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GUEST COMMENTARY

Bacterial Identification for Publication: When Is Enough Enough?

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The accurate and definitive identification of microorganisms, including bacteria, is one of the cornerstones forming the joint foundation of the fields of microbiology and infectious diseases. Identification is based upon the labeling of bacteria, parasites, and fungi with appropriate binomial names of Latin or Greek origin. Bacterial nomenclature thus provides the foundation from which host-parasite disease relationships are defined, therapeutic regimens are developed, and epidemiological investigations (e.g., comparative analysis of bacterial strains involved in outbreaks) are instigated.

A recent correspondence in *Clinical Infectious Diseases* (W. Frederiksen and B. Tenning, Letter, Clin. Infect. Dis. **32:**987-989, 2001) highlighted problems concerning the potential misidentification of bacteria when commercial identification systems are used. However, this concern is only the tip of an iceberg of a potentially larger problem with more important ramifications. With the advent of the availability of commercial systems for rapid analysis and the use of molecular methods to provide genus and species identifications, the number of case reports linking old agents to new diseases and new or unusual (rare) agents to infectious processes has risen dramatically. However, equally important with regard to these published case reports describing new pathogenic taxa is the scientific accuracy of the identification of each species. Reliance on a single identification method when publishing can lead to misidentification, which can translate into an inaccurate body of information in the medical literature concerning the clinical significance of many microbial species. A compounding problem is the dramatic increase in the number of new bacterial species of medical or veterinary importance that are described on the basis of the sequence analysis of the 16S ribosomal DNA (rDNA) genes of one or two strains (3, 32). It is the goal of this article to briefly review pertinent aspects of this topic and to discuss the evolving fields of bacterial taxonomy and nomenclature as they relate to microbiology and infectious diseases.

BACTERIAL NOMENCLATURE

The first credible approaches to the systematic classification of bacteria began in the latter part of the 19th century. These early studies separated groups of bacteria primarily on the basis of morphology, size, and motility. A pioneer investigator during this period was Ferdinand Cohn, who supported the concept of a diversity of microorganisms and argued that, within species, varieties emerged and transmitted their characteristics to the next generation (21). The subsequent development of agar-based media led to the in vitro isolation and propagation of pure cultures. This singular event fueled the first substantive biochemical investigations of bacterial species that occurred during the late 19th and early 20th centuries. However, because of the limited number of biochemical and phenotypic tests available, characterization of proposed species was inadequate and imprecise. This problem often led to confusion and resulted in the discovery and rediscovery of the same bacterial species by different investigators who gave the same taxa new names based upon slightly different morphological, cultural, and phenotypic criteria. The gram-negative bacterium *Klebsiella pneumoniae* provides an example of this taxonomic quagmire. This member of the family *Enterobacteriaceae* was listed under six different genera, with seven different species epithets, between 1885 and 1928. Between 1918 and 1960, a total of 84 descriptions of new motile species of bacteria or emendations of previously described motile species were published in the *Journal of Bacteriology*, yet of the 84 species, only 5 (6%) were properly described.

Over the first half of the 20th century, a number of approaches to the identification and classification of bacteria were entertained. Among these were the use of physiologic tests to assess the diversity of bacteria, the first codification of biochemical test results, and the initial systematic approaches to classifying different groups of bacteria. In 1957, P. H. A. Sneath introduced the use of computers to analyze morphological and biochemical characteristics as an approach to classifying bacteria through numerical taxonomy methods. This groundbreaking work eventually led to the evolution of phenetic analysis as a means to assess the relatedness of different bacteria or groups of bacteria to one another (29). Complex computer programs and large data matrices consisting of independent covariant characters can be used to assess similarity or the likelihood that an unknown organism belongs to a given taxon. For instance, it is generally accepted that for an isolate to belong to a given species there must be at least 80 to 85% similarity based upon unweighted-pair-group method analysis. Many other different numerical methods have been proposed to identify taxa based upon probability and nonprobalistic data matrices and companion identification matrices. These methods are reviewed in Wilcox et al. (29).

A major innovation in laboratory sciences occurred in the late 1960s, when manual miniaturized identification systems

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were first introduced into the clinical microbiology laboratory. These early systems included the nine-test Enterotube (Roche, Basel, Switzerland) and two-tube R-B systems for identifying members of *Enterobacteriaceae*. These systems offered several advantages over conventional testing, namely, all inocula for a set of tests performed sequentially were prepared from a single colony, preparation costs and the amount of storage space required for standard reagents and media were reduced, less incubation space was required, and in some instances identification times were reduced. However, biochemical reactions observed with commercial systems often did not correlate well with conventional test results, and because of the limited number of tests employed in these systems, the percentage of strains correctly identified to the species level was less than satisfactory. These kits were rapidly followed by the landmark API 20E strip test (bioMérieux-Vitek, Hazelwood, Mo.), which was a micromethod employing 20 different biochemical tests. The 20E strip generated a septyl (7-digit) code in 18 to 21 h based upon biochemical reactions plus a screening oxidase test. The numeric code could then be located in a logbook that converted septyl codes into a final bacterial identification. The system identified species in the family *Enterobacteriaceae* and common nonfermenters, such as *Pseudomonas aeruginosa*. Although a manual method, the API 20E strip test was so advanced for its time that it is still commonly used in clinical microbiology labs throughout the world and is considered by many to be the "gold standard" commercial system against which all other such systems are measured (16).

In 1978, MicroScan (now Dade Behring MicroScan, West Sacramento, Calif.) developed the first combination panel providing both organism identification and susceptibility profile determination (MIC determination) simultaneously. The 1980s saw continued improvements in diagnostic bacteriology with the introduction of automation into the laboratory (23). In 1983, MicroScan released the AS-3/touchSCAN system, the first widely accepted automated system for microbial identification and drug susceptibility testing in the clinical laboratory. Other systems, such as the Vitek AutoMicrobic system, quickly followed (23). These automated systems transformed processes for bacterial identification by reducing the time required to identify rapidly growing bacteria to as little as 2 to 4 h, in contrast to the 1 or more days that had been required previously for a final identification by conventional test methodologies.

The use of molecular biology and molecular techniques as an aid to bacterial taxonomy and identification was in its infancy in the 1960s. Early studies using DNA base composition could clearly distinguish between genomes that were unrelated based upon differences in G+C content (in moles percent) (21). However, the first major leap in molecular taxonomy and identification applicable to diagnostic microbiology occurred with the introduction of DNA-DNA hybridization studies pioneered by Don Brenner and his colleagues at the Walter Reed Army Institute of Research and later at the Centers for Disease Control and Prevention (3). The value of DNA hybridization was that it provided a quantitative definition of what constituted a species, ~70% or greater DNA-DNA relatedness with a $\triangle T_m$ of 5°C or lower (26, 30). In cases where new species were identified via DNA hybridization, it was also observed that in most instances the results of simple biochemical tests would clearly separate newly recognized or redesignated genera or species from other established groups. This allowed for taxonomic advances made through DNA hybridization studies to be easily adapted to the diagnostic laboratory through the use of new phenotypic identification schemes. However, not all newly recognized taxa that had been identified by molecular techniques could be readily identified by biochemical tests in clinical laboratories.

The downside of DNA hybridization is that it is an expensive, technically complex, and labor-intensive procedure that, at its zenith, was restricted to a small number of research or public health centers around the world. Today, very few laboratories perform DNA hybridization by classic methods. The world of molecular taxonomy was revolutionized, however, in the mid-1980s with the advent of full sequence analysis of molecular chronometers such as rRNA (21). By the mid-1990s, sequencing of the small subunit (16S) rDNA genes had become commonplace, considered a standard tool of microbial taxonomists not only for elucidating phylogenetic relatedness but also as a means of bacterial identification (15, 21). The automation of 16S rDNA gene sequencing with such instruments as the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.) allowed for a quick comparative analysis of published sequences deposited in microbial genome databases (14). Today, bacterial strains that defy identification by conventional commercial methodologies are often subjected to 16S rDNA sequence analysis so that a useful label can be placed on the isolate in question (31).

IDENTIFICATION SYSTEM LIMITATIONS

All systems used to identify bacteria, whether phenotypic or genotypic, have limitations, because no single test methodology will provide results that are 100% accurate. Phenotypic systems, the most common approach used in clinical laboratories to identify bacteria, have several drawbacks. In contrast to the properties determined by molecular methods such as DNA hybridization, biochemical properties do not accurately reflect the entire extent of the genomic complexity of a given species. Furthermore, phenotypic properties can be unstable at times and expression can be dependent upon changes in environmental conditions, e.g., growth substrate, temperature, and pH levels (21). A further problem with commercial systems is the construction of databases, not to mention which biochemical tests are included on panels. Once biochemical tests are commercially produced, panel configurations are rarely changed, although reformulation of tests does occasionally occur. For instance, the tests included on the API 20E strip in 1975 were still the same tests on the strip in 2001. Yet the number of newly described taxa increased substantially between 1975 and 2001 (7). That means that when new taxa are added to existing commercial databases they must be identified on the basis of results of tests available on these preconfigured strips or panels. Often the best tests available to identify these newer species are not on the panels or cannot be formulated to fit a micromethod. This can lead to less reliable identification results. The accuracy of commercial in-house databases used in the identification of bacterial species is dependent upon both the number of strains included in the database and the phenotypic diversity of strains tested. For species encountered less

frequently, even rarely, in the clinical laboratory, commercial databases may not have a sufficient number of bona fide strains to accurately provide a definitive identification for these isolates. Finally, most commercial systems do not consider clinical frequency or site of isolation in their bacterial identification matrices. One of the consequences of all of these limitations is that some commercial systems have great difficulty identifying certain groups of bacteria, such as *Pasteurella* species (10; Frederiksen and Tenning, letter) or *Haemophilus* species (11). Despite all of these shortcomings, commercial systems have served clinical microbiology laboratories well in the routine identification of infectious agents of medical importance. Given the number of isolates tested with these high-throughput systems, commercial systems are very accurate for the more common species and provide quick test results in a cost-effective manner.

16S rDNA gene sequencing is a powerful tool and by far the single most common molecular technique presently used for bacterial species identification (30). Although this technique relies on sequencing of the DNA that encodes the 16S rRNA subunit, like phenotypic tests, it surveys only a small portion of the microbial genome. Since ribosomal genes are highly conserved, sequence variation between strains belonging to different species from distinct genera is less evident with rDNA gene sequencing than with DNA pairing studies, where DNA relatedness values are used to compare strains. Another problem, one not faced in DNA hybridization studies (26), is that there are no consensus guidelines that define what constitutes a species based upon 16S rDNA gene sequence similarity or divergence. It is generally accepted that an unidentified isolate whose rDNA gene sequence is <97% similar to those of the isolate's closest phylogenetic neighbors constitutes a new taxon. The general availability and ease with which ribosomal genes can be sequenced has fueled an explosion in the description of new taxa. Over the past decade, approximately 40% of all newly described species have been described based upon the analysis of a single strain (4) (see, e.g., an article published in this journal) (32). This tendency is of immense concern when these species are of medical and/or veterinary importance, since the published phenotype of the single (type) strain may or may not accurately reflect the typical biochemical profile of that species. Since clinical and veterinary laboratories continue to rely upon biochemical properties and not upon 16S rDNA gene sequencing to identify bacterial strains, reports of the biochemical properties of a new species that are based upon analysis of a single strain recovered from an infected site are essentially meaningless. Strains with 97% or greater similarity may or may not belong to the same species, and DNA pairing studies should be performed to resolve these issues (22). Because of the expense involved and the general lack of availability of DNA hybridization, various researchers or investigators have attempted to establish cutoff values for strain relatedness based upon sequencing of 16S rDNA. One recent investigation (6) characterizing 177 unidentifiable environmental, veterinary, and clinical bacterial isolates defined a species match as a strain exhibiting ≥99% 16S rDNA gene sequence similarity (1% divergence) to strains previously deposited in GenBank. In another case report, 99.5% sequence relatedness was used to identify a strain as *Enterobacter cloacae* (31). However, these arbitrary values may not always accurately reflect taxonomic relatedness. Sequencing of the 16S subunit (~1539 bp) of both *Edwardsiella hoshinae* ATCC 33379^T and *Edwardsiella ictaluri* ATCC 33202^T in our laboratory revealed only 0.19 to 0.65% divergence from the sequence of the type strain of *Edwardsiella tarda*, ATCC 15947^T. By 16S rDNA gene sequence analysis alone, many established breakpoints might recognize these as the same species, yet each of these three species is recognized as genetically distinct not only on the basis of DNA hybridization results but also because each group occupies a distinct ecologic niche.

A final issue regarding 16S identification concerns deposition of sequences into established microbial genome databases. The accuracy of a partial or complete sequence of a 16S rDNA gene or of any other bacterial gene(s) deposited in GenBank or other databases is dependent upon how extensively the bacterial strain from which the sequences were derived has been characterized. Sequence heterogeneity in the literature can result from strain misidentification (5, 17). Since biochemical characteristics are still the touchstone for bacterial identifications, the choice in the use of commercial versus conventional methodologies for the identification of strains undergoing genetic characterization may largely influence how accurate the resulting label is. Thus, while the sequence data may be correct, if it is incorrectly associated with the wrong taxa, major errors in the literature can result.

ISSUES

Almost a decade ago, Weaver (27) raised the issue that case reports involving infrequently isolated bacteria should be accompanied by at least a minimum of data regarding the biochemical characteristics of the organism and the methods used in identifying the species. A recent example of this possible problem can be seen in the case report of Varghese et al. (25) describing the recovery of an isolate identified as *Vibrio fluvialis* from the wound of a 67-year-old woman subsequent to medicinal leech therapy. In this case report, reputed to be the first describing *V. fluvialis* as a wound pathogen after leech therapy, no information was provided on the methods used to identify this vibrio. In contrast to the authors' findings, several things suggest this isolate was probably not *V. fluvialis. Aeromonas* species live as symbionts in the gut of the medicinal leech, *Hirudo medicinalis* (8), and there are numerous reports in the literature documenting aeromonad-associated wound infections following leech therapy. Furthermore, *Aeromonas* species live in this environ apart from any other species (most often isolated in pure culture); rarely has any other microbial species ever been isolated from the gut of the leech. As indicated previously, there are problems associated with certain commercial systems in the identification of *Pasteurella* and *Haemophilus* species (10, 11, 27; Frederiksen and Tenning, letter), and the same situation exists with *Aeromonas* species and *V. fluvialis*. The results obtained with most commercial systems for these species are nearly identical, and they often generate the same septyl or biotype codes. To distinguish these organisms, tests such as salt tolerance, susceptibility to 2,4-diamino-6,7-diisopropylpteridine, production of gas from D-glucose, and the string test need to be performed. These tests are not available on any commercial bacterial identification system. Finally, *V. fluvialis* is a halophilic vibrio and requires

TABLE 1. Proposed guidelines for identification of bacterial species for publication purposes

Type of study	Circumstances	Minimum requirements[a]
Description of new species	Description of an organism and proposal of a new bacterial species	Identification based upon at least five strains (i) that have been demonstrated to be different by at least one molecular technique (e.g., PFGE) and (ii) that are not related temporally, geographically, or epidemiologically (see reference 4)
Case report (single)	Isolation of a species identified as unusual or rare (e.g., unusual biotype of a common species or a common species associated with a new disease, disease syndrome, or anatomic site of isolation)	(i) Species or biotype identification confirmed by two independent laboratories (preferably, one of the two serves in a reference capacity); (ii) identification methods must differ (e.g., a commercial system and a traditional [tube] method) and must generate results indicating very good to excellent identification likelihood; and (iii) relevant phenotype(s) or genotype(s) used for identification and method(s) of detection (e.g., AP120E, septyl code, and identification probability [results showing excellent, very good, or good likelihood], and/or percent similarity or divergence from a published 16S rDNA gene sequence already in a database) must be reported
Case report (series)	Isolation of a single agent or multiple agents, some or all of which are species that are uncommon or at least rarely identified in the clinical laboratory	(i) For a single species, identification of at least two isolates confirmed by two independent methods; (ii) for multiple species, identification of at least one isolate of each species confirmed by two or more methods; and (iii) laboratory data indicating that isolates of the same species do not represent the same strain must be provided
Population studies	Isolation of multiple strains belonging to multiple taxa (e.g., in epidemiological investigations or validation studies of identification systems)	Identification of each strain by at least a single universally available (i.e., commercial) method

[a] PFGE, pulsed-field gel electrophoresis.

media with salt supplementation. This species typically lives, in its natural environment, only in marine habitats, including estuaries. These cumulative facts strongly suggest that the isolate described by Varghese et al. (27) was not *V. fluvialis* but rather an *Aeromonas* species.

There are many more examples in the literature of the misidentification of bacteria by commercial systems. When these misidentifications are associated with the description of new infectious disease syndromes (either when a previously recognized disease agent is associated with a new syndrome or when a newly recognized disease agent is associated with a new disease), they take on added importance. A good example of this problem can be seen in the original descriptions by Albert and coinvestigators (1, 2) of several strains of *Hafnia alvei* that possessed some *Escherichia coli*-like virulence factors (attaching-effacing genes), produced diarrhea in animal models, and apparently caused gastroenteritis in young children. The reports by Albert et al. (1, 2) fueled a number of follow-up case studies concerning *H. alvei*-associated diarrhea (18, 28; J. Reina, J. Hervas, and N. Borrell, Letter, Clin. Infect. Dis. **16:**443, 1993), and subsequent reports recognized *H. alvei* as a causative agent of intestinal disorders, including diarrhea (9, 20). However, in the original studies of Albert et al. (1, 2), the phenotypic properties of these strains were not reported and only a general reference to use of the API 20E strip and conventional test methodologies was made. Studies from several different laboratories have demonstrated that these strains do not possess typical phenotypic or genotypic properties consistent with the genus *Hafnia* (12, 13), and 16S rRNA gene sequencing and *phoE* gene probe assays indicate that these strains belong to the genus *Escherichia* (13, 19). Thus, there is no credible evidence at present that *H. alvei* is an enteropathogenic member of the family *Enterobacteriaceae*.

PROPOSALS AND POSSIBLE SOLUTIONS

Concerns regarding appropriate methods to identify bacteria in published reports will become an increasingly difficult issue in the future unless standards are developed for the description of new taxa, reporting of DNA sequences into databases, and the submission of case reports involving unusual (rare) bacterial species. Between 1980 and the end of 1996 there was a 238% increase in the total number of approved names in the literature, with the number rising to 5,569 taxa (7). With the advent of the availability of automated molecular techniques to identify bacteria (e.g., 16S rDNA sequencing), more species are being described based upon examinations of five or fewer strains and a limited number of differential biochemical characteristics (21). Reliance on a single identification system, phenotypic or genotypic, to identify an organism provides more opportunity for misidentifying bacterial species, and if the trend described above continues, the risk will become even greater in the future.

Recently, Christensen and colleagues (4) made a formal

proposal in this arena. They proposed that Recommendation 30b of the *Bacteriological Code* (1990 Revision) be revised so that proposals to recognize new species are based not upon a single strain but rather upon a minimum of 5 to 10 strains from geographically and epidemiologically unrelated areas. We agree with this proposal and believe that similar (and perhaps expanded) guidelines should be applied to case reports or a limited series of case reports involving unusual (rare) bacterial species or infrequent biotypes (genotypes) of established (traditional) pathogens. In addition, under these guidelines, proposals to recognize new species would require confirmation of the bacterial species or unusual phenotype or genotype by two independent laboratories (Table 1). In the case of species identification, secondary confirmation would be conducted by a recognized reference laboratory by a different methodology, preferably a nonautomated (commercial) one. The biochemical results on any newly described strain need to be published in sufficient detail so that the reader can be confident of the accurate identification of the species and/or the phenotype or genotype.

Ideally, identification of any taxon is based upon a polyphasic approach (24) that includes a combination of phenotypic testing methods (e.g., biochemical testing, cellular fatty acid analysis, and numerical analysis) and genotypic testing methods (e.g., DNA-DNA hybridization, analysis of G+C content [in moles percent], and 16S rDNA gene sequencing). However, such methods are time consuming, expensive, and not easily adaptable to workflow in clinical microbiology laboratories. Table 1 lists an alternative proposal that should be both technically and financially feasible and would help to reduce the number of publications with misidentifications. In all cases, as suggested by Weaver (27), critical information regarding the strain in question needs to be provided so that a assessment of the validity of the identification can be made. Only in this way can we attempt to ensure both the scientific and medical accuracy of the association of pathogenic bacteria with infectious processes in the medical literature.

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