tube results*

Bacterium	No. studied	Test ^a										
		An	GLU	ADH	N ₂	H ₂ S	IND	XYL	GLU	U	CIT	OX
<i>Achromobacter xylosoxidans</i>	8	—		6/8— ^b	6/8+	—	—	5/8+	—	—	+	+
<i>Acinetobacter</i> var. <i>anitratus</i> ^c	40	—	—	—	—	—	—	+	+	10/40+	25/40+	—
<i>Acinetobacter</i> var. <i>lwoffi</i> ^c	42	—	—	—	—	—	—	—	—	38/42—	—	—
<i>Aeromonas hydrophila</i>	6	+	+	—	—	—	+	5/6—	+	—	4/6—	+
<i>Alcaligenes denitrificans</i>	3	—	—	3/7+	—	—	—	—	—	—	—	+
<i>Alcaligenes faecalis</i>	7	—	—	—	—	—	—	—	5/7—	5/7+	+	+
<i>Alcaligenes odorans</i>	9	—	—	—	—	—	—	—	—	8/9+	+	+
<i>Flavobacterium</i> sp.	5	—	—	—	—	—	+	—	+	—	—	+
<i>Moraxella osloensis</i>	3	—	—	—	—	—	—	—	—	—	—	+
<i>Pseudomonas aeruginosa</i>	65	—	64/65+	22/65+	—	—	—	+	+	57/65—	+	+
<i>Pseudomonas acedovorans</i>	6	—	—	—	—	—	—	—	—	—	+	+
<i>Pseudomonas alcaligenes</i>	7	—	—	—	—	—	—	—	—	6/7—	+	+
<i>Pseudomonas cepacia</i>	1	—	—	—	—	—	—	+	+	+	+	+
<i>Pseudomonas diminuta</i>	11	—	—	—	—	—	—	—	—	—	—	+
<i>Pseudomonas fluorescens</i>	10	—	—	+	—	—	—	+	9/10+	—	+	+
<i>Pseudomonas maltophilia</i>	6	—	—	—	—	—	—	—	—	—	—	—
<i>Pseudomonas pseudoalcaligenes</i>	3	—	—	—	—	—	—	—	—	—	2/3+	+
<i>Pseudomonas putida</i>	12	—	—	+	—	—	—	10/12+	+	2/12w	+	+
<i>Pseudomonas putrefaciens</i>	2	—	—	—	—	+	—	—	—	+	—	+
<i>Pseudomonas stutzeri</i>	13	—	—	—	7/13+	—	—	+	9/13+	—	+	12/13+
<i>Pseudomonas testosteronei</i>	6	—	—	—	—	—	—	—	—	—	—	+

^a Abbreviations: An, anaerobic; ADH, arginine dihydrolase; N₂, nitrogen gas; H₂S, hydrogen sulfide; IND, indole; XYL, xylose; GLU, glucose; U, urease; CIT, citrate; OX, oxidase; w, weak reaction. Symbols: —, negative; +, positive.

^b Number of strains reacting as indicated per number of bacteria studied.

^c Abbreviated form for *A. calcoaceticus* var. *anitratus* and *A. calcoaceticus* var. *lwoffi*, respectively.

duction by two representatives of this species was evident in both approaches. Not all *Pseudomonas fluorescens* and *Pseudomonas stutzeri* produced acid on glucose oxidatively in the Oxi/ferm tube, an observation that led to the variable designation for these reactions in the manufacturer's chart listing the reactions of this system with bacteria of established identity. This variation did not obscure the identification efforts.

One strain of *P. stutzeri* did not produce oxidase in 48 h, but subcultures reacted with 0.5% aqueous solution of tetramethyl-*p*-phenylenediaminedihydrochloride after 3 to 4 days. The results of the routine procedures were in agreement with published reactions (2, 3, 10). The few discrepancies between the reactions of the Oxi/ferm tube and corresponding routine tests consisted of the aforementioned failure of *A. denitrificans* to produce N₂ and ADH produc-

tion with one strain of *P. aeruginosa*. In addition, CIT utilization in the Oxi/ferm tube was not confirmed with routine methods for two strains of *A. calcoaceticus* var. *lwoffii*.

The Roche system misidentified nine of the bacteria on first trial. Three strains of *A. xyloxydans* were among this group. These cultures failed to produce acid from XYL; two did not elaborate N_2 and ADH. One strain reacted correctly with XYL acid and ADH production on reexamination with the Oxi/ferm tube. The routine test regimen suggests that the remaining cultures belonged to group IIIa in which the bacterial action is limited to nitratase, an activity not discerned by the Oxi/ferm tube. Correct identification of these bacteria by the routine tests required additional examinations, especially staining of flagella (9). Two bacteria designated as *A. faecalis* were misidentified by the Oxi/ferm tube. These bacteria utilized CIT and U. On the basis of these reactions, Roche's Computer Identification Data suggested three additional tests: (i) the determination of a peculiar "sweet-apple" odor, (ii) the presence of wet, confluent growth, and (iii) growth in nutrient broth within 24 h. None of the four bacteria differentiated by these three supplemental tests was *A. faecalis*. Although the reaction chart which accompanies the Oxi/ferm tube lists reactions on CIT and U as variable for *A. faecalis*, Tatum et al. (10) define the genus *Alcaligenes* as U negative and non-saccharolytic to differentiate it from *Bordetella bronchiseptica* and the genus *Achromobacter*. Since the routine tests confirmed the Oxi/ferm findings, these bacteria cannot be considered *A. faecalis* by this definition. Unfortunately, the basis for listing *Alcaligenes* reactions as variable or positive for CIT and U in the Oxi/ferm reaction chart is not provided. A culture of *A. odorans* was identified by the Roche system as *A. faecalis* on the basis of its lack of CIT utilization; a repeated examination with the Oxi/ferm tube led to proper reactions for identification. The Oxi/ferm tube failed repeatedly to recognize a culture of *A. denitrificans* because it did not reduce nitrate to gas within the 48 h specified for the reactions. An *A. hydrophila* could not be identified by the computer identification system, since it generated an ID value that was not included in the data manual. This bacterium produced acid from XYL in both systems, although the 113 strains, the reactions of which form the basis for the authoritative chapter by Ewing and Hugh (2), did not attack this carbohydrate. It must be pointed out that the reactions elicited by this bacterium in the Oxi/ferm tube made the diagnosis of *A. hydrophila* a very likely one. Failure to identify in this in-

stance was the result of the computer data base. A *P. stutzeri* was initially misidentified as *P. fluorescens* primarily because of the failure of a supplemental test. Thus, the nine initial misidentifications were reduced to six by repeated examinations, which were diminished to five actual failures when one takes into account the XYL-utilizing *Aeromonas*.

No supplemental tests were required for the identification of 42 of the 265 bacteria in this study. The remaining 223 required one to eight supplemental tests.

All bacteria were examined in duplicate with both test systems. Reproducibility was very good; reaction discrepancies were encountered in 37 instances in the 5,300 opportunities provided. The duplicate routine tests disagreed 42 times during the performance of 9,010 individual examinations.

DISCUSSION

The kit or system approach has broadened the ability of many clinical laboratories to recognize and properly characterize members of *Enterobacteriaceae* (5). This study indicates that the Oxi/ferm tube has extended this capability to the nonfermentative, gram-negative rods. This system allows each and every clinical laboratory to categorize these organisms within 24 to 48 h and to recognize them, most often at the species level, expeditiously.

The Oxi/ferm tube is one answer for a dire need that has prevented adequate laboratory recognition of these clinically significant bacteria during the past decade. In that time, representatives of this large group of protista have been isolated with increasing frequency from the specimens of patients (4). Although the ubiquity of these microorganisms in nature cannot be denied, their involvement in human disease, especially within hospitals, has only been recently observed and reflects the changing ecology resulting from advances in medical practices (6). Their clinical relevance when repeatedly recovered from blood, postoperative wounds, spinal fluid, transtracheal aspirates, and urine specimens cannot be denied. At the same time, it is not acceptable to merely lump them into major groups. These bacteria possess differing antibiotic profiles; many survive in moisture reservoirs frequently required by modern patient support equipment, and all may participate in local "cluster" epidemics. The protection of susceptible hosts requires identification to levels that are readily attained by the use of the Oxi/ferm tube and its simple supplemental tests.

Of course, the nature of these bacteria, i.e.,

their somewhat slower growth and carbohydrate metabolism as compared to *Enterobacteriaceae*, extends the time required for their identification. The supplemental tests are very simple and require materials that are readily available in clinical microbiology laboratories. Actually, many of the supplemental tests are performed simultaneously with the Oxi/ferm tube inoculum or require only a few hours for results. This quasimodular approach has obvious advantages. One can expect that additional substrates to categorize these organisms may become available as indicated by the recent studies of Otto and Pickett (8). These advances may drastically alter the spectrum of supplemental tests while preserving the usefulness of the Oxi/ferm tube.

The reaction chart accompanying the Oxi/ferm tube suggests that this system will provide guidance in identifying 33 species, 2 genera, 2 biotypes, and 12 groups, a total of 47 categories; this study dealt with only 21. However, these 21 representatives encompass bacteria that are isolated in the clinical laboratory with some degree of frequency. Although the capability to direct the laboratorian toward the identification of rare organisms is an advantage of this system, it was our intention to evaluate it for its performance in the ordinary situation. The type and numbers of the less commonly encountered representatives included in this study do not reflect the frequency of their isolation from clinical specimens. The purpose of the system or kit approach is to enable every clinical microbiology laboratory to report significant organisms expeditiously. Since the Oxi/ferm tube provides intimations of identity in 24 to 48 h, it fulfills this basic requirement. Since it also provides an excellent guide to the judicious use of simple supplemental tests, the proper identification of nonfermentative, gram-negative rods is within the

capability of all clinical microbiology laboratories.

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