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Clinical Evaluation of the Vitek ANI Card for Identification of Anaerobic Bacteria

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An evaluation of the Vitek Anaerobe Identification (ANI) card was performed with 341 bacterial isolates, including 313 clinical isolates and 28 stock strains of anaerobic microorganisms. Identifications obtained with the ANI card were compared with those determined by conventional methods. The card identified 73.2% of 149 anaerobic gram-negative bacilli, 63.6% of 44 *Clostridium* spp., 65.8% of 38 anaerobic nonsporeforming gram-positive bacilli, and 69.1% of 110 anaerobic cocci, with no further testing required. When genus-level identifications were included, 83.9% of the anaerobic gram-negative bacilli, 70.5% of *Clostridium* spp., 73.7% of the anaerobic nonsporeforming gram-positive bacilli, and 73.6% of the anaerobic cocci were identified. Nineteen isolates (5.6%) produced identifications of good confidence but marginal separation or questionable biotype, in which the correct identification was listed with one or two other possible choices and extra tests were required and suggested. A total of 28 (8.2%) were not identified and 29 isolates (8.5%) were misidentified by the ANI card. Among the commonly isolated clinically significant anaerobes, the ANI card identified 100% of 55 *Bacteroides fragilis* and 100% of 8 *Clostridium perfringens*. Use of supplemental tests and expansion of the data base to include additional strains of organisms that are difficult to separate even with conventional methods may improve the accuracy of the ANI card as a method for identification of anaerobic bacteria in the clinical laboratory.

Methods for identifying anaerobic bacteria have traditionally involved both time-consuming biochemical testing under strict anaerobic conditions and gas-liquid chromatographic analyses of short-chained fatty acid metabolites of glucose fermentation (12). These conventional methods, as well as some of the packaged commercial identification kits, require growth of the organisms and need 24 to 48 h or more of anaerobic incubation before test results can be read and interpreted (3).

In recent years, several systems have become commercially available or have been adapted for rapid identification of clinically significant anaerobic bacteria without the requirement for anaerobic incubation. These systems are all based on the detection of preformed bacterial enzymes through their action on modified conventional substrates or novel chromogenic enzyme substrates. Initially, these techniques involved the adaptation of readily available products to anaerobe identification and included use of Patho-Tec strips (General Diagnostics, Morris Plains, N.J.) (23) and the API ZYM system (Analytab Products, Plainview, N.Y.) (2, 11, 17–21, 24, 25, 27, 28). More recently, kit systems specifically designed for identification of anaerobic bacteria have become available and include the RapID-ANA (Innovative Diagnostic Systems Inc., Atlanta, Ga.) (1, 4–6, 8–10, 14–16, 22) and the AN-Ident (Analytab Products) (5, 6, 22, 26, 27) systems.

The Anaerobe Identification (ANI) card (Vitek Systems, Hazelwood, Mo.) is a qualitative micromethod that uses modified conventional and chromogenic enzyme substrate tests for identifying 76 species of anaerobic bacteria. This card, which was developed for Vitek by Innovative Diagnostic Systems, uses 12 of the tests currently included in the manual RapID-ANA system, plus 16 additional substrates. The purpose of this study was to evaluate the accuracy of the Vitek ANI card for the identification of clinically significant anaerobic bacteria. Results obtained with the ANI card were compared with those obtained by the methods described by the Virginia Polytechnic Institute and State University, Blacksburg (12, 13).

MATERIALS AND METHODS

ANI card. The ANI card is a molded plastic card containing 30 wells; 28 of these contain substrates for biochemical reaction determinations. The card contains 20 chromogenic substrate tests and 8 modified conventional tests. Chromogenic substrates include 12 *p*-nitrophenyl carbohydrate derivatives, 6 *p*-nitroanilide derivatives of amino acids, *p*-nitrophenyl phosphate, and *p*-nitrophenyl phosphatidylcholine. These substrates detect specific bacterial glycosidases, aminopeptidases, phosphatase, and esterase, respectively. Hydrolysis of these colorless compounds by the appropriate enzyme yields a yellow nitrophenol or nitroaniline product. Modified conventional carbohydrate fermentation tests include acid production from glucose, trehalose, arabinose, raffinose, and xylose. Triphenyl tetrazolium reduction, rapid arginine dihydrolase, and urease complete the 28-test battery.

To inoculate the card, a suspension of a pure culture equivalent to or greater than a no. 3 McFarland turbidity standard is prepared in sterile saline from 24- to 48-h growth on an anaerobic blood agar plate. The card is inoculated automatically by the Vitek system filling module as for the other Vitek identification and susceptibility test cards, but it is incubated for 4 h at 35°C in an air incubator instead of in the Vitek reader-incubator module. Since the ANI test reactions occur in the red-yellow part of the spectrum, the cards cannot be read in the Vitek reader-incubator module, which can only detect colors in the blue-green range. Therefore, the cards are read manually with the Vitek card reader, which allows magnified visualization of card reactions under

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incident light against a reflective background. A comparator template showing the colors of positive tests along the appropriate test wells is inserted into the reader to facilitate test interpretation. Test results are entered into the Vitek computer module, along with the Gram-stain reaction, bacterial morphology, and spot indole results. Reaction results are indicated as positive (+), negative (−), or indeterminate (?).

The ANI computer program provides an organism identification or several possible identifications, along with identification confidence levels and probabilities, comments, and any contraindicated test results. The program identifies a major discrepancy when a negative biochemical test result with ≥90% positive probability or a positive biochemical test result with ≤10% positive probability occurs. A minor biochemical discrepancy is noted when a positive result with 11 to 25% positive probability or a negative test result with 75 to 89% positive probability occurs. Confidence levels for identifications are expressed as (i) excellent, very good, or acceptable (organism biopatterns are either typical, have only minor biochemical discrepancies, or generally have no more than one major biochemical discrepancy that differs from the typical biopattern of the first-choice organism); (ii) good confidence but marginal separation (GCMS; typical organism biopatterns but insufficient separation of the two or three listed species does not permit definitive identification); (iii) questionable biopattern (QB; organism biopattern resembles two species in the data base, but the probability that the isolate may belong to a third taxon not listed by the program is too great to provide an unqualified identification); (iv) unidentified organism (biochemical test pattern does not resemble any organism in the data base enough to produce an identification). In cases in which the ANI card test results are not discriminatory enough to provide a species identification, a genus-level identification is provided, with a list of suggested species and conventional tests for species resolution.

Taxa contained in the ANI data base include 20 *Bacteroides* spp., 4 *Fusobacterium* spp., and *Capnocytophaga* spp. *Clostridium clostridiiforme* and *Clostridium ramosum* are programmed in the data base as both gram-positive and gram-negative bacilli. The data base includes 20 *Clostridium* spp., 6 *Actinomyces* spp., 3 *Eubacterium* spp., 7 *Lactobacillus* spp., 2 *Propionibacterium* spp., and *Bifidobacterium* spp. No attempt is made to differentiate among the bifidobacteria. Seven *Peptostreptococcus* spp., three *Streptococcus* spp., *Staphylococcus saccharolyticus*, and *Veillonella parvula* complete the data base.

Bacterial strains. The 341 organisms evaluated in this study were both clinical isolates (313 strains) and stock strains (28 strains). Clinical isolates were obtained from specimens submitted to the University of Illinois Hospital Anaerobic Bacteriology Laboratory and represented members of the genera *Actinomyces*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptostreptococcus*, *Propionibacterium*, *Streptococcus*, *Staphylococcus*, and *Veillonella*. Stock organisms were American Type Culture Collection (Rockville, Md.) strains maintained as quality control organisms in the laboratory. Stock organisms were subcultured on agar media at least three times before testing with the ANI card and conventional laboratory procedures.

Conventional identification procedures included Gram stain, aerotolerance, growth on selective and differential media (e.g., kanamycin-vancomycin-laked blood agar, *Bacteroides* bile esculin agar, cycloserine-cefoxitin-fructose agar, egg yolk agar, and peptone-yeast extract-glucose broth with bile), and gas-liquid chromatography. Biochemical tests and carbohydrate fermentation reactions were performed with prereduced, anaerobically sterilized media (Carr-Scarborough Microbiologicals, Stone Mountain, Ga.) by methods described in the Virginia Polytechnic Institute anaerobic bacteriology manual (12), in the latest edition of *Bergey's Manual* (13), and in unpublished Virginia Polytechnic Institute materials (Clinical Anaerobic Bacteriology Course, 11 to 22 August 1986). Identifications with both conventional tests and the ANI card were performed in a blinded fashion.

RESULTS

Table 1 shows the ANI card results for the anaerobic gram-negative bacilli. Of the 99 *Bacteroides fragilis* group isolates tested, the ANI card correctly identified 82 (82.8%); all 55 *B. fragilis* isolates were identified correctly. Three *B. fragilis* group isolates produced GCMS or QB biotypes. The correct identifications were among the choices listed by the ANI program for two of the isolates. Five isolates (two *Bacteroides thetaiotaomicron*, two *Bacteroides vulgatus*, and one *Bacteroides uniformis*) were not identified. Nine *B. fragilis* group organisms were misidentified by the ANI card; eight of these were *Bacteroides ovatus* isolates that were called *B. thetaiotaomicron*. A single *B. uniformis* strain was also misidentified as *B. thetaiotaomicron*. Of the 19 *Bacteroides melaninogenicus* group organisms, 11 (57.9%) were correctly identified (Table 1). An additional three strains (15.8%) were identified to genus level only, and three isolates were not identified by the card. One *B. melaninogenicus* strain produced a GCMS identification, and a second was misidentified as *Bacteroides oralis*. Among 20 other *Bacteroides* spp., 13 (65.0%) were identified, with an additional 5 strains (25.0%) being identified to genus level only. One *Bacteroides bivius* strain was misidentified as *Fusobacterium varium*, and the single *B. oralis* strain was called *Bacteroides buccae* by the ANI card. All eight *Fusobacterium nucleatum* isolates were identified to genus level only, whereas the three other strains tested were correctly identified to the species level. Single isolates of *Bacteroides zoogleoformans* and *Fusobacterium gonidiaformans*, which are not included in the ANI data base, were called an unidentified organism and genus group *Fusobacterium* spp., respectively, by the ANI computer program. These were the only two organisms tested that were not in the ANI data base and, as such, were not included in the statistical evaluation. Of the 149 anaerobic gram-negative bacilli tested, the ANI card identified 109 (73.2%) to species and an additional 16 (10.7%) to genus level, with 12 strains (8.1%) being misidentified.

Table 2 presents ANI card results for sporeforming and nonsporeforming anaerobic gram-positive bacilli. Seven (63.6%) of 11 *Clostridium difficile* isolates and all *Clostridium perfringens* isolates were correctly identified. Four *C. difficile* isolates produced GCMS biotypes, with *C. difficile* being among the three possible identifications. GCMS or QB or unidentified organism identifications were obtained with *Clostridium bifermentans* (two strains), *Clostridium sporogenes* (two strains), *Clostridium sordellii* (one strain), *C. clostridiiforme* (one strain), and *C. ramosum* (one strain). Both of the *Clostridium innocuum* strains tested were misidentified as *Clostridium tetani*. In all, 28 (63.6%) and 3 (6.8%) of the *Clostridium* species were identified to species and genus levels, respectively.

Among the 38 nonsporeforming gram-positive bacilli tested, 25 (65.8%) were correctly identified to species, with

TABLE 1. Identification of anaerobic gram-negative bacilli with the ANI card

Organism	No. (%) of isolates					
	Tested	Identified to species[a]	Identified to genus[b]	Identified GCMS or QB[c]	Unidentified[d]	Misidentified
Bacteroides fragilis group	99	82 (82.8)	0	3 (3.0)	5 (5.1)	9 (9.1)
B. fragilis	55	55 (100)	0	0	0	0
B. thetaiotaomicron	19	16 (84.2)	0	1 (5.3)	2 (10.5)	0
B. ovatus	12	3 (25.0)	0	1 (8.3)	0	8 (66.7)
B. vulgatus	7	5 (71.4)	0	0	2 (28.6)	0
B. uniformis	4	1 (25.0)	0	1 (25.0)	1 (25.0)	1 (25.0)
B. distasonis	2	2 (100)	0	0	0	0
Bacteroides melaninogenicus group	19	11 (58.0)	3 (15.8)	1 (5.2)	3 (15.8)	1 (5.2)
B. intermedius	7	6 (85.7)	0	0	1 (14.3)	0
B. melaninogenicus	6	1 (16.7)	1 (16.7)	1 (16.7)	2 (33.2)	1 (16.7)
B. asaccharolyticus	4	2 (50.0)	2 (50.0)	0	0	0
B. corporis	1	1 (100)	0	0	0	0
B. gingivalis	1	1 (100)	0	0	0	0
Other *Bacteroides* spp.	20	13 (65.0)	5 (25.0)	0	0	2 (10.0)
B. bivius	9	5 (55.6)	3 (33.3)	0	0	1 (11.1)
B. capillosus	4	3 (75.0)	1 (25.0)	0	0	0
B. disiens	3	2 (66.7)	1 (33.3)	0	0	0
B. ureolyticus	2	2 (100)	0	0	0	0
B. oralis	1	0	0	0	0	1 (100)
B. buccae	1	1 (100)	0	0	0	0
Fusobacterium spp.	11	3 (27.3)	8 (72.7)	0	0	0
F. nucleatum	8	0	8 (100)	0	0	0
F. mortiferum	2	2 (100)	0	0	0	0
F. necrophorum	1	1 (100)	0	0	0	0

[a] Excellent, very good, or acceptable confidence level species identification.
[b] Supplemental tests required for resolution of species identification.
[c] Correct identification may or may not be among the choices listed.
[d] Biopattern not represented in the data base.

TABLE 2. Identification of anaerobic sporeforming and nonsporeforming gram-positive bacilli with the ANI card[a]

Organism	No. (%) of isolates					
	Tested	Identified to species	Identified to genus	Identified GCMS or QB	Unidentified	Misidentified
Clostridium spp.	44	28 (63.7)	3 (6.8)	6 (13.6)	5 (11.4)	2 (4.5)
C. difficile	11	7 (63.6)	0	4 (36.4)	0	0
C. perfringens	8	8 (100)	0	0	0	0
C. bifermentans	5	3 (60.0)	0	1 (20.0)	1 (20.0)	0
C. sporogenes	4	1 (25.0)	1 (25.0)	1 (25.0)	1 (25.0)	0
C. sordellii	3	2 (66.7)	0	0	1 (33.3)	0
C. histolyticum	3	3 (100)	0	0	0	0
C. septicum	2	1 (50.0)	1 (50.0)	0	0	0
C. clostridiiforme	2	1 (50.0)	0	0	1 (50.0)	0
C. cadaveris	2	2 (100)	0	0	0	0
C. innocuum	2	0	0	0	0	2 (100)
C. ramosum	1	0	0	0	1 (100)	0
C. subterminale	1	0	1 (100)	0	0	0
Nonsporeforming spp.	38	25 (65.8)	3 (7.9)	4 (10.5)	3 (7.9)	3 (7.9)
Actinomyces odontolyticus	3	0	1 (33.3)	0	1 (33.3)	1 (33.3)
A. viscosus	2	2 (100)	0	0	0	0
A. israelii	1	1 (100)	0	0	0	0
Propionibacterium acnes	18	16 (88.8)	0	2 (11.2)	0	0
Eubacterium lentum	6	2 (33.3)	0	1 (16.7)	1 (16.7)	2 (33.3)
Lactobacillus spp.	4	1 (25.0)	2 (50.0)	0	1 (25.0)	0
Bifidobacterium spp.	4	3 (75.0)	0	1 (25.0)	0	0

[a] See Materials and Methods and footnotes of Table 1 for explanation of column headings.

TABLE 3. Identification of anaerobic gram-positive and gram-negative cocci with the ANI card[a]

Organism	No. (%) of isolates					
	Tested	Identified to species	Identified to genus	Identified GCMS or QB	Unidentified	Misidentified
Peptostreptococcus spp.	58	44 (75.9)	5 (8.6)	2 (3.4)	5 (8.6)	2 (3.5)
P. asaccharolyticus	15	14 (93.3)	0	0	1 (6.7)	0
P. magnus	14	12 (85.8)	0	1 (7.1)	1 (7.1)	0
P. micros	13	8 (61.5)	2 (15.4)	0	2 (15.4)	1 (7.7)
P. anaerobius	9	8 (88.9)	1 (11.1)	0	0	0
P. prevotii	7	2 (28.6)	2 (28.6)	1 (14.3)	1 (14.3)	1 (14.3)
Streptococcus spp.	40	20 (50.0)	0	3 (7.5)	7 (17.5)	10 (25.0)
S. intermedius	36	19 (52.8)	0	3 (8.3)	5 (13.9)	9 (25.0)
S. morbillorum	2	0	0	0	2 (100)	0
S. constellatus	2	1 (50.0)	0	0	0	1 (50.0)
Veillonella parvula	11	11 (100)	0	0	0	0
Staphylococcus saccharolyticus	1	1 (100)	0	0	0	0

[a] See Materials and Methods and footnotes of Table 1 for explanation of column headings.

an additional 3 strains (7.9%) being identified to the genus level (Table 2). Four organisms produced GCMS or QB identifications, and in one case (*Eubacterium lentum*), the correct organism identification was not listed by the computer program as a possible choice. One *Actinomyces odontolyticus* and two *E. lentum* strains were misidentified as *Propionibacterium acnes* and *Clostridium hastiforme*, respectively.

Table 3 summarizes the ANI card results for the anaerobic cocci. A total of 44 (75.9%), 20 (50%), and 11 (100%) of the peptostreptococci, streptococci, and *V. parvula* were correctly identified to species, respectively. Five peptostreptococci were identified to genus level, and another five strains were not identified. Of the two isolates producing GCMS or QB identifications, the correct identification was among the choices for one of them. One *Peptostreptococcus micros* was misidentified as *Peptostreptococcus anaerobius* and one *Peptostreptococcus prevotii* was called *Peptostreptococcus magnus* by the ANI computer program. Of the streptococci included in the data base, 20 (50%) were correctly identified and 10 (25%) were misidentified. Of the 10 misidentifications, 9 occurred with *Streptococcus intermedius* strains that were called *Streptococcus constellatus* by the card; the other was an *S. constellatus* that was misidentified as *P. micros*. The single *Staphylococcus saccharolyticus* strain was correctly identified.

Of the 341 isolates tested in this evaluation, 28 (8.2%) were stock strains and included several species that were also represented among the clinical isolates. No differences in biochemical reactivity with the ANI card or in the frequencies of correct or incorrect identifications were noted between the clinical isolates and the stock strains examined in the study.

DISCUSSION

In this study, the Vitek ANI card was evaluated for its ability to identify a wide variety of anaerobic bacteria recovered in the clinical laboratory. After familiarization with the types of color reactions produced by organisms on the various substrates, the interpretation of most of the tests on the card was not difficult. The yellow colors resulting from hydrolysis of the *p*-nitrophenyl and *p*-nitroanilide substrates were generally clear-cut, although the reactions for the aminopeptidase substrates were usually stronger than those for the glycosidase substrates. Positive carbohydrate fermentation reactions were yellow, with red or orange colors considered negative, and both tetrazolium reduction and urease tests were easily interpreted. Equivocal reactions were rarely encountered. When such results were entered, the ANI computer program excluded these tests from consideration in the identification. The computer program also afforded the opportunity to rapidly check whether positive or negative interpretations of individual tests changed the identification(s) provided. In many cases, exclusion of equivocal test results did not make a difference in the computer-assisted identification.

All data in this study were based on results obtained with the Vitek ANI software version R1.02. After completion of the study, it was learned that Vitek had made data base modifications involving six species. These species were *B. bivius*, *Bacteroides disiens*, *C. difficile*, *C. perfringens*, *C. ramosum*, and *C. sordellii*. The ANI card results obtained for the 35 strains belonging to these species were rerun on the updated ANI software version, R3.01. The new program resulted in no significant changes in the results. Two *B. bivius* strains that produced GCMS identifications with the previous software were correctly identified, whereas two other *B. bivius* strains that were correctly identified originally resulted in GCMS identifications with the new program. Consequently, the overall performance of the ANI card in the present study was not altered by the changes incorporated into the new ANI software program.

The two currently available rapid systems for identification of anaerobic bacteria, the RapID-ANA and the AN-Ident, have been evaluated by several investigators and have demonstrated widely varying abilities for identifying anaerobic bacteria. In these various studies, 59 to 93% of anaerobic gram-negative bacilli, 67 to 100% of clostridia, 50 to 100% of nonsporeforming gram-positive bacilli, and 78 to 96% of anaerobic cocci were identified by these rapid systems when compared with various conventional methods (1, 4–6, 8–10, 14–16, 22, 26, 27). The wide variations in the performance of the RapID-ANA and the AN-Ident in these evaluations are related to the numbers, types, and sources (e.g., stock strains, human clinical isolates, and veterinary strains) of organisms tested, whether additional tests suggested by computer-generated code books of the systems were included as a part of the identification procedure, and

whether genus-level identifications were considered correct identifications. In the present evaluation, the ANI card correctly identified 109 (73.2%) of 149 anaerobic gram-negative bacilli, 28 (63.6%) of 44 *Clostridium* spp., 25 (65.8%) of 38 anaerobic nonsporeforming gram-positive bacilli, and 76 (69.1%) of 110 anaerobic cocci. When genus-level identifications are included, 83.9% of the gram-negative bacilli, 70.5% of the clostridia, 73.7% of the nonsporeforming gram-positive bacilli, and 73.6% of the anaerobic cocci were identified. In addition, 19 (5.6%) of the 341 isolates tested produced GCMS or QB identifications; in most cases, the correct identification was among the two or three choices listed by the computer program.

For GCMS and genus-group identifications, the ANI software program is designed to aid the user so that a species identification may be expeditiously obtained. When the biochemical pattern of ANI card reactions results in a GCMS or a genus-group identification, the ANI program lists those conventional tests and the corresponding reactions for the organism choices that may be used for separation. For example, in a GCMS identification listing *B. melaninogenicus* (66%) and *B. bivius* (34%), the computer-assisted report lists the production of black pigment and the hydrolysis of esculin as tests that can be used to make a species identification. Since the ANI card is unable to identify *F. nucleatum* to species level, the program suggests examination of a Gram stain and a lipase test for confirmation of this species.

Of 12 *B. ovatus* strains, 8 were misidentified as *B. thetaiotaomicron* by the ANI card. These two species are difficult to separate with conventional methods as well. According to the Virginia Polytechnic Institute manual (12), most *B. ovatus* isolates utilize trehalose and salicin, whereas *B. thetaiotaomicron* produces variable reactions with these carbohydrates. Therefore, positive trehalose or salicin fermentation reactions may or may not be helpful in discriminating between the two species. The catalase reaction is also variable for these species (12). Fermentation of xylan (7), the discriminatory test used in this study, arginine and histidine aminopeptidase activities in the AN-Ident system, and arginine aminopeptidase activity in the RapID-ANA system (22) are helpful in distinguishing these two species. Incorporation of xylan or chromogenic substrates for detection of arginine or histidine aminopeptidase into the ANI card may improve performance with these species.

Of the 36 *S. intermedius* strains, 9 were misidentified as *S. constellatus* by the ANI card. This was primarily because of false-negative or equivocal reactions in the ANI β-galactosidase test well. As with the organism pair discussed above, *S. intermedius* and *S. constellatus* are also difficult to distinguish by conventional methods; in the present study, acid production from lactose and raffinose and a conventional *o*-nitrophenyl-β-D-galactopyranoside test were used for species separation. The current repertoire of chromogenic substrates incorporated in the ANI card may need to be altered to accommodate substrates that are more reliable for separation of these and other closely related organisms.

In summary, the ANI card holds promise as yet another rapid method for identification of anaerobic bacteria for those laboratories that utilize the Vitek identification-susceptibility test instrument. Improvement is needed in certain aspects of the data base and, perhaps, in the selection of chromogenic substrates or modified conventional tests for separation of closely related species. Optimal use of the card and the supplemental information generated by the computer program depends, however, on basic knowledge and recognition of certain key characteristics of anaerobic bacteria encountered in the clinical microbiology laboratory.

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