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The Role of 16S rRNA Gene Sequencing in Identification of Microorganisms Misidentified by Conventional Methods

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Traditional methods for microbial identification require the recognition of differences in morphology, growth, enzymatic activity, and metabolism to define genera and species. Full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms. We report on three bacterial blood isolates from three different College of American Pathologists-certified laboratories that were referred to ARUP Laboratories for definitive identification. Because phenotypic identification suggested unusual organisms not typically associated with the submitted clinical diagnosis, consultation with the Medical Director was sought and further testing was performed including partial 16S rRNA gene sequencing. All three patients had endocarditis, and conventional methods identified isolates from patients A, B, and C as a *Facklamia* sp., *Eubacterium tenue*, and a *Bifidobacterium* sp. 16S rRNA gene sequencing identified the isolates as *Enterococcus faecalis*, *Cardiobacterium valvarum*, and *Streptococcus mutans*, respectively. We conclude that the initial identifications of these three isolates were erroneous, may have misled clinicians, and potentially impacted patient care. 16S rRNA gene sequencing is a more objective identification tool, unaffected by phenotypic variation or technologist bias, and has the potential to reduce laboratory errors.

Most clinical laboratories rely on manual, automated, or semiautomated phenotypic methods and commercial systems for identification of bacterial pathogens. Algorithms and databases used for the interpretation of results from these methods are based on characteristics observed in known and reference strains, with predictable biochemical and physical properties under optimal growth conditions. Phenotypic profiles including Gram stain results, colony morphologies, growth requirements, and enzymatic and/or metabolic activities are generated, but these characteristics are not static and can change with stress or evolution (5). Thus, when common microorganisms present with uncommon phenotypes, when unusual microorganisms are not present in reference databases, or when databases are out of date, reliance on phenotypes can compromise accurate identification. Technologist bias or inexperience with an unusual phenotype or isolate may similarly compromise identification when results of biochemical tests are interpreted to fit expectations. Although not perfect, genotypic identification of microorganisms by 16S rRNA gene sequencing has emerged as a more objective, accurate, and reliable method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria (2).

There is scarce data in the clinical microbiology literature evaluating the frequency of laboratory errors or their impact on patient care, and only recently have Yuan et al. demonstrated the potential value of collecting these data prospectively (8). Furthermore, we are aware of no published data examining the frequency of errors resulting from phenotypic misidentification or how often these "errors" adversely impact patient care. We identified three cases of endocarditis where the initial identification by phenotypic methods was erroneous and genotypic identification by 16S rRNA gene sequencing provided clinicians with a more accurate and meaningful result.

Three bacterial blood isolates from three different College of American Pathologists-certified laboratories were referred to ARUP Laboratories for definitive identification. All three isolates were reviewed by multiple technologists and underwent a full battery of phenotypic tests including manual biochemical testing, Biolog (GP2 and GN2 MicroPlates; Biolog, Inc., Hayward, CA), RapID ANA II (Remel, Lenexa, KS), and API 20A (bioMerieux, Inc., Durham, NC) methods. In each case, concern that the phenotypic identification was not consistent with the clinical diagnosis of endocarditis as indicated on the requisition slip led to Medical Director consultation. Subsequently, all three isolates were referred for 16S rRNA gene sequencing analysis. Bacterial colonies were resuspended in 50 μl of DNA extraction reagent and incubated for 10 min at 95°C, after which 2 μl of lysate was combined with 10 μl of PCR master mix and 8 μl of reagent-grade water in a 0.2-ml tube (MicroSeq bacterial identification kit; Applied Biosystems, Foster City, CA). Following an initial 5-min hold at 94°C, a 500-bp 16S rRNA gene fragment was amplified by 30 cycles of denaturing at 94°C (30 s), annealing at 60°C (30 s), and extension at 72°C (45 s), with a final 2-min extension at 72°C and a 4°C hold. PCR products were purified using ExoSAP-IT reagent (USB Corporation, Cleveland, Ohio) as per manufacturer's instructions and then sequenced using the original amplification primers. Forward and reverse sequencing reactions were performed with BigDye Terminator cycle sequencing ready reaction kit reagents on an ABI Prism 310 or 377 DNA sequencer (Applied Biosystems, Foster City, CA) by standard protocols. Sequences were assembled, edited, and identified using the MicroSeq software, with comparison to reference

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TABLE 1. Phenotypic and genotypic identification of three patients with endocarditis

Bloodstream isolate	Species identification (% similarity)					
	Conventional methods				16S rRNA gene sequencing	
	Manual	Biolog	RapID ANA	API 20A	MicroSeq	GenBank
A	*Facklamia* sp.	*Pediococcus* sp., *Lactococcus* sp., or *Gemella* sp.			*Enterococcus faecalis* (99.82)	*Enterococcus faecalis* (100)
B	*Eubacterium tenue*	*Pasteurella volantium*	*Fusobacterium mortiferum*		*Cardiobacterium valvarum* (98.6)	*Cardiobacterium valvarum* (98)
C			*Bifidobacterium* sp. or *Streptococcus constellatus*	*Bifidobacterium* sp.	*Streptococcus mutans* (99.82)	*Streptococcus mutans* (99)

sequences in the MicroSeq 500 bacterial database, version 1.4.3, and GenBank reference sequences. Alignments and phylogenic trees were constructed using AlignX software (Vector NTI Suite; Invitrogen, Carlsbad, CA) and type strains (when available) from GenBank reference sequences.

For patient A, Gram stain morphology and a manual biochemical profile appeared most consistent with identification as a *Facklamia* sp. Several attempts at a more definitive identification with Biolog tools created further ambiguity, in which the microorganism was identified on three different attempts as three different microorganisms, namely, a *Pediococcus* sp., a *Lactococcus* sp., and a *Gemella* sp. Partial and full 16S rRNA gene sequencing identified the microorganism as *Enterococcus faecalis*, with 99.82% similarity compared to the reference strain in the MicroSeq database and 100% similarity compared to the top five isolates in GenBank (Table 1). Phylogenetic analysis demonstrated that our isolate aligned most closely with *Enterococcus faecalis* (JCM 5803) (99.8%), sharing less similarity with *Enterococcus faecium* (ATCC 19434) (92.3%), *Pediococcus inopinatus* (DSM 20285) (81.9%), *Lactococcus lactis* (ATCC 19435) (81.5%), *Gemella morbillorum* (ATCC 27824) (81%), and *Facklamia hominis* (ATCC 700628) (80.6%). In patient B, the referring laboratory thought the patient's isolate was most consistent with the aerotolerant anaerobe *Eubacterium tenue.* A Gram stain of isolated colonies on sheep blood agar appeared most consistent with overdecolorized gram-positive bacilli with terminal swellings. RapID ANA identified the microorganism as *Fusobacterium mortiferum* (99.9% similarity) while Biolog identified the microorganism as *Pasteurella volantium*. Partial 16S rRNA gene sequencing identified the isolate as *Cardiobacterium valvarum* (98.6% similarity to MicroSeq database, 98% to GenBank). Phylogenetic analysis demonstrated that our isolate aligned most closely with four GenBank entries identified as *C. valvarum* isolates (AY596470, 98.4% similarity; AY506987, 98.2%; AY596469, 98.2%; and AY596468, 97.7%), and shared less similarity with *C. hominis* (ATCC 15826) (92.6%), *Pasteurella volantium* (NCTC 3438T) (81.8%), and *Fusobacterium mortiferum* (ATCC 25557T) (67.4%). Similarly, in patient C, a Gram stain of the isolate from direct colonies was described as showing gram-positive bacilli and the microorganism had improved growth in increased CO_2 conditions. The microorganism was insufficiently characterized by RapID ANA, by which identification varied with the selected reference database as either a *Bifidobacterium* sp. (gram-positive rod database: similarity of >99%, bioscore 1/11,965, probability "questionable") or *Streptococcus constellatus* (gram-positive cocci database: similarity of 92.3%, bioscore 1/1,812, probability "inadequate"). API 20A identified the microorganism as a *Bifidobacterium* sp. (96.5% similarity) while partial 16S rRNA gene sequencing identified it as *Streptococcus mutans* (99.82% similarity with MicroSeq, 99% with GenBank). Phylogenetic analysis demonstrated that our isolate aligned most closely with *S. mutans* (NCTC 10449) (99.6%), sharing less similarity with *S. constellatus* (ATCC 27823) (89.7%) and *Bifidobacterium dentium* (ATCC 27534) (66.5%) type strains.

Phenotypic methods have numerous strengths but often fail because the phenotype is inherently mutable and subject to biases of interpretation. 16S rRNA gene sequencing is a more accurate and objective method of identification of microorganisms (1, 3, 4, 6, 7) with particular utility in the clinical laboratory. It also reduces the interpretive bias and obviates the need for a "pretest" probability regarding a microorganism's classification to direct workup and database selection. Medical technologists may pursue an erroneous identification algorithm based on their phenotypic "intuition," such that when unusual microorganisms are encountered, they are made to "fit" with technologist expectations, or when common microorganisms with atypical phenotypes are encountered, they are made to "fit" characteristics of extremely unusual pathogens. Conventional automated identification systems often rely on technologists' interpretations of a microorganism's Gram stain morphology (e.g., RapID ANA) or oxidase result (e.g., Biolog) for selecting the correct reference database. This case series demonstrates that seemingly simple biochemical or Gram reactions are not unquestionably foolproof and may lead to inappropriate use of comparative databases. Such exhaustive phenotypic testing potentially delays turnaround time without the added benefit of accuracy.

For serious infections such as endocarditis, the putative pathogen often suggests the underlying pathophysiology of disease. When phenotypic methods identify a microorganism that is not typically associated with a specific infectious disease, 16S rRNA gene sequencing can offer a more consistently reliable and accurate method for identification. Although we were unable to determine if phenotypic misidentification for these

three cases adversely affected patient care, 16S rRNA gene sequencing has the potential to prevent laboratory errors and provide more meaningful results for clinicians. The frequency of misidentification by phenotypic methods compared to 16S rRNA gene sequencing and its impact on patient care are unknown and warrant further study.

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