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Multicenter Evaluation of the Vitek 2 Anaerobe and *Corynebacterium* Identification Card▽

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The new anaerobe and *Corynebacterium* (ANC) identification card for Vitek 2 was compared with a 16S rRNA gene sequencing (16S) reference method for accuracy in the identification of corynebacteria and anaerobic species. Testing was performed on a Vitek 2 XL system with modified software at three clinical trial laboratories. Reproducibility was determined with nine ATCC quality control strains that were tested 20 times over a minimum of 10 days at all three sites. A challenge set of 50 well-characterized strains and 365 recent fresh and frozen clinical isolates were included in the study. The expected positive and negative biochemical well reactions were also evaluated for substrate reproducibility. All strains were tested with the ANC card, and clinical isolates were saved for 16S rRNA gene sequencing. All reproducibility tests yielded expected results within a 95% confidence interval, except for that with *Corynebacterium striatum* ATCC 6940, for which identification failed at one trial site. For the challenge isolates, there was 98% correct identification, 5% low discrimination, and 2% incorrect identification, and 0% were unidentified. For clinical strains, there was 95.1% correct identification, 4.9% low discrimination, and 4.6% incorrect identification, and 0.3% were unidentified. The 4.6% (17/365) of clinical isolates that were incorrectly identified consisted of 14 isolates that were correct at the genus level and three that were incorrect at the genus level. The new ANC card met all performance criteria within a 95% confidence interval compared to the identification performance by 16S rRNA gene sequencing.

Identification of some bacterial species in the clinical laboratory involves either extensive testing or submitting species to a reference laboratory. For identifying anaerobes, this choice has required the use of multiple techniques including gram-staining morphology, biochemical testing in prereduced anaerobic systems, and gas-liquid chromatography of volatile and nonvolatile fatty acids (7). Most laboratories simply do not have these capabilities or cannot maintain the expertise in this area, and so anaerobic pathogens often go unidentified. For microorganisms such as the corynebacteria, of which the major pathogen is *Corynebacterium diphtheriae*, many laboratories look for this pathogen only under the appropriate clinical circumstances. Other species may be combined as coryneforms or diphtheroids. Important pathogens within the genus *Corynebacterium*, particularly the antibiotic-resistant species, may be missed.

With the commonplace use of automated and semiautomated systems for the identification of isolates in most clinical laboratories, commercial emphasis has moved to expanding the capabilities of these systems to identify a wider variety of bacterial pathogens. Until recently, however, for identification of the coryneform bacteria and for anaerobes, most of the systems were confined to manual miniwell strip test systems. For identifying the coryneforms, these systems were reasonably accurate (5, 6, 9, 15, 17), but for identifying anaerobes, there were significant gaps, mainly with the biochemically inactive clostridia (3, 13, 16). For the identification of other anaerobic genera, results have been variable. Although the systems appear to perform well at the genus level (2, 8), correct identification at the species level may vary from 30 to 80%, depending on the taxa (4, 10, 12, 14, 18).

To expand the capabilities of the Vitek 2 automated identification system, bioMérieux, Inc. has developed a new anaerobe and *Corynebacterium* (ANC) identification card. The ANC card has a database that includes 63 taxa of anaerobes and corynebacteria belonging to the genera *Actinomyces*, *Arcanobacterium*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Corynebacterium*, *Eggerthella*, *Eubacterium*, *Finegoldia*, *Fusobacterium*, *Lactobacillus*, *Microbacterium*, *Micromonas*, *Peptoniphilus*, *Peptostreptococcus*, *Prevotella*, *Propionibacterium*, *Staphylococcus*, and *Veillonella*. The ANC card is based on colorimetric technology utilizing dehydrated media containing chromogenic substrates. Identification is accomplished with a 64-well card that utilizes a heavy inoculum and a short incubation period of approximately 6 h. For identifying anaerobes, most systems require similar or greater numbers of bacterial cells in the inoculum. Species identification is based on an algorithm of substrate reactions from data collected from known strains of the claimed species.

The present study was designed to test the capabilities of the ANC card to rapidly identify those species whose identities are more difficult to determine in three large tertiary care laboratories. Successful identification of such species may have im-

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portant benefits such as separating pathogens from commensal species and choosing appropriate therapies when required.

MATERIALS AND METHODS

Clinical trial sites. Three tertiary care hospital laboratories participated in the investigation. These sites were Medical Microbiology Laboratory, University of Alberta Hospital, Edmonton, Alberta, Canada; Clinical Microbiology Laboratory, Duke University Hospital, Durham, NC; and University of Illinois Medical Center, Chicago, IL.

Test methodology. All isolates were stored frozen prior to testing, except for the available clinical isolates that were recovered directly from clinical specimens and tested with the ANC card after they were subcultured. The corynebacterial strains were subcultured on tryptic soy blood agar, and anaerobes were subcultured on Columbia blood agar. The plates were incubated either in 5 to 7% carbon dioxide or in an anaerobic chamber for 24 to 48 h at 35°C. The growth was suspended in 0.45% aqueous NaCl to a turbidity value equivalent to a 3 McFarland standard. For each isolate, the inoculum was then incorporated into an ANC card in the Vitek 2 XL automated identification system. The Vitek 2 contained research software to accommodate the ANC card requirements. The cards were incubated for approximately 6 h, and then the Vitek 2 XL algorithm made the interpretation for the final identification.

Each clinical isolate at all three study sites was stored as a heavy suspension at 4°C in sterile physiological saline. These strains were then shipped directly to MIDI Labs (Newark, DE) for 16S rRNA gene sequencing.

Quality control/reproducibility testing. Nine microorganisms comprised the quality control and reproducibility sets. These strains were tested 20 times over a minimum of 10 days at each of the three clinical trial sites. The results were used to evaluate system and substrate reproducibility. One manufacturing lot of ANC cards was used for the entire study. The strains tested included *Parabacteroides* (*Bacteroides*) *distasonis* ATCC BAA-1295, *Bacteroides ovatus* ATCC BAA-1304, *B. ovatus* ATCC BAA-1296, *B. vulgatus* ATCC 8482, *Clostridium perfringens* ATCC 13124, *C. septicum* ATCC 12464, *C. sordellii* ATCC 9714, *Corynebacterium striatum* ATCC 6940, and *C. striatum* ATCC BAA-1293. Each clinical trial site received the same set of quality control isolates from bioMérieux, Inc.

Evaluation of system reproducibility was based on the number of correct identifications. Correct identification was defined as a microorganism accurately identified by the system as the only choice (with any level of confidence) or as one of the choices within a multichoice identification or a low-discrimination result. The expected system reproducibility performance criteria, as set by the clinical trial monitors, was to obtain at least 95% correct identification within a 95% confidence interval.

Evaluation of substrate reproducibility was based on a microorganism and biochemical combination with a distinct, expected reaction of positive or negative. The substrates for the ANC card were developed by the manufacturer and were not comparable to other substrates used in manual tests. It was, therefore, not possible to compare the results of the ANC card reactions with those of other commercial systems for the identification of these microorganisms. The codes for the substrates were proprietary and have meaning only within the overall context of microorganism identification in the test algorithms. The expected substrate reproducibility performance criterion was to achieve agreement at least 95% of the time tested within a 95% confidence interval for the individual well reactions with distinct positive or negative reactions.

Challenge testing. Each clinical study site tested a panel of 50 well-characterized isolates, once with the ANC card. These strains were provided by bioMérieux, Inc., and were tested in a blinded manner by the study sites. The isolates were not used to create the ANC database and were selected to represent the identification claims of the ANC product. There were 13 genera and 28 species in the challenge set that included both corynebacteria and anaerobes. Definitions for the levels of correct identification were as follows: (i) overall correct identification, the microorganism was accurately identified by the system as the only choice (with any level of confidence) or was one of the choices within a multichoice identification or a low-discrimination result; (ii) low discrimination, the algorithm gave two or three single or multichoice identification results for the isolate; (iii) incorrect identification, this was a final identification in which the genus or species was incorrect compared to that of the reference 16S rRNA result; and (iv) unidentified organisms, a final identification of "unidentified organism," "inconclusive identification," or "nonreactive biopattern" could be observed. Either the isolate was not one of the claimed genera and species, the reactions did not fit any known species and the 16S result did not resolve the ANC card result, or there were no reactions in the ANC card that would provide any identification. There were, in addition, unclaimed species that were excluded from the composite results collation.

The minimum performance requirements for the clinical trial for the ANC card were at least 90% overall correct identification for claimed species, less than 40% low discrimination, less than 5% incorrect identification, and less than 5% unidentified organisms within a 95% confidence interval.

Clinical isolate testing. The three clinical sites collected data by testing 365 fresh and frozen clinical isolates. All isolates were tested with the ANC card and with 16S rRNA gene sequencing (16S), utilizing approximately 500 base pairs. The 16S sequencing was considered the reference identification method. MIDI Labs (Newark, DE) performed the 16S sequencing and utilized Applied Biosystems' MicroSeq microbial analysis software and database to evaluate genetic similarities (11, 19). When a genetic match was not found in the MicroSeq library, MIDI performed a BLAST search of the GenBank public database (1). Sequences from isolates with a genus-level 16S identification from MIDI or GenBank were also compared to those in the ANC manufacturer's proprietary database. This level of comparison to 16S rRNA analysis provided a greater level of accuracy than a comparison of genera and species to another card or substrate type.

The 16S results were interpreted as follows. (i) All 16S results were reviewed to confirm that the isolate identified was listed on the ANC claim list; if a microorganism was not listed, it was excluded from the data set. (ii) A 16S result consisting of a single-choice species-level genetic match to the ANC identification was a complete, correct result. (iii) A 16S result with no percentage of genetic difference between multiple species was confirmed through supplemental biochemical testing to determine if one of the species listed matched the ANC identification. (iv) A 16S result that showed a percentage of genetic differences among species but was flagged as a species that was closely related to one or more species within the genus was reviewed to determine if additional testing was required to confirm the 16S result. (v) A 16S result with a species-level identification but one that was discrepant from the ANC identification was evaluated to determine if the microorganism listed was part of the ANC claim list; if the microorganism was listed, the discrepant result was accepted if the combination was observed during development or the isolate was retested and the discrepancy was confirmed or resolved as a correct identification. (vi) A 16S result reported by MIDI as a genus-level identification and/or a match to a sequence in the GenBank public database was sent to bioMérieux Research and Development Microbiology group in La Balme, France, for review. Definitions used for the level of correct identification and the performance criteria were the same as those used for the challenge testing.

Excluded/unclaimed genera/species. The strains that were not included as claimed genera or species by the ANC card were recorded but necessarily excluded from the overall data set compilations. The16S sequence was used to arbitrate the ANC card results for these strains. The criterion for an excluded or unclaimed strain was an identification from the ANC card that was not in the database and was supported by 16S rRNA sequencing or no identification given by the ANC card and a 16S sequence identification that was not one of the claimed species or no 16S identification.

RESULTS

Quality control and reproducibility testing. The performance requirements within a 95% confidence interval were met for the quality control and reproducibility isolates tested with the ANC card at the three test sites, both cumulatively and by individual trial site, except for the identification of *Corynebacterium striatum* ATCC 6940, which failed at one trial site (Table 1).

The combination of a distinct microorganism and a biochemical reaction of positive or negative, by which substrate reproducibility was evaluated, also indicated that the 95% confidence level was met both cumulatively and by individual sites, except for the following combinations: *B. ovatus* ATCC BAA-1304 (beta-D-fucopyranoside and alpha-L-arabinofuranoside wells) failed cumulatively; *Clostridium septicum* ATCC 12464 (L-pyrollidine-arylamidase well) failed at one site; *Corynebacterium striatum* ATCC 6940 (D-mannose well) failed cumulatively; and *C. striatum* ATCC BAA-1293 (Ellman well) failed

TABLE 1. ANC card quality control and reproducibility cumulative results for all sites

ATCC accession no.	Species	% of isolates correctly identified (no. correct/ total no. of tests)[a]	Results within 95% CI (interval/total)[b]
BAA-1295	*Parabacteroides* (*Bacteroides*) *distasonis*	100 (62/62)	Yes (≥55/62)
BAA-1304	*Bacteroides ovatus*	100 (62/62)	Yes (≥55/62)
BAA-1296	*Bacteroides ovatus*	100 (63/63)	Yes (≥56/63)
8482	*Bacteroides vulgatus*	100 (62/62)	Yes (≥55/62)
13124	*Clostridium perfringens*	100 (64/64)	Yes (≥57/64)
12464	*Clostridium septicum*	100 (62/62)	Yes (≥55/62)
9714	*Clostridium sordellii*	100 (60/60)	Yes (≥53/60)
6940	*Corynebacterium striatum*	87 (54/62)	No (≥55/62)
BAA-1293	*Corynebacterium striatum*	100 (65/65)	Yes (≥58/65)

[a] Some trial sites set up more than 20 tests.
[b] CI, confidence interval.

cumulatively. Cumulative failures occurred when the results for an isolate did not meet the 95% acceptance criteria when the data for all three sites were put together, even though one or more sites may have had an acceptable result.

Challenge testing. The challenge set of 50 strains was identified correctly at all sites 98% of the time (147 of 150 results). Of the 147 correct results, six strains gave a total of seven low-discrimination results. The same strains of *Fusobacterium necrophorum* gave incorrect species but correct genus identifications of *F. nucleatum* at all three sites. Reference identifications were provided by bioMérieux, Inc., for all challenge set strains.

Clinical isolate testing. As shown in Table 2, for those strains for which the system had a genus and species claim, the ANC card identified the clinical isolates correctly 95.1% of the time (347 of 365 strains tested). This included a total of 18 strains (4.9%) that gave low-discrimination identifications. Only 1 strain was not identified, and 17 (4.6%) gave incorrect identifications. All performance criteria set by the clinical trial protocol were met for the clinical isolates. The discrepant identifications are shown in Table 3. Of the 17 incorrect identifications, 14 were correct to the genus level but the wrong species was listed. Six of these were *Clostridium* species other than *C. perfringens*. There were three strains with the wrong genus; two of these strains were identified as *Actinomyces naeslundii* by the ANC card but were identified as *Bifidobacterium* and *Propionibacterium* species by 16S rRNA analysis. One *F. nucleatum* isolate was identified by 16S analysis as *C. clostridioforme*.

The overall performance summary by all sites for all clinical strains tested is shown in Table 4. A total of 51 species were included among the clinical isolates tested. Of these, 38 species were anaerobes. There were seven species of *Corynebacterium* identified. Identification of *Clostridium* species and *Bacteroides* species was very high overall. A small number of species were less easily identified. These included *Clostridium tertium* (two of seven strains misidentified) and *F. necrophorum* (two of seven strains misidentified). In many cases, there were only small numbers of each species in the clinical trial isolates.

Excluded/unclaimed isolates. There were 169 clinical strains excluded from the data set, 60 (36%) of which were isolated from normal flora. These included 85 isolates from one site, 39 from another site, and 45 from the third site. Of the 169 strains, 16 were mixed cultures or incomplete tests. Forty-nine strains did not have a 16S species-level match; of these, 34 were correct to the genus level, 10 were incorrect at the genus level, and 5 were not identified. Another 86 isolates were species not claimed by the ANC card. Of these, 70 were correct to the genus level, 6 were incorrect at the genus level, and 10 were unidentified. Finally, 18 isolates belonged to genera not claimed by the ANC card. The genera that were observed to give only a 16S result were *Brevibacterium*, *Campylobacter*, *Capnocytophaga*, *Desulfovibrio*, *Dialister*, *Enterococcus*, *Erysipelothrix*, *Pantoea*, *Pasteurella*, *Porphyromonas*, and *Turicella*. This resulted in 8 unidentified strains and 10 strains that were misidentified. Six of these 11 unclaimed genera are claimed by other Vitek 2 identification cards.

DISCUSSION

This study was designed to accurately validate the performance of the latest Vitek 2 combined ANC identification card in high-volume tertiary clinical laboratories that perform routine identification of pathogens with automated platforms. The performance of the ANC identification card

TABLE 2. ANC card overall performance summary by site for clinical isolate testing for genera and species claimed by the system

Testing laboratory site	Total no. of isolates tested by site	Overall % correctly identified	% of low discrimination	% Incorrectly identified	% Unidentified
A	121	95	4.1	4.1	0.8
B	143	95.8	6.3	4.2	0
C	101	94	4.0	6.0	0
Combined total	365	95.1	4.9	4.6	0.3

TABLE 3. ANC card discrepant identifications in the clinical strain set at all sites

ANC identification	Vitek 2 confidence level	16S rRNA gene sequencing identification
Bacteroides caccae	Very good	*Bacteroides thetaiotaomicron*
Actinomyces naeslundii	Very good	*Bifidobacterium* spp.
Actinomyces naeslundii	Acceptable	*Propionibacterium acnes*
Clostridium paraputrificum	Acceptable	*Clostridium septicum*
Clostridium bifermentans	Low discrimination	*Clostridium sordellii*
Fusobacterium nucleatum	Excellent	*Fusobacterium necrophorum*
Clostridium barati, *C. clostridioforme*, *C. septicum*	Low discrimination	*Clostridium tertium*
Clostridium clostridioforme	Excellent	*Clostridium ramosum*
Fusobacterium nucleatum	Excellent	*Fusobacterium necrophorum*
Prevotella bivia	Excellent	*Prevotella melaninogenica*
Actinomyces naeslundii	Acceptable	*Actinomyces israelii*
Prevotella disiens	Excellent	*Prevotella intermedia*
Clostridium subterminale	Excellent	*Clostridium difficile*
Bacteroides ovatus	Excellent	*Bacteroides fragilis*
Fusobacterium nucleatum	Excellent	*Clostridium clostridioforme*
Corynebacterium pseudodiphtheriticum	Good	*Corynebacterium urealyticum*
Clostridium butyricum, *C. clostridioforme*	Low discrimination	*Clostridium tertium*

met the validation requirements for the three components tested, as described in the study protocol. All microorganism/biochemical combinations having distinct expected reactions of positive or negative, in which quality control was evaluated, met the performance criteria when they were measured cumulatively across sites, with the exception of five biochemical reactions among four quality control microorganisms. These exceptions did not affect the final identification of these species.

However, due to the apparently inconsistent performance observed for the identification of *C. striatum* ATCC 6940 in this study, the manufacturer has removed it from the recommended list of quality control isolates found in the product information. Of interest, in the clinical trial isolate set, 20 strains of *C. striatum* were discovered. All were identified correctly, and only one was a low-discrimination-but-correct-identification result. Based on these observations, this ATCC strain appears to have characteristics that are unusual for the species.

For the corynebacteria, the ANC card performed very well. Only one strain of *C. urealyticum* was misidentified, and the remainder were correctly identified. The ANC card readily separated the more antibiotic-resistant species *C. jeikeium* and *C. amycolatum*. Two of the three strains of *C. diphtheriae* gave low-discrimination results, but were correctly identified. Additional strains would be required for the identification of some of these species to ensure that the observations for the genus hold up over time.

The separation of species among the anaerobic isolates tested was excellent for those strains for which the ANC card had a claim. Previous comparisons using other automated and manual commercial biochemical card systems have been variable (2, 4, 8, 10, 12, 14, 18) but disappointing for the identification of clostridia (3, 13, 16). These microorganisms are notoriously nonreactive in standard biochemical tests, and it is often necessary to perform analysis of volatile and nonvolatile acids by gas-liquid chromatography or perform sequence analyses. With the ANC card, errors were rare, and for identification of the clostridia, errors occurred only at the species level. This is the first automated system that allows identification of these microorganisms on a single card, without the need for extensive, time-consuming, and expensive tests.

The quality control and reproducibility data show that the ANC card performance was consistent within a site and across three different laboratory test sites. For the clinical data set, the data show that the ANC card consistently provided correct identifications wherever the strain was isolated. Like many of these studies, the data sets have limitations. During the trial, there were few isolates of some species recovered from clinical specimens. It is not possible to observe all claimed species in a clinical trial, but as the study was conducted in three tertiary care hospital laboratories, it would be expected that the most important clinical species would be observed. There were also isolates recovered for which the ANC card had no claim by the system. At the genus level, these isolates were few in number. A total of 31 isolates were misidentified. For those microorganisms not claimed by the ANC card, there were some species that might be considered clinically significant. These included 17 strains identified as *Corynebacterium xerosis*, two strains as *Actinomyces odontolyticus*, and 10 isolates as *C. pseudogenitalium* of uncertain clinical significance. There were a small number of other isolates, usually recovered from oral infections, that are not commonly observed. It will be important, as this new ANC card is utilized in clinical laboratories, to expand the system to include more of those species.

This investigation has shown that the ANC card identifies the large majority of isolates of corynebacteria and anaerobes observed in clinical specimens. However, like all systems, not every species is included in the database. Microorganisms such as *Listeria* and *Erysipelothrix* spp. or other less frequently occurring coryneform gram-positive bacilli such as *Turicella*, *Rothia*, *Oerskovia*, and *Brevibacterium* spp. should be identified by other methods to avoid delay. Users should consult the product package insert for those genera and species that are included in the database.

The data for which the card has a claim indicated that the ANC card met all performance criteria within a 95% confidence interval. The results from this clinical trial performed in three large tertiary care centers indicate that the Vitek 2

TABLE 4. ANC card performance summary by species for the clinical isolate test set: all sites

Bacterial species	Total no. of isolates tested	Overall % correctly identified	% of low discrimination	% Incorrectly identified	% Unidentified
Actinomyces israelii	2	50	0	50	0
Actinomyces meyeri	2	100	0	0	0
Arcanobacterium haemolyticum	4	100	0	0	0
Arcanobacterium pyogenes	2	100	0	0	0
Bacteroides caccae	3	100	0	0	0
Parabacteroides (*Bacteroides*) *distasonis*	7	100	0	0	0
Bacteroides fragilis	40	97.5	0	2.5	0
Bacteroides ovatus	8	100	0	0	0
Bacteroides stercoris	1	100	0	0	0
Bacteroides thetaiotaomicron	21	95.2	4.8	4.8	0
Bacteroides uniformis	6	100	0	0	0
Bacteroides ureolyticus	1	100	0	0	0
Bacteroides vulgatus	13	100	0	0	0
Bifidobacterium spp.	4	75	25	25	0
Clostridium barati	1	100	0	0	0
Clostridium bifermentans	5	100	100	0	0
Clostridium butyricum	1	100	100	0	0
Clostridium cadaveris	3	100	0	0	0
Clostridium clostridioforme	4	75	25	25	0
Clostridium difficile	4	75	0	25	0
Clostridium paraputrificum	5	100	0	0	0
Clostridium perfringens	32	100	0	0	0
Clostridium ramosum	11	90.9	0	9.1	0
Clostridium septicum	8	87.5	0	12.5	0
Clostridium sordellii	2	0	0	50	50
Clostridium sporogenes	2	100	100	0	0
Clostridium subterminale	1	100	100	0	0
Clostridium tertium	7	71.4	0	28.6	0
Corynebacterium amycolatum	2	100	0	0	0
Corynebacterium diphtheriae	3	100	66.7	0	0
Corynebacterium jeikeium	10	100	0	0	0
Corynebacterium pseudodiphtheriticum	9	100	0	0	0
Corynebacterium striatum	20	100	5	0	0
Corynebacterium ulcerans	2	100	0	0	0
Corynebacterium urealyticum	5	80	0	20	0
Eggerthella lenta	9	100	0	0	0
Eubacterium limosum	3	100	0	0	0
Finegoldia magna	14	100	0	0	0
Fusobacterium mortiferum	3	100	0	0	0
Fusobacterium necrophorum	7	71.4	0	28.6	0
Fusobacterium nucleatum	7	100	14.3	0	0
Lactobacillus gasseri	2	100	0	0	0
Micromonas micros	7	100	0	0	0
Peptostreptococcus anaerobius	3	100	0	0	0
Prevotella bivia	7	100	14.3	0	0
Prevotella buccae	3	100	0	0	0
Prevotella intermedia	3	66.7	0	33.3	0
Prevotella melaninogenica	4	75	25	25	0
Prevotella oralis	1	100	0	0	0
Propionibacterium acnes	35	97.1	0	2.9	0
Veillonella spp.	6	100	0	0	0
Cumulative totals	365	95.1	4.9	4.6	0.3

ANC card is acceptable for routine use in a clinical microbiology laboratory.

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