Identification of Clinical Coryneform Bacterial Isolates: Comparison of Biochemical Methods and Sequence Analysis of 16S rRNA and *rpoB* Genes[▽]

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We compared the relative levels of effectiveness of three commercial identification kits and three nucleic acid amplification tests for the identification of coryneform bacteria by testing 50 diverse isolates, including 12 well-characterized control strains and 38 organisms obtained from pediatric oncology patients at our institution. Between 33.3 and 75.0% of control strains were correctly identified to the species level by phenotypic systems or nucleic acid amplification assays. The most sensitive tests were the API Coryne system and amplification and sequencing of the 16S rRNA gene using primers optimized for coryneform bacteria, which correctly identified 9 of 12 control isolates to the species level, and all strains with a high-confidence call were correctly identified. Organisms not correctly identified were species not included in the test kit databases or not producing a pattern of reactions included in kit databases or which could not be differentiated among several genospecies based on reaction patterns. Nucleic acid amplification assays had limited abilities to identify some bacteria to the species level, and comparison of sequence homologies was complicated by the inclusion of allele sequences obtained from uncultivated and uncharacterized strains in databases. The utility of *rpoB* genotyping was limited by the small number of representative gene sequences that are currently available for comparison. The correlation between identifications produced by different classification systems was poor, particularly for clinical isolates.

The coryneform bacteria consist of a heterogeneous group of aerobically growing, asporogenous, non-partially-acid-fast, irregularly shaped, gram-positive rods (9). Medically relevant members of this group include, among others, *Brevibacterium* spp., *Corynebacterium* spp., *Curtobacterium* spp., *Leifsonia* spp., *Microbacterium* spp., and *Oerskovia* spp. (4, 6).

The importance of the coryneform bacteria to clinical microbiologists and physicians has increased in recent decades. The most prevalent human pathogen among these bacteria, *Corynebacterium diphtheriae*, continues to be a major cause of morbidity and mortality in developing countries and increasingly in developing countries where medical and public health systems function poorly because of political conflict and economic decline (10). Coryneform bacteria other than those belonging to the *C. diphtheriae* group are mostly opportunistic or nosocomial pathogens of humans. These infections are no longer rare, largely due to the dramatic increase in the numbers of susceptible persons, including those receiving immunosuppressive therapy for cancer, autoimmune disorders, and solid-organ transplant.

Accurate and timely identification of coryneform bacteria causing human infection is warranted for several reasons. Al-

though these bacteria are generally of low virulence, the infections that they cause may be severe or fatal. Prompt, effective antimicrobial therapy is necessary to optimize disease outcomes (23). The precise identification of coryneform bacterial isolates is important because the antimicrobial susceptibilities of these bacteria are quite variable (22). Resistance to multiple antimicrobial agents has increased over the past several decades and is characteristic of certain species such as *Corynebacterium jeikeium*, *Corynebacterium urealyticum*, and *Brevibacterium* spp. (9, 22). Resistance to vancomycin, an agent commonly used for empirical therapy of gram-positive infections in immunocompromised hosts, has recently been reported (17). Finally, these studies may lead to the identification of novel organisms and new associations of established species with human disease.

Identification of coryneform bacteria has been traditionally performed by a combination of Gram stain appearance and biochemical characteristics (9). Several commercial systems have been developed for use in the clinical diagnostic laboratory, including BBL Crystal, API (Rapid) Coryne, and RapID CB Plus. The performances of these systems have been evaluated in a limited number of studies, with variable but generally positive results, although additional tests may be required for the definitive identification of isolates to the species level (2, 3, 7, 8, 11, 13). More recently, genotypic classification systems based on nucleic acid sequence polymorphisms of 16S rRNA and DNA-dependent RNA polymerase β -subunit (*rpoB*) genes have been developed (14, 15, 18, 21). Only one

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16S rRNA gene sequencing system (MicroSeq 500; Applied Biosystems, Foster City, CA) is commercially available, validated, and cleared by the Food and Drug Administration for clinical diagnostic use. Many laboratories, however, also perform these studies for research purposes using proprietary primers and amplification protocols. Since the primary goal of these assays is the identification of a large number of potential pathogens, most primers are "broad range," designed to amplify partial or complete ribosomal genes from a wide range of pathogenic bacteria (12). Although these amplification targets are highly conserved among bacteria, nucleotide sequence differences may reduce both the sensitivities and specificities of tests. Limited homology between target genes and primers may also preclude adequate gene amplification, or, on the other hand, gene sequences from related bacteria may be insufficiently polymorphic to permit their distinction. This may be the case with coryneform bacteria. Khamis and colleagues previously found that accurate molecular identification of *Corynebacterium* species was possible only by sequencing of the complete 16S rRNA gene rather than the smaller fragments amplified by the MicroSeq 500 system and many laboratories (15). As a consequence, the use of targets that are potentially more discriminatory, such as *rpoB*, has been proposed (14). Since no studies to date have compared the relative effectiveness of these phenotypic and genotypic systems, we tested a recently isolated group of diverse coryneform bacteria including well-characterized control strains and organisms isolated from pediatric oncology patients at our institution.

MATERIALS AND METHODS

Control bacterial isolates. Controls included the following reference strains obtained from the American Type Culture Collection (ATCC) (Manassas, VA): *Corynebacterium striatum* (ATCC 6940), *Corynebacterium urealyticum* (ATCC 43043), *Corynebacterium pseudotuberculosis* (ATCC 43924), *Corynebacterium jeikeium* (ATCC 43734), *Leifsonia aquatica* (ATCC 14665, previously known as *Corynebacterium aquaticum*), *Corynebacterium amycolatum* (ATCC 700206), *Microbacterium testaceum* (ATCC 15829), *Rhodococcus equi* (ATCC 6939), *Corynebacterium minutissimum* (ATCC 23348), *Corynebacterium pseudodiphtheriticum* (ATCC 10701), *Arcanobacterium pyogenes* (ATCC 19411), and *Corynebacterium renale* (ATCC 19412). Each lyophilized isolate was reconstituted according to the manufacturer's directions, passed twice, and tested for purity prior to testing.

Clinical bacterial isolates. A total of 38 bacterial strains isolated from clinical specimens obtained from immunocompromised children and young adults between 1992 and 2004 were included for study. Using strict clinical criteria, about half of these isolates represented true pathogens (1). All work was approved by the St. Jude Children's Research Hospital Office of Human Subjects Protection. Isolates were identified as coryneform bacteria by a combination of Gram stain, colony morphology, and catalase reaction. Each isolate was retrieved from an aliquot of brain heart infusion or trypticase soy broth supplemented with 20% glycerol. All isolates had been maintained at -80°C prior to recovery and were deidentified and coded prior to inclusion. Each aliquot was transferred onto a trypticase soy agar plate with 5% sheep blood, passed a second time, and incubated at 35°C for 18 to 24 h prior to testing.

Identification of coryneform bacteria by biochemical identification panels. Each isolate was tested in duplicate by three commercial biochemical kit methods (BBL Crystal Rapid Gram Positive ID [Diagnostic Systems, Sparks, MD], API Coryne V2.0 [bioMérieux, Durham, NC], and RapID CB Plus [Remel, Lenexa, KS]) using package insert directions. If replicates agreed, the consensus result was used for comparative analyses. If there was disagreement between replicates, the more definitive result (result with a higher confidence level) was used for comparative analysis. Quality control testing was performed for each commercial kit as suggested by the manufacturer. A low confidence level of identification by API Coryne was given if the result showed a doubtful profile, low discrimination, an unacceptable profile, or an unreliable identification; a high confidence level was determined for the API Coryne system if there was an acceptable identification, a good identification, or a very good identification. For

the RapID CB Plus system, a low confidence level was assigned if there was inadequate identification, and a high confidence level was assigned if there was acceptable identification.

BBL Crystal. Panels were inoculated with isolated colonies of a pure culture of each isolate grown for 18 to 24 h that were suspended in BBL inoculum fluid to achieve visual turbidity equal to a 0.5 McFarland standard. The substrate panel was incubated face down in a 35°C non- CO_2 incubator for 24 h and read using the BBL Crystal Panel viewer based on package insert criteria and the provided color reaction chart. The resulting profile number for each isolate was entered into BBL Crystal Mind software to obtain identification.

API Coryne. Using an ampoule of API suspension medium provided with the testing kit, a pure culture of each isolate grown for 18 to 24 h was inoculated to achieve visual turbidity equal to a 6 McFarland standard. The culture suspension (100 μl) was distributed into each of the first 11 receptacles of the test strip. API GP medium was added to the remaining suspension and distributed to the last nine test receptacles only. The underlined tests were covered with mineral oil to create a slightly convex meniscus. Each tray was covered and incubated for 24 h at 35°C in an aerobic, non-carbon dioxide environment. After the appropriate addition of reagents provided with the kit (nitrates 1 and 2, PYZ, and ZYM A and B), a profile number for each isolate was determined using color-based reactions. Final identification of isolates was performed via an automated telephone database.

RapID CB Plus. A turbid suspension equivalent to a 4 McFarland standard was prepared from a pure culture of each isolate grown for 18 to 24 h using RapID inoculation fluid. The resulting culture suspension was evenly inoculated among the RapID CB Plus panel test cavities. Each panel was incubated at 35°C in a non- CO_2 incubator for 4 h according package insert instructions. After adding RapID CB Plus, nitrate A, and nitrate B reagents to select cavities, a numeric profile was determined based on observed color changes in the test cavities. Identification of isolates was performed using the Electronic RapID Compendium.

Identification of coryneform bacteria by 16S rRNA and *rpoB* gene sequencing. Both sense and antisense DNA strands were sequenced for each strain. Two sets of primers were used to amplify 16S rRNA genes: broad-range bacterial primers (5'-GTTAAGTCCCGCAACGA-3' and 5'-AGGAGGTGATCCAGCC-3') and primers optimized for the amplification of coryneform bacterial 16S rRNA genes. The latter primers (5'-CGTAGGGTGCAGCGTTGTCCG-3' and 5'-CGTTGCGGGAAGTAAACCAACATCTC-3') correspond to the consensus sequence of an approximately 516-bp fragment of the 16S rRNA genes of *Corynebacterium diphtheriae* strain NCTC 11397 (GenBank accession no. X84248), *Corynebacterium efficiens* strain YS-155 (accession no. AB055965), *Corynebacterium glutamicum* strain CICC10226 (accession no. AY794056) *Bifidobacterium longum* strain SB11 (accession no. AY850359), *Leifsonia xyli* strain CV86 (accession no. AJ17351), and *Propionibacterium acnes* strain JP1B (accession no. AY642053). The previously described *Corynebacterium* consensus *rpoB* primers C2700F and C3130R were used to amplify 440-bp fragments from each bacterial strain (15).

Reaction mixtures for the amplification of broad-range 16S rRNA genes included 1 μg of genomic DNA, 3.2 pmol each of reverse and forward primers, 1 $\times$ ready reaction premix (Applied Biosystems), and 1 $\times$ BigDye Terminator sequencing buffer v1.1/3.1 (Applied Biosystems). PCR amplification was performed on one of two cyclers: an MJ Research DNA Engine Tetrad cycler (Bio-Rad, Hercules, CA) or an MJ Research PTC-225 Peltier gradient thermal cycler (Bio-Rad, Hercules, CA). Amplification conditions included a denaturation step at 96°C for 30 s, an annealing step at 50°C for 15 s, and an extension step at 60°C for 4 min. Twenty-five cycles of amplification were performed for each reaction. Sequences were analyzed using ABI 3730xl DNA analyzers (Applied Biosystems, Foster City, CA).

Reaction mixtures for coryneform-optimized 16S rRNA and *rpoB* gene amplifications included 1 μg of genomic DNA or a single bacterial colony from an agar plate in 1 $\times$ buffer containing 0.2 mM deoxynucleoside triphosphates, 4.5 mM MgCl_2 , and 5 units of BioExact DNA polymerase (Bioline, Canton, MA) with denaturation at 94°C for 1 min, annealing at 45°C for 30 s, and extension at 72°C for 1 min. Thirty-five cycles of amplification were performed for each reaction. Both strands of all amplification products were sequenced using amplification primers and an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

Partial 16S rRNA gene sequences were compared to those entered into each of three databases: GenBank (www.ncbi.nlm.nih.gov/GenBank/index.html), the European rRNA Database (www.psb.rug.ac.be/rRNA/index.html), and the Ribosomal Database Project II (<http://rdp.cme.msu.edu/index.jsp>). *rpoB* sequences were compared to those entered in GenBank. Isolates were identified to the genus level if gene sequences exhibited homology equivalent to those of more

TABLE 1. Identification of 12 control isolates

Assay	No. (%) of correctly identified isolates	
	Species level	Genus level
API Coryne	9 (75.0)	9 (75.0)
BBL Crystal	7 (58.3)	7 (58.3)
RapID CB Plus	5 (41.7)	5 (41.7)
Broad-range 16S rRNA gene sequencing	4 (33.3)	9 (75.0)
Coryneform-optimized 16S rRNA gene sequencing	9 (75.0)	11 (1.7)
<i>rpoB</i> gene sequencing	8 (66.7)	8 (66.7)

than one member species of a genus of bacteria and to the species level if the isolate was homologous to a single bacterial species. Isolates with gene sequence homology to a sequence entered into any database of less than 96% or for which interpretable nucleotide sequence could not be obtained were considered to be not identifiable.

Statistics. Binomial distribution was used to compute confidence intervals (CIs) for the proportion of clinical isolates with an identification made with high confidence. Definitions of high confidence for each assay are given above. Moesteller's test, also known as the exact McNemar test, was used to perform pairwise comparisons of the high-confidence identification rates of assays on clinical samples. Descriptive analyses were performed for the control isolates, which could not be considered to be a random sample. No multiple testing adjustments were performed. All analyses were performed using SAS software (SAS Institute, Cary, NC) and Windows, version 9.1.3.

RESULTS

Identification of control strains. When each test was evaluated on an individual basis, species-level identification was successful in 9 of 12 isolates (75.0%) using API Coryne, 5 of 12 isolates (41.7%) using RapID CB Plus, and 7 of 12 isolates (58.3%) using BBL Crystal (Table 1 and Table 2). Correct phenotypic identification of control strains to the species level was achieved by all three testing methods for only three isolates and by two methods for six isolates. No phenotypic method correctly identified *Corynebacterium minutissimum*, *Corynebacterium amycolatum*, or *Microbacterium testaceum* to the species or genus level. Genus-level identifications that were correct and that had a high assigned confidence level were achieved by API Coryne in 9 of 12 isolates (83.3%), by RapID CB Plus in 5 of 12 isolates (66.7%), and by BBL Crystal in 7 of 12 isolates (58.3%). Those organisms not correctly identified consisted predominantly of control species not included in the test kit databases, isolates that did not produce a pattern of reactions that could be "coded out" using kit databases, and isolates that could not be differentiated among several possible genospecies based on reaction patterns. Few incorrect identifications were seen among control isolates; that is, when a single identification was produced, it was usually correct.

Species-level identification was successful for 4 of 12 isolates (33.3%) and genus-level identification was successful for 8 of 12 isolates (66.7%) using broad-range 16S rRNA gene primers. The use of primers optimized for amplification of coryneform bacterial 16S rRNA genes gave the correct identification more frequently, with 9 of 12 (75.0%) strains identified to the species level and 11 of 12 (91.7%) strains identified to the genus level. Amplification and genotyping of *rpoB* were intermediate in sensitivity, correctly classifying 8 of 12 (66.7%) strains to the

TABLE 2. Identification of 12 control strains of coryneform bacteria by automated phenotypic and genotypic systems^a

Identification of control strain by:						
Source	API	RapID	BBL Crystal	Broad-range 16S rRNA gene sequencing	Coryneform-optimized 16S rRNA gene sequencing	rpoB gene sequencing
<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. urealyticum</i>	<i>C. urealyticum</i>	<i>C. urealyticum</i>	No ID	<i>C. urealyticum</i>	<i>C. urealyticum</i>	<i>C. urealyticum</i>
<i>C. pseudotuberculosis</i>	<i>C. pseudotuberculosis</i>	No ID	<i>C. pseudotuberculosis</i>	<i>C. ulcerans</i>	<i>Corynebacterium</i> sp.	<i>C. pseudotuberculosis</i>
<i>C. jeikeium</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>	No ID	<i>Corynebacterium</i> sp.	<i>C. jeikeium</i>	<i>C. jeikeium</i>
<i>C. aquaticum</i>	<i>C. aquaticum/Aureobacterium</i> sp.	<i>L. aquatica</i>	<i>C. aquaticum</i>	No ID	No ID	No ID
<i>C. amycolatum</i>	No ID	<i>Brevibacterium</i> sp.	No ID	No ID	<i>C. amycolatum</i>	<i>C. amycolatum</i>
<i>M. testaceum</i>	<i>Aureobacterium</i> sp./ <i>Arthrobacterium</i> sp./ <i>Cellulomonas</i> sp./ <i>Microbacterium</i> sp.	<i>L. aquatica</i>	No ID	<i>Microbacterium</i> sp.	<i>Microbacterium</i> sp.	No ID
<i>R. equi</i>	<i>R. equi</i>	No ID	<i>R. equi</i>	No ID	<i>R. equi</i>	<i>R. equi</i>
<i>C. minutissimum</i>	No ID	<i>Brevibacterium</i> sp.	No ID	No ID	<i>C. minutissimum</i>	<i>Corynebacterium</i> sp.
<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>	<i>C. pseudodiphtheriticum</i>
<i>A. pyogenes</i>	<i>A. pyogenes</i>	No ID	<i>A. pyogenes</i>	<i>Arcanobacterium</i> sp.	<i>A. pyogenes</i>	No ID
<i>C. renale</i>	<i>C. renale</i> group	No ID	<i>C. renale</i> group	<i>C. renale</i>	<i>C. renale</i>	<i>C. renale</i>

^a No ID, no identification.

^a No ID, no identification.

TABLE 3. Comparison of identifications of 38 clinical isolates using phenotypic and genotypic systems^a

Identification of isolates by API	Identification of isolates by:				
	RapID	BBL Crystal	Broad-range 16S rRNA gene sequencing	Coryneform-optimized 16S rRNA gene sequencing	<i>rpoB</i> gene sequencing
<i>C. striatum</i>	No ID	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. jeikeium</i>	<i>C. pseudotuberculosis</i>	No ID	<i>C. jeikeium</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>
<i>C. jeikeium</i>	No ID	No ID	<i>C. amycolatum</i>	<i>C. amycolatum</i>	<i>C. amycolatum</i>
Corynebacterium group G	<i>C. minutissimum</i>	<i>C. striatum</i>	No ID	Corynebacterium sp.	<i>C. aurimucosum</i>
<i>Lacterius</i> sp.	No ID	No ID	No ID	<i>Lactobacillus</i> sp.	No ID
<i>C. aquaticum</i> / <i>C. amycolatum</i>	<i>C. amycolatum</i>	<i>C. aquaticum</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>
<i>C. striatum</i> / <i>C. amycolatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	No ID	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. pseudotuberculosis</i>	<i>C. pseudotuberculosis</i>	No ID	<i>C. riegeli</i>	<i>C. riegeli</i>	<i>C. riegeli</i>
Corynebacterium group G	<i>C. minutissimum</i>	No ID	<i>C. imitans</i>	<i>C. appendices</i>	<i>C. imitans</i>
<i>C. aquaticum</i> / <i>Aureobacterium</i> sp.	<i>Oerskovia</i> sp.	<i>C. aquaticum</i>	No ID	No ID	No ID
<i>C. striatum</i> / <i>C. amycolatum</i>	Corynebacterium group G	<i>C. striatum</i>	<i>C. amycolatum</i>	<i>C. amycolatum</i>	<i>C. amycolatum</i>
Corynebacterium group G	No ID	<i>C. striatum</i>	No ID	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. propinquum</i>	No ID	<i>C. pseudodiphtheriticum</i>	No ID	<i>C. striatum</i>	No ID
<i>C. urealyticum</i>	No ID	<i>C. pseudotuberculosis</i>	No ID	<i>Rhodococcus erythropolis</i>	No ID
<i>C. aquaticum</i>	<i>C. aquaticum</i>	<i>C. aquaticum</i>	<i>Microbacterium</i> sp.	<i>Microbacterium</i> sp.	No ID
No ID	<i>C. aquaticum</i>	<i>C. aquaticum</i>	<i>Microbacterium</i> sp.	<i>Microbacterium</i> sp.	No ID
<i>C. accolens</i>	No ID	<i>C. striatum</i>	<i>C. simulans</i>	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. propinquum</i>	<i>C. striatum</i>	<i>C. ulcerans</i>	No ID	<i>C. amycolatum</i>	<i>C. amycolatum</i>
<i>C. propinquum</i>	No ID	<i>C. striatum</i>	<i>C. imitans</i>	Corynebacterium sp.	Corynebacterium sp.
<i>C. urealyticum</i>	No ID	<i>C. pseudotuberculosis</i>	No ID	<i>C. amycolatum</i>	No ID
No ID	<i>C. xerosis</i>	<i>C. striatum</i>	No ID	<i>Leucobacter</i> sp.	No ID
<i>C. propinquum</i>	<i>Oerskovia</i> sp.	<i>C. propinquum</i>	No ID	No ID	No ID
No ID	<i>Oerskovia</i> sp.	No ID	No ID	<i>Microbacterium</i> sp.	No ID
<i>C. aquaticum</i>	<i>Leifsonia aquatica</i>	<i>C. aquaticum</i>	No ID	<i>Microbacterium</i> sp.	No ID
<i>C. aquaticum</i> / <i>Aureobacterium</i> sp.	No ID	<i>C. aquaticum</i>	No ID	<i>Actinomyces europaeus</i>	No ID
<i>C. afermentans</i>	<i>C. minutissimum</i>	<i>C. propinquum</i>	No ID	<i>C. mucofaciens</i>	No ID
<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	No ID	<i>C. amycolatum</i>	<i>C. amycolatum</i>
Corynebacterium group G	<i>C. striatum</i>	<i>C. striatum</i>	No ID	<i>C. striatum</i>	<i>C. striatum</i>
<i>C. jeikeium</i>	<i>C. minutissimum</i>	Corynebacterium sp.	No ID	Corynebacterium sp.	No ID
No ID	No ID	<i>C. aquaticum</i>	<i>Microbacterium</i> sp.	<i>Microbacterium</i> sp.	No ID
No ID	Corynebacterium group G	<i>C. renale</i> group	No ID	<i>Kocuria kristinae</i>	No ID
No ID	No ID	<i>C. aquaticum</i>	<i>Microbacterium</i> sp.	<i>Microbacterium</i> sp.	No ID
No ID	<i>C. jeikeium</i>	<i>C. bovis</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>	<i>C. jeikeium</i>
<i>C. striatum</i> / <i>C. amycolatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	<i>C. bovis</i>	<i>C. bovis</i>	<i>C. bovis</i>
<i>C. striatum</i>	<i>C. striatum</i>	<i>C. striatum</i>	No ID	<i>Leucobacter</i> sp.	No ID
No ID	No ID	<i>C. aquaticum</i>	<i>Curtobacterium</i> sp.	<i>Curtobacterium</i> sp.	No ID
No ID	<i>Leifsonia aquatica</i>	<i>C. aquaticum</i>	No ID	<i>Curtobacterium</i> sp.	No ID
No ID	No ID	<i>C. aquaticum</i>	<i>Clavibacter xyli</i>	<i>Leifsonia xyli</i>	No ID

^a No ID, no identification.

species level and 9 of 12 (75%) strains to the genus level. Identification of control strains to the species level was achieved by all three testing methods in four cases (33.3%), only one more case than was achieved by using phenotypic methods. As with phenotypic classifications, no method successfully identified *Microbacterium testaceum* to the species level. *Corynebacterium minutissimum* and *Corynebacterium amycolatum* (missed by all phenotypic methods) were identified to the species level by coryneform-optimized 16S rRNA gene primers. *Leifsonia aquatica*, correctly identified by all phenotypic methods, was not identified using any of the sequencing methods. Organisms not correctly identified consisted of those producing only genus-level sequence matches, those whose sequence did not match any sequences in the three databases that were searched, and those with poor amplification reactions. There were no instances wherein a high-confidence sequence-derived species- or genus-level identification did not agree with its corresponding control strain.

Identification of clinical isolates. All clinical isolates appeared to be unique (Table 3). The limited ability of each method to provide a definitive identification from clinical bacterial isolates was notable. Among phenotypic methods, API Coryne, BBL Crystal, and RapID CB Plus produced high-confidence genus-level identifications in 28 of 38 (73.7% [95% CI, 56.9% to 86.6%]), 32 of 38 (84.2% [95% CI, 68.8% to 94.0%]), and 24 of

38 (63.2% [95% CI, 46.0 to 78.2%]) cases, respectively (Table 4). Among genotypic methods, coryneform-optimized 16S rRNA gene sequencing produced a genus-level identification in 36 of 38 (94.7% [95% CI, 82.2% to 99.4%]) samples. The use of broad-range 16S rRNA or *rpoB* gene sequencing yielded genus-level identification in 17 of 38 cases (44.7% [95% CI, 28.6% to 61.7%]) each. The use of API Coryne provided a species-level identification for 27 of 38 (71.1% [95% CI, 54.1 to 84.6%]) isolates. RapID CB Plus identified 21 of 38 (55.3% [95% CI, 38.3 to 71.4%]) isolates to the species level. Using BBL Crystal, identification to the species level was possible for 31 of 38 (81.6% [95% CI, 65.7 to

TABLE 4. High-confidence identification of 38 clinical isolates

Assay	No. (%) of isolates with high-confidence identification	
	Species level	Genus level
API Coryne	27 (71.1)	28 (73.7)
BBL Crystal	31 (81.6)	32 (84.2)
RapID CB Plus	21 (55.3)	24 (63.2)
Broad-range 16S rRNA gene sequencing	12 (31.6)	17 (44.7)
Coryneform-optimized 16S rRNA gene sequencing	22 (57.9)	36 (94.7)
<i>rpoB</i> gene sequencing	16 (42.1)	17 (44.7)

TABLE 5. Identification of a common species (clinical isolates)^a

Assay	No. of samples in common or % of all samples for assay:					
	API Coryne	BBL Crystal	RapidID CB Plus	<i>rpoB</i> gene sequencing	Coryneform-optimized 16S rRNA gene sequencing	Broad-range 16S rRNA gene sequencing
API Coryne	27	31.6	21.1	10.5	10.5	7.9
BBL Crystal	12	31	23.7	13.2	13.2	2.6
RapidID CB Plus	8	9	21	7.9	7.9	2.6
<i>rpoB</i> gene sequencing	4	5	3	16	36.8	23.7
Coryneform-optimized 16S rRNA gene sequencing	4	5	3	14	22	21.1
Broad-range 16S rRNA gene sequencing	3	1	1	9	8	12

^a Lightface table entries represent numbers of samples in common; boldface entries represent percentages among all samples.

92.3%]) isolates. RapID CB Plus identified significantly fewer isolates to the species level with high confidence than did BBL Crystal ($P = 0.0309$). There was not a statistically significant difference between the species-level identification rates of RapID CB Plus and those of API Coryne ($P = 0.2101$) or between those of BBL Crystal and those of API Coryne ($P = 0.4240$). No pair of methods showed significant differences in genus identification rates ($P = 0.0768$ for RapID CB Plus versus BBL Crystal; $P > 0.4$ for other comparisons).

Amplification and sequencing of 16S rRNA genes using broad-range 16S rRNA gene primers identified 12 of 38 (31.6% [95% CI, 17.5 to 48.7%]) isolates to the species level and 17 of 38 (44.7% [95% CI, 28.6 to 61.7%]) isolates to the genus level. The use of primers optimized for coryneform bacterial 16S rRNA genes was more frequently successful in classifying these isolates, identifying 22 of 38 (57.9% [95% CI, 40.8 to 73.7%]) strains to the species level and 36 of 38 (94.7% [95% CI, 82.2 to 99.4%]) strains to the genus level. Identification based on partial *rpoB* sequences was intermediate in sensitivity, identifying 16 of 38 (42.1% [95% CI, 26.3 to 59.2%]) strains to the species level and 17 of 38 (44.7% [95% CI, 28.6 to 61.7%]) strains to the genus level.

Agreement between identification systems. Compared to the case for control isolates, concordance between systems was generally reduced when results using clinical isolates were compared (Table 5). API Coryne and BBL Crystal showed species-level agreement for 12 of 38 isolates (31.6%), API Coryne and RapID CB Plus agreed in 8 of 38 cases (21.1%), and RapID CB Plus and BBL Crystal agreed in 9 of 38 cases (23.7%). Among genotypic methods, broad-range and coryneform-optimized 16S rRNA gene sequencing agreed for 8 of 38 (21.1%) isolates, broad-range 16S rRNA and *rpoB* gene sequencing agreed for 9 of 38 (23.7%) isolates, and optimized 16S rRNA and *rpoB* gene sequencing agreed for 14 of 38 (36.8%) isolates. Six isolates received common species-level identifications using all phenotypic methods; eight were identified the same with all genotypic methods. The concordance of results between phenotypic and genotypic methods was even poorer than that seen among each of these groups, with only one isolate showing the same result among all six methods used in the study. Three isolates identified by all phenotypic methods as being *Leifsonia aquatica* were identified as being *C. jeikeium* or *Microbacterium* sp. by sequencing methods. Genus-level identifications produced a

slightly higher level of agreement between methods (data not shown).

DISCUSSION

This study illustrates the difficulty in the identification of clinical coryneform bacterial isolates. While the availability of commercial biochemical panels and nucleic acid amplification techniques has enabled diagnostic laboratories to identify most bacterial pathogens with great accuracy and expediency, their relative efficacies in classifying more unusual opportunistic pathogens are less well described. Findings here demonstrate that commercial and nucleic acid sequencing-based methods have severe limitations in their abilities to identify coryneform bacterial isolates. These data offer a relatively comprehensive perspective on available technologies, complementing other more focused studies.

In the present study, both phenotypic and genotypic systems correctly identified 41.7 to 75.0% of control isolates to the species level, with the phenotypic systems appearing less effective than in previous reports. Certain isolates presented particular challenges. *C. amycolatum*, *M. testaceum*, and *C. minutissimum* were misidentified or not contained in databases (Table 2).

Our observations are consistent with the experiences of other investigators. Most previously published studies found that the efficacy of biochemical identification systems is limited by the need to perform additional tests to definitively identify strains to the species level and the absence of some species from relevant databases. In an evaluation of the API Coryne system, 90.5% of 407 strains of coryneform bacteria were correctly identified (8). Additional tests were required to definitively identify 55.1% of these isolates; however, 5.6% of strains were not identified, and 3.8% of strains were misidentified (8). Those findings were comparable to those of evaluations of an earlier version of the API Coryne database, although additional tests were required for only 16 to 45% of strains using the older version of the kit (5, 11, 20). Two previous studies evaluated the RapID CB Plus system. Hudspeth and colleagues previously tested 115 diverse clinical and reference strains of coryneform and phenotypically similar gram-positive rods (13). A total of 90.5% of *Corynebacterium* species and 75.0% of other coryneform bacteria were accurately identified to the species level without additional testing. Funke and co-

workers tested a larger panel of 378 strains, noting the correct identification of 73.5% of isolates to the species level with >95% probability, the correct identification of an additional 7.4% of strains with <95% probability, and classification of an additional 12.2% of strains to the genus level (7). In the latter study, 3.7% of isolates were misidentified. No previously published studies evaluated the accuracy of the BBL Crystal system for the identification of coryneform organisms. The lower rates of successful identification with commercially available systems in the current study may reflect the greater phenotypic and genotypic diversity of organisms tested. Whereas previous studies used well-characterized isolates, many of which were drawn from culture collections of laboratories, clinical strains tested in this work were obtained from highly immunocompromised patients and more likely to represent rare, opportunistic pathogens.

The accuracies of phenotypic identification systems may be limited by the entries present in their respective databases. In many instances, rare or recently described species are not always included in a particular kit's identification matrix. This can lead to a failure in identification, or it can lead to erroneous identification, wherein an isolate is matched with the closest phenotypic pattern among database entries. The latter can result in high-confidence identification that is inconsistent with either sequence-based results or results of kits that include other strains in their databases. Finally, the reliability of current database entries can limit the accuracy of either genotypic or phenotypic designations. Many entries are based on a limited number of type strains, and in some cases, phenotype may vary among strains of a given species. Such inconsistencies can also lead to disparate identifications either when different kit-based phenotypic methods are compared or when phenotypic results are compared with genotypic results. Additional difficulties when depending upon biochemical results from organisms that were particularly slow growing and relatively biochemically inert (even when propagating in logarithmic phase) may have arisen. Such characteristics may make it hard to achieve sufficient inocula for testing and may result in weak biochemical reactions that are difficult to discriminate. The latter may affect the reproducibility and accuracy of test results.

Few studies have evaluated the ability of molecular diagnostic testing to identify coryneform bacteria. In one evaluation, the MicroSeq 500 16S bacterial sequencing system correctly identified 27 of 42 (64.3%) *Corynebacterium* spp. and 5 of 6 (83.3%) related bacteria (21). Using conventional 16S rRNA gene sequencing as the "gold standard," Lau and coworkers previously noted that the most common explanation for the failure of the MicroSeq 500 16S rRNA gene identification system was a lack of a highly homologous 16S rRNA gene sequence in the database (16). In a second study, Roux et al. found that 16S rRNA gene sequencing identified 28 of 31 *Corynebacterium* spp. isolated from bone and joint infections, whereas the API Coryne system led to the correct identification of only 8 strains (19). The abilities of 16S rRNA and *rpoB* gene sequencing to identify 168 *Corynebacterium* isolates from clinical specimens were directly compared by Khamis and colleagues, who found *rpoB* sequencing to be the most sensitive assay, positively identifying 91% of isolates, compared to 81% by 16S rRNA gene sequencing (14). The variation in rates of

successful identification with molecular identification systems may reflect the phenotypic and genotypic diversity of organisms, the presence of representative strains in databases, or differences in individual assays. As in those studies, the most common problem that we encountered with broad-range 16S rRNA gene sequencing was a failure to amplify target genes or to generate a high-quality template for sequencing reactions. Using optimized 16S rRNA gene primers, difficulties regarding a limited ability to discriminate between some bacterial species, especially *Microbacterium*, were encountered. For example, those portions of the *Microbacterium lacticum*, *M. aerolatum*, and *M. paraoxydans* ribosomal alleles amplified by this primer set shared identical nucleotide sequences (GenBank accession numbers AB007415.1, AJ309929.1, and AJ491806.1, respectively). In addition, the inclusion of large numbers of 16S rRNA gene sequences from uncultivated and incompletely annotated bacterial strains in databases complicated the assignment of some isolates. In several instances, clinical isolates had 16S rRNA gene sequences that were more homologous to those of a *Corynebacterium* isolate entered into databases that was not identified to the species level than to well-characterized bacteria. When *rpoB* sequencing failed to identify an isolate, in most cases, this was because the amplification and sequencing of genes were possible but closely homologous gene sequences were not available in the GenBank database. It is likely that the availability of data from newly reported bacterial genomes and from projects designed to evaluate the degree of genetic diversity among bacteria will increase the robustness of genotyping systems.

This study is the first to directly compare the relative effectiveness of commonly used phenotypic and genotypic systems for the identification of coryneform bacteria. Our results indicate that not only did all identification systems have relatively low rates of identifying coryneform bacteria, but different classification systems resulted in a high proportion of discordant identifications. A relatively low percentage of well-characterized control isolates in this study was accurately identified to the species level by any given assay, and in only 2 of 12 cases did all assays successfully classify an isolate. Furthermore, for the strains tested in this study, the use of a combination of two systems would not be superior for all isolates compared to a single test.

In contrast to previous reports, we found that the ability of nucleotide acid amplification-based identification systems to correctly identify control isolates was comparable to that of phenotypic assays in this study but that, collectively, genotypic assays were less likely to identify clinical isolates and were about as likely to concur with one another as were phenotypic assays. Our data also demonstrate that an assay that amplifies and sequences the 16S rRNA gene using relatively broad-range primers is inferior to one using primers optimized for coryneform bacteria. The development of a procedure incorporating several different primer sets that target groups of genetically related bacteria may be required for optimal sensitivity. The specificity of 16S rRNA gene amplification-based systems would likely be improved by the amplification and sequencing of a larger segment of the gene, but technical constraints on the lengths of amplification products that can be conveniently sequenced by automated systems limit this approach. The efficacy of using alternative amplification targets

for genetic identification is currently limited by the inclusiveness of genetic databases. It is possible, however, that *rpoB*, other housekeeping genes, or a multilocus sequence strategy may provide optimal sensitivity and specificity. For all nucleotide amplification-based assays, the rapid proliferation of sequence data available for environmental and other uncharacterized bacteria will necessitate the development of algorithms that more effectively gate searches for homologous gene sequences.

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