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Accuracy and Reproducibility of the 4-Hour ATB 32A Method for Anaerobe Identification

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The ATB 32A system (API System SA, La Balme les Grottes, Montalieu-Vercieu, France) was evaluated for use in the identification of 214 anaerobes. Organisms included 73 isolates of the *Bacteroides fragilis* group, 24 *Bacteroides* spp., 10 fusobacteria, 43 clostridia, 28 cocci, and 36 gram-positive, nonsporeforming rods. With the concomitant use of Gram stain, pigmentation, catalase testing, and aerobic growth, the ATB 32A system correctly identified 97% of the *B. fragilis* group isolates, 88% of *Bacteroides* spp., 50% of fusobacteria, 74% of clostridia, 100% of cocci, and 86% of the gram-positive, nonsporeforming rods. Overall, 188 strains (88%) were correctly identified, with 18 (8%) requiring extra tests, other than the four mentioned above, for correct identification. Eight strains were misidentified, including one *Bacteroides* sp., three fusobacteria, one *Clostridium* sp., and three gram-positive, nonsporeforming rods. Reproducibility was very good, with 12 of 14 strains (86%) tested in triplicate yielding identical correct results on each of three occasions and 2 strains (14%) yielding identical correct results on two occasions. There was a low-probability identification (including the correct species) on the third testing. The ATB 32A system represents a worthwhile advance in systems used for the identification of clinically significant anaerobic bacteria.

The role played by anaerobes in the development of bacterial infections is firmly established. The pathogenic potential of these organisms and the development of species- and group-specific differences in antimicrobial susceptibility patterns point to the need for preliminary or definitive identification of these organisms, especially in cases of severe infections (1). Conventional biochemical testing, together with gas-liquid chromatography, is the most accurate identification method (1, 16, 28), but it is time-consuming, expensive, and beyond the means of many clinical microbiology laboratories.

Commercial methods which have been developed for anaerobe identification can be divided into the following two groups: (i) methods that depend upon detection of products after growth for at least 24 h and (ii) methods that detect preformed enzymes a few hours after inoculation. API 20A (Analytab Products, Plainview, N.Y.), the Minitek system (BBL Microbiology Systems, Cockeysville, Md.), and the Anaerobe-Tek system (marketed by Flow Laboratories, Inc., McLean, Va., but subsequently withdrawn from the U.S. market) depend upon analysis of reactions after growth of the organisms (3–6, 9–12, 14, 17–19, 21–23, 26, 27). The RapID-ANA (Innovative Diagnostic Systems, Inc., Atlanta, Ga.) and AN-Ident (Analytab) systems are both commonly used and commercially available 4-h systems (2, 4, 7, 8, 10, 13, 14, 17, 18, 22, 23, 27, 29). These methods are especially applicable in smaller laboratories without facilities for extended conventional testing. API ZYM (Analytab) has been available as a research tool for several years, but the method lacks a computerized data base and is not yet commercially available (14, 15, 20, 29).

Other rapid methods include the 4-h ANI Card (Vitek Systems, Hazelwood, Mo. [25]); the 2-h ABL system (Austin Biological Systems, Austin, Tex. [G. Ortisi, M. Cernuschi, P. Scarpellini, A. Pagano, and G. Privitera, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C298, p. 381]); and a newly released, rapid, 2-h microtiter identification system (American Microscan, Sacramento, Calif.) which supersedes a previously released 48-h microtiter method (24) which has since been withdrawn.

The ATB 32A system (API System SA, La Balme les Grottes, Montalieu-Vercieu, France) is a newly released, 32-well, 4-h qualitative micromethod based on the detection of preformed enzymes by use of chromogenic substrates for the identification of clinically significant anaerobes. The method has been preliminarily evaluated for rapid characterization of oral and nonoral pigmented *Bacteroides* species (30). This study evaluates the accuracy and reproducibility of the ATB 32A system in the identification of a spectrum of clinically isolated anaerobes.

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MATERIALS AND METHODS

Strains. All bacteria tested (Table 1) were clinical strains isolated from the Hershey Medical Center and a variety of other centers (see Acknowledgments). Organisms were identified by a combination of biochemical tests and gas-liquid chromatography, as recommended in established texts (1, 16, 28), and were tested blindly without prior knowledge of their identities. Isolates were frozen in sterile defibrinated sheep blood at −70°C, plated onto enriched anaerobic blood agar plates (28), and incubated for 24 to 72 h at 37°C in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) to check for purity and viability. Immediately prior to the inoculation of kits, cultures were replated from the latter medium onto enriched anaerobic blood agar plates with Columbia agar base (Difco Laboratories, Detroit, Mich.) which were prepared in house within 24 h of use. Columbia agar plates were incubated for 48 h in a glove box as described above.

The API 32A system. The kit for the API 32A system consisted of 32 cupules, 29 of which contained dehydrated substrates and 3 of which were empty. Tests in the system

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detected the following reactions: urease, arginine dihydrolase, α-galactosidase, β-galactosidase, β-galactosidase 6-phosphate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, β-*N*-acetylglucosaminidase, fermentation of mannose and raffinose, glutamic acid decarboxylase, α-fucosidase, nitrate reduction, indole production, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, and serine arylamidase.

Inocula were prepared by suspending growth from Columbia agar plates in ATB 32A suspension medium to a density of a McFarland no. 4 standard. Strips were inoculated with approximately 55 μl of a culture suspension according to the instructions of the manufacturer and incubated aerobically at 37°C for 4 h. Reactions in the upper row of tests were then read without the addition of reagent, and those in the lower row were read after the addition of reagents for the detection of nitrate reduction, indole, and production of all arylamidase reactions. Color reactions were recorded as positive, negative, or questionable according to the instructions of the manufacturer. Equivocal tests yielded color reactions between negative and positive, as described in the package insert and color chart. At the time that the study was initiated, a code book was not yet available, and all work sheets were sent to the API System in France for identification with their data base. A code book as well as an automated reader are now available. The first 24 tests were used to construct an eight-digit profile by combining the four digits of the upper row with the four digits of the lower row. The five other tests on the right part of the strip were used in cases of low-discrimination identifications.

When multiple organism identifications with low confidence values occurred, supplemental tests, as recommended by the data base of the manufacturer, were performed. Catalase testing (28) was performed in one of the three filled cupules without test reagents. Identifications were classified as (i) correct without additional tests, (ii) correct with additional tests, or (iii) incorrect. For reproducibility studies, organisms were tested in triplicate on successive days.

RESULTS

Reactions with the ATB 32A system were relatively clear-cut. However, equivocal reactions had to be reported as questionable; failure to do this resulted in high numbers of unacceptable profiles, especially with the *Bacteroides fragilis* group of organisms. As the method now stands, the automated reader ignores equivocal reactions; if the code book is used, both possibilities must be looked up. In spite of these potential problems, however, we did not encounter difficulties in the identification of strains with equivocal reactions.

Results of anaerobe identification with the ATB 32A system are given in Tables 1 and 2. The ATB 32A system identifies *Veillonella* spp. and some *Bifidobacterium* and *Capnocytophaga* spp. to the genus level only; *Peptostreptococcus asaccharolyticus* is identified as *Peptostreptococcus asaccharolyticus* or *Peptostreptococcus indolicus*, and *Clostridium bifermentans* is identified as *Clostridium bifermentans* or *Clostridium beijerinckii*. For the purposes of this study, these identifications were taken as correct. When Gram stain, aerobic growth, catalase testing, and pigmentation were taken as primary characteristics, correct identification rates improved markedly, as shown in Table 2.

TABLE 1. Identifications with the ATB 32A system

Organism (no. of strains tested)	No. identified: Correctly without extra tests	Correctly with extra tests	Incorrectly
Bacteroides fragilis (28)	26	2[a]	0
Bacteroides thetaiotaomicron (17)	15	2	0
Bacteroides distasonis (9)	9	0	0
Bacteroides vulgatus (5)	5	0	0
Bacteroides ovatus (11)	0	11[a]	0
Bacteroides caccae (3)	2	1[a]	0
Bacteroides intermedius (4)	2	1	1
Bacteroides melaninogenicus (1)	0	1	0
Bacteroides oralis (3)	0	3[a]	0
Bacteroides bivius (5)	5	0	0
Bacteroides disiens (8)	8	0	0
Bacteroides buccae (2)	2	0	0
Capnocytophaga sp. (1)	1	0	0
Fusobacterium nucleatum (3)	0	2[a]	1
Fusobacterium necrophorum (1)	0	1[a]	0
Fusobacterium mortiferum (4)	1	2	1
Fusobacterium varium (2)	0	1[a]	1
Clostridium perfringens (7)	7	0	0
Clostridium bifermentans (2)	1	1	0
Clostridium tertium (5)	0	5[a]	0
Clostridium paraputrificum (2)	0	2	0
Clostridium difficile (4)	1	3	0
Clostridium sporogenes (3)	2	1	0
Clostridium sordellii (6)	6	0	0
Clostridium septicum (1)	0	1	0
Clostridium botulinum (1)	1[b]	0	0
Clostridium ramosum (6)	5	1	0
Clostridium butyricum (3)	1[c]	1	1
Clostridium innocuum (1)	1	0	0
Clostridium subterminale (1)	0	1[a]	0
Clostridium cadaveris (1)	1	0	0
Peptostreptococcus magnus (7)	7	0	0
Peptostreptococcus anaerobius (6)	6	0	0
Peptostreptococcus asaccharolyticus (3)	3[d]	0	0
Peptostreptococcus micros (4)	4	0	0
Veillonella spp. (8)	7	1[a]	0
Propionibacterium acnes (11)	11	0	0
Lactobacillus acidophilus (2)	0	1	1
Bifidobacterium spp. (5)	4	1	0
Actinomyces israelii (2)	2	0	0
Actinomyces odontolyticus (2)	0	0	2
Actinomyces viscosus (4)	4	0	0
Arachnia propionica (1)	1	0	0
Eubacterium lentum (8)	0	8[a]	0
Eubacterium limosum (1)	0	1[a]	0

[a] Required Gram stain, catalase, aerobic growth testing, or pigmentation for correct identification (see Table 2).
[b] Presumptive identification.
[c] Identified as *Clostridium butyricum* or *Clostridium beijerinckii* with the ATB 32A system.
[d] Identified as *Peptostreptococcus asaccharolyticus* or *Peptostreptococcus indolicus* with the ATB 32A system.

Table 3 lists additional tests that were required for low-probability identifications (including the four tests mentioned above). Extra tests were required for several members of the *Bacteroides fragilis* group, *Bacteroides* spp., fusobacteria, clostridia, and gram-positive, nonsporeforming rods. However, Gram stain, aerobic growth, pigmentation, and catalase testing delineated the correct identification in 37 of 55 (67%) of these cases. All 11 *Bacteroides ovatus* strains could be satisfactorily differentiated from *Bacteroides uniformis* strains by positive catalase reactions, while pigmentation differentiated all three *Bacteroides oralis* strains from

TABLE 2. Identifications with the ATB 32A system by using original[a] and amended[b] data bases

Organism (no. of strains tested)	No. (%) identified:					
	Correctly without extra tests		Correctly with extra tests		Incorrectly	
	Original	Amended	Original	Amended	Original	Amended
Bacteroides fragilis group (73)	57 (78)	71 (97)	16 (22)	2 (3)	0 (0)	0 (0)
Bacteroides spp. (24)	18 (75)	21 (88)	5 (21)	2 (8)	1 (4)	1 (4)
Fusobacteria (10)	1 (10)	5 (50)	6 (60)	2 (20)	3 (30)	3 (30)
Clostridia (43)	26 (61)	32 (74)	16 (37)	10 (23)	1 (2)	1 (2)
Cocci (28)	27 (96)	28 (100)	1 (4)	0 (0)	0 (0)	0 (0)
Gram-positive, nonsporeforming rods (36)	22 (61)	31 (86)	11 (31)	2 (6)	3 (8)	3 (8)
Total (214)	151 (71)	188 (88)	55 (26)	18 (8)	8 (4)	8 (4)

[a] As used by API 32A in the current study.
[b] Including Gram stain, catalase testing, aerobic growth, and pigmentation as primary characteristics.

black-pigmented *Bacteroides* spp. The Gram stain reaction accurately identified four *Fusobacterium* isolates, while aerobic growth differentiated all five *Clostridium tertium* strains from the other clostridia. The Gram stain differentiated all nine eubacteria from the other anaerobic organisms. In all 37 of the strains described above, one of these four tests was all that was necessary for accurate identification (1, 16, 28); other tests listed in the data base of the manufacturer of the system were useful only for confirmatory identification. Ribose fermentation was used to differentiate *Clostridium butyricum* from *Clostridium beijerinckii*, and coagulase testing was used to differentiate *Peptostreptococcus asaccharolyticus* from *Peptostreptococcus indolicus*.

Eight organisms were misidentified by the ATB 32A system. These included one *Bacteroides intermedius* (misidentified as *Bacteroides asaccharolyticus*), one *Fusobacterium nucleatum* (misidentified as *Fusobacterium varium*), one *Fusobacterium mortiferum* (misidentified as *Bacteroides oralis/Bacteroides denticola/Bacteroides loescheii/Bacteroides melaninogenicus*), one *Fusobacterium varium* (misiden-

TABLE 3. Low-discrimination identifications with the ATB 32A system

Organism identified by ATB 32A (no. of strains tested)	Additional required test(s)[b]
Bacteroides fragilis[a]/*Bacteroides caccae* (1)	*CAT*, TRE, ARA, RIB
Bacteroides fragilis[a]/*Bacteroides melaninogenicus* (1)	*PIGM*, CAT, GEL
Bacteroides ovatus/Bacteroides uniformis/Bacteroides thetaiotaomicron[a]/*Bacteroides eggerthii/Bacteroides oralis* (2)	CAT, TRE, RIB, XYLAN, SAL
Bacteroides ovatus[a]/*Bacteroides uniformis* (11)	*CAT*, TRE, RIB, SAL
Bacteroides caccae[a]/*Bacteroides thetaiotaomicron/Bacteroides distasonis* (1)	*CAT*, ARA
Bacteroides intermedius[a]/*Bacteroides asaccharolyticus* (1)	GLU, MLT, AMD[h], FRU
Bacteroides melaninogenicus[a]/*Bacteroides denticola/Bacteroides oralis/Bacteroides loescheii* (1)	PIGM, CELLOB, ESC[f], MEL, RIB
Bacteroides oralis[a]/*Bacteroides loescheii/Bacteroides denticola* (3)	*PIGM*
Fusobacterium nucleatum[a]/*Clostridium tetani/Clostridium bifermentans* (2)	*GRAM*, SPORE, GLU, LEC
Fusobacterium necrophorum[a]/*Clostridium tetani* (1)	*GRAM*, SPORE
Fusobacterium mortiferum[a]/*Leptotrichia buccalis* (2)	MLT
Eubacterium limosum/Clostridium innocuum/Fusobacterium varium[a] (1)	*GRAM*, SPORE, ESC[h], TRE
Clostridium bifermentans[a]/*Clostridium difficile* (1)	LEC, HEM
Clostridium tertium[a]/*Clostridium ramosum/Clostridium beijerinckii-C. butyricum* (3)	*AER*, MOT, ARA, RIB, AMD[h]
Clostridium tertium[a]/*Clostridium ramosum* (2)	*AER*, MOT
Clostridium ramosum/Clostridium tertium/Clostridium paraputrificum[a] (1)	MOT, RIB, AMD[h]
Clostridium ramosum/Clostridium paraputrificum[a]/*Clostridium tertium* (1)	MOT, RIB, AMD[h]
Clostridium difficile[a]/*Clostridium bifermentans/Clostridium glycolicum* (3)	GEL, LEC, HEM, ESC[h]
Clostridium botulinum/Clostridium sporogenes[a] (1)	TOX
Clostridium septicum[a]/*Clostridium paraputrificum* (1)	GEL, AMD[h], HEM, TRE
Clostridium ramosum[a]/*Clostridium tertium/Clostridium paraputrificum* (1)	MOT, AER, RIB, AMD[h]
Clostridium tertium/Clostridium ramosum/Clostridium beijerinckii-Clostridium butyricum[a] (1)	RIB, AER, MOT, ARA, RIB, AMD[h]
Clostridium subterminale[a]/*Peptostreptococcus magnus* (1)	*GRAM*, SPORE, HEM, MOT
Clostridium tyrobutyricum/Veillonella sp.[a] (1)	*GRAM*, SPORE, GLU
Lactobacillus acidophilus[a]/*Bifidobacterium* sp./*Actinomyces meyeri* (1)	AMD[h], RIB, HEM, ARA
Bifidobacterium sp.[a]/*Actinomyces meyeri* (1)	AMD[h], HEM, MLT, ARA
Clostridium limosum/Clostridium botulinum/Eubacterium lentum[a] (8)	*GRAM*, SPORE, GEL, GLU, LIP
Clostridium botulinum/Clostridium tetani/Eubacterium limosum[a]/*Fusobacterium nucleatum/Bacteroides ureolyticus* (1)	*GRAM*, SPORE, GLU, GEL

[a] Correctly identified organism.
[b] Italicized tests are those tests (CAT, GRAM, PIGM, and AER) that should be included in the primary data base. Abbreviations: CAT, catalase; GRAM, Gram stain; AER, aerobic growth; PIGM, pigment; MOT, motility; SPORE, spore formation; GEL, gelatinase; HEM, hemolysis; LEC, lecithinase; LIP, lipase; TOX, toxin testing; AMD[h], amygdalin hydrolysis; ESC[h], esculin hydrolysis; GLU, glucose fermentation; TRE, trehalose fermentation; ARA, arabinose fermentation; RIB, ribose fermentation; FRU, fructose fermentation; XYLAN, xylan fermentation; ESC[f], esculin fermentation; MLT, maltose fermentation; CELLOB, cellobiose fermentation; MEL, melibiose fermentation; SAL, salicin fermentation.

tified as *Fusobacterium necrophorum*), one *Clostridium butyricum* (misidentified as *Clostridium bifermentans*), and one *Lactobacillus acidophilus* and two *Actinomyces odontolyticus* (all three of which were misidentified as *Actinomyces meyeri*).

To test the reproducibility of the ATB 32A system, 14 strains were tested in triplicate, each on successive days. In 12 of 14 cases (86%), results of all 3 identifications were identical, with 10 correct identifications and 2 probability overlaps. One strain of *Bacteroides fragilis* yielded the correct identification on the first two testings but yielded *Bacteroides fragilis/Bacteroides distasonis/Capnocytophaga* sp. on the third testing, and one *Bacteroides thetaiotaomicron* yielded the correct identification on the first two testings but *Bacteroides thetaiotaomicron/Bacteroides ovatus* on the third testing.

DISCUSSION

Previously published evaluative studies on the API 20A and Minitek systems have documented the adequacy of these methods for identification of reactive organisms such as the *Bacteroides fragilis* group and clostridia. However, accurate identification is not possible with these systems without the use of gas-liquid chromatography and numerous additional tests (3-5, 9–12, 14, 17, 18, 21–23, 26, 27).

Both the AN-Ident and RapID-ANA systems have proved accurate in the identification of a wide variety of saccharolytic as well as weakly or nonsaccharolytic anaerobes. However, both systems must be supplemented with additional tests for the complete identification of selected organism groups. The additional tests required are, in most cases, simple and within the scope of smaller microbiology laboratories. Kits are easy to inoculate and read with a little experience, and results are superior to those of commercial methods that require growth of the organisms. However, because of rapid changes in taxonomy, it is still difficult, and in some instances impossible, for either system to differentiate between many species of pigmented and nonpigmented *Bacteroides* that do not grow well in 20% bile medium (2, 4, 7, 8, 10, 13, 14, 17, 18, 22, 23, 27, 29). Although the 4-h ANI card (Vitek) holds promise as another rapid method for anaerobe identification, improvement is required in certain aspects of the data base and, possibly, in the selection of chromogenic substrates or modified conventional tests for the separation of closely related species (25).

In the one published report of the ATB 32A method (30), the system yielded results which distinguished between 10 pigmented *Bacteroides* spp., including asaccharolytic strains such as *Bacteroides gingivalis*, *Bacteroides asaccharolyticus*, and *Bacteroides endodontalis*. However, additional tests were necessary in some cases to differentiate between the last two organisms (30).

In the present study, the ATB 32A system performed well in the identification of a wide variety of clinically isolated anaerobic strains. However, as the data base now stands, Gram stain, catalase, aerotolerance, and pigmentation represent additional tests that are required to delineate the correct identification from a spectrum of identifications with low probabilities. These four tests should be incorporated into the primary data base of the ATB 32A system; if this were done, 88% of strains would be correctly identified (up from 71%, as it was here), and only 8% of strains would require additional testing (down from 26%, as it was here). Catalase testing may be done conveniently either in one of the carbohydrate fermentation wells or in one of the cupules without reagent (as in the current system).

In summary, the ATB 32A system represents a rapid and accurate method for the identification of clinically isolated anaerobes, especially with the modifications in the data base suggested above. The method has the added advantage of flexibility, in that three cupules are empty. These could be filled and the data base could be expanded even further, should the need arise.

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