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Identification of Coryneform Bacterial Isolates by Ribosomal DNA Sequence Analysis

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Identification of coryneform bacteria to the species level is important in certain circumstances for differentiating contamination and/or colonization from infection, which influences decisions regarding clinical intervention. However, methods currently used in clinical microbiology laboratories for the species identification of coryneform bacteria are often inadequate. We evaluated the MicroSeq 500 16S bacterial sequencing kit (Perkin-Elmer Biosystems, Foster City, Calif.), which is designed to sequence the first 527 bp of the 16S rRNA gene for bacterial identification, by using 52 coryneform gram-positive bacilli from clinical specimens isolated from January through June 1993 at the Mayo Clinic. Compared to conventional and supplemented phenotypic methods, MicroSeq provided concordant results for identification to the genus level for all isolates. At the species level, MicroSeq provided concordant results for 27 of 42 (64.3%) *Corynebacterium* isolates and 5 of 6 (83.3%) *Corynebacterium*-related isolates, respectively. Within the *Corynebacterium* genus, MicroSeq gave identical species-level identifications for the clinically significant *Corynebacterium diphtheriae* (4 of 4) and *Corynebacterium jeikeium* (8 of 8), but it identified only 50.0% (15 of 30) of other species ($P < 0.01$). Four isolates from the genera *Arthrobacter*, *Brevibacterium*, and *Microbacterium*, which could not be identified to the species level by conventional methods, were assigned a species-level identification by MicroSeq. The total elapsed time for running a MicroSeq identification was 15.5 to 18.5 h. These data demonstrate that the MicroSeq 500 16S bacterial sequencing kit provides a potentially powerful method for the definitive identification of clinical coryneform bacterium isolates.

Coryneform bacteria include variety groups of aerobically growing, asporogenous, non-acid-fast, irregularly shaped, gram-positive rods which are very diverse not only morphologically but also metabolically and structurally (8). Within the last few years, clinically relevant coryneform bacteria have been encountered with increasing frequency in human specimens, and many new taxa of coryneform bacteria have been described (11, 12). As a result, clinical microbiologists are often confronted with making identifications within this heterogeneous group as well as with determining the clinical significance of such isolates. However, the majority of coryneform bacteria are environmental residents and/or normal flora, and they are isolated very frequently in clinical laboratories. Thus, it is often difficult to determine their potential clinical significance. Whereas anatomical site, organism predominance, and organism concentration have been used to determine clinical significance, identification of coryneform bacteria to the species level might be useful for distinguishing sources of contamination, colonization, or infection, thereby determining the need for clinical intervention.

However, currently used methods for coryneform bacterial identification in clinical microbiology laboratories are substantially deficient when faced with the diversity of organisms they are expected to identify. A new genotypic identification system, the MicroSeq 500 16S bacterial sequencing kit (Perkin-Elmer [PE] Biosystems, Foster City, Calif.), is designed to sequence the first 527 bp of the 16S rRNA gene (2) for bacterial identification. The system is a simplified version of the original MicroSeq system (17, 18), which uses only two sequencing primers to analyze a single PCR product, thereby significantly reducing the cost and labor required for identification. Due to the difficulty of identifying coryneform bacteria with standard phenotypic methods, we compared this system's ability to differentiate clinical coryneform isolates with that of conventional phenotypic identification methods.

Clinical isolates and conventional identification. Coryneform gram-positive bacilli evaluated in this study were human isolates received by the Mayo Referral Bacteriology Laboratory from January through June 1993. All isolates were subcultured on Columbia agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood for 24 h at 37°C in a 5% CO_2 atmosphere. They were identified by conventional biochemical methods (6), supplemented with the API (RAPID) Coryne system version 1 (5, 7, 9, 16), the Biolog GP microstation system (13), and cellular fatty acid profiles (1, 19). Isolates for which discordant results were obtained were sent to the Department of Medical Microbiology at the University of Zurich for further identification (6, 7, 19). Four *Corynebacterium diphtheriae* isolates were provided by the College of American Pathologists through proficiency tests. Taxonomy was based on newly published reviews (8, 11, 12).

Genotypic identification. The MicroSeq 500 16S bacterial sequencing kit (PE Biosystems) contains a PCR and cycle

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TABLE 1. Genotypic identification of clinical coryneform bacterial isolates compared with conventional phenotypic methods

Organism	Genus level		Species level	
	No. tested	No. matched (%)	No. tested	No. matched (%)
Corynebacterium species	42	42 (100.0)	42	27 (64.3)
C. diphtheriae	4	4 (100.0)	4	4 (100.0)
C. jeikeium	8	8 (100.0)	8	8 (100.0)
Other	30	30 (100.0)	30	15 (50.0)[b]
Corynebacterium-related species[a]	10	10 (100.0)	6	5 (83.3)
Total	52	52 (100.0)	48	32 (66.7)

[a] See text and reference 8 for definition.
[b] Fifteen unmatched *Corynebacterium* isolates. Eight phenotypically identified as *C. amycolatum* were identified as *C. xerosis* by MicroSeq, 1 *C. glucuronolyticum* was identified as *C. seminale*, 1 *C. pseudodiphtheriticum* isolate was identified as *C. pseudotuberculosis*, 1 *C. minutissimum* isolate was identified as *C. xerosis*, 1 *C. afermentans* isolate was identified as *C. urealyticum*, 1 CDC group G isolate was identified as *C. tuberculostearicum*, 1 *C. striatum* isolate was identified as *C. minutissimum*, and 1 *C. imitans* isolate was identified as *C. xerosis*.

TABLE 2. Species identification by 16S sequencing of gram-positive bacilli not identified to species level by phenotypic methods

Isolate	Phenotypic identification	Genotypic identification	% Difference from ATCC strain[a]
M17	*Arthrobacter* sp.	*Arthrobacter globiformis*	0.6
M25	*Brevibacterium* sp.	*Brevibacterium casei*	0.2
M32	*Microbacterium* sp.	*Microbacterium lacticum*	0.8
M89	*Microbacterium* sp.	*Microbacterium lacticum*	3.2

[a] ATCC, American Type Culture Collection.

sequencing module, bacterial identification and analysis software, and a 16S ribosomal DNA sequence database library. Bacterial genomic DNA isolation and PCR amplification of the first 527 bp of the 16S rRNA gene (2) were performed according to the manufacturer's instructions. Double-stranded sequence analysis of the first 527 bp was completed by using the ABI PRISM 377 or 310 DNA sequencer (PE Biosystems). Using the MicroSeq microbial identification and analysis software, sequence sample files were assembled, and the final consensus sequence was compared with over 1,100 validated 16S ribosomal DNA sequences in the MicroSeq database library. Polymorphic positions present in those organisms containing multiple copies of the gene were included to ensure the highest degree of accuracy (17).

From 56 coryneform isolates available, 37 (66.1%) were sent to Zurich for further identification and confirmation. Eight isolates were withdrawn because of discrepant results between Mayo and Zurich. Therefore, a total of 52 coryneform isolates, including 4 *C. diphtheriae*, were included in our study. Among them, 48 were identified to species level by conventional phenotypic methods, which served as our evaluation standard. The isolates identified to the species level included 42 *Corynebacterium* strains (3 *C. afermentans*, 9 *C. amycolatum*, 1 *Corynebacterium* CDC group G, 1 *C. coyleae*, 4 *C. diphtheriae*, 1 *C. glucuronolyticum*, 1 *C. imitans*, 8 *C. jeikeium*, 3 *C. minutissimum*, 7 *C. pseudodiphtheriticum*, 2 *C. striatum*, and 2 *C. xerosis* strains) and 6 *Corynebacterium*-related strains (1 *Actinomyces neuii*, 1 *Arcanobacterium pyogenes*, 1 *Curtobacterium pusillum*, 1 *Exiguobacterium acetylicum*, and 2 *Microbacterium luteolum* strains).

A total of 15.5 to 18.5 h was needed to run the MicroSeq system, including 1 h for DNA extraction, 3 h for PCR amplification, 0.5 h for sequence reaction preparation, 10 to 15 h for cycling sequencing, and 1 h for data analysis. Compared to the conventional, phenotypic method, MicroSeq provided identical genus-level identification for all isolates. At the species level, MicroSeq matched 27 of 42 (64.3%) *Corynebacterium* isolates and 5 of 6 (83.3%) *Corynebacterium*-related isolates, respectively (Table 1). Within the *Corynebacterium* isolates, MicroSeq correctly identified the known clinically significant species, *C. diphtheriae* (4 of 4) and *C. jeikeium* (8 of 8), but only 50.0% (15 of 30) correlation was achieved for other *Corynebacterium* species ($\chi^2 = 9.33$, $P < 0.01$). MicroSeq apparently performed better with the *Corynebacterium*-related species than with the *Corynebacterium* species itself, but the difference did not achieve statistical significance ($\chi^2 = 0.86$, $P = 0.35$).

In our study, four *Corynebacterium*-related isolates were not identified to the species level by conventional methods due to technical inability or complexity. In contrast, species-level identification was provided by MicroSeq with an average sequence difference of 1.20% (95% confidence interval is 0.12 to 2.18%) relative to the American Type Culture Collection prototype strains (Table 2). These data suggest that unrecognizable isolates can be assigned at least tentatively to a phylogenetic branch at the genus or species level in the absence of clearly defined biochemical parameters.

The classification of coryneform bacteria, which is based primarily on complicated, often overlapping, biochemical reactions, has been significantly improved by gradually incorporating genetic analyses such as the 16S rRNA gene sequence analysis and nucleic acid hybridization (8, 14, 15). The low (64.3%) rate of agreement between standard phenotypic and MicroSeq genotypic methods for identification of *Corynebacterium* isolates probably shows the uncertainty of the taxonomy assessed by subjective parameters (11, 12). While the limits of the currently used identification systems are evident, two clinically significant species, *C. diphtheriae* and *C. jeikeium*, were consistently identified correctly by all the systems we used, thus demonstrating the accuracy of both phenotypic and genotypic methods in identifying clinically significant pathogenic *Corynebacterium* isolates encountered in the clinical microbiology laboratory. While *C. diphtheriae* is rarely seen, rapid, definitive identification of *C. jeikeium* is important in the clinical setting, since the majority of *C. jeikeium* isolates have been reported to be resistant to most antibiotics except vancomycin (10).

One limitation of 16S rRNA gene sequencing-based identification is its inability to assign a species to representatives of recently diverged species, such as some *Bacillus* and *Neisseria* strains (3, 4, 20). Thus, the suitability of the 16S rRNA gene sequence should be determined on a case-by-case basis. Sequencing studies have demonstrated that coryneform bacteria evolved at an early stage, thereby leading to a significant differentiation (14, 15). These data suggest that 16S rRNA gene sequence analysis can not only play a significant role in the assignment of species designations but also provide an unambiguous species-level identification of coryneform bacteria in the clinical laboratory. Even more important, sequence analysis may provide a degree of resolution that allows discrimination of *Corynebacterium* and related species isolated from the same patient, which may help to determine the clinical significance of such multiple isolates.

Bacterial identification based on the MicroSeq is also faster than conventional methods. Phenotypic identification of coryneform bacteria routinely requires 3 to 7 days; however, some of these supplemented techniques can take weeks to complete.

Identification based on the MicroSeq can be completed within 48 h and can potentially be performed directly on blood cultures. Meanwhile, driven in part by technological progress in the human and microbial genome projects, sequencing costs will probably continue to fall rapidly, bringing this technology within the reach of many microbiology laboratories (18). Currently, based on our assessment, the direct cost of running the MicroSeq system is $84.25 per test, which includes test kits, materials, reagents, and laboratory personnel salaries (unpublished data). As the MicroSeq database becomes more comprehensive, this genotypic identification system may soon become a cost-effective alternative to conventional methods in identifying difficult-to-identify organisms, extending to rapid diagnosis.

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