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Conventional Methods versus 16S Ribosomal DNA Sequencing for Identification of Nontuberculous Mycobacteria: Cost Analysis

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The clinical profile of nontuberculous mycobacteria (NTM) has been raised by the human immunodeficiency virus and AIDS pandemic. Different laboratory techniques, often molecular based, are available to facilitate the rapid and accurate identification of NTM. The expense of these advanced techniques has been questioned. At the National Reference Center for Mycobacteriology and the Health Sciences Center, University of Manitoba, in Winnipeg, Canada, we performed a direct cost analysis of laboratory techniques for commercial DNA probe-negative (Gen-Probe, Inc., San Diego, Calif.), difficult-to-identify NTM. We compared the costs associated with conventional phenotypic methodology (biochemical testing, pigment production, growth, and colony characteristics) and genotypic methodology (16S ribosomal DNA [rDNA] sequence-based identification). We revealed a higher cost per sample with conventional methods, and this cost varied with organism characteristics: $80.93 for slowly growing, biochemically active NTM; $173.23 for slowly growing, biochemically inert NTM; and $129.40 for rapidly growing NTM. The cost per sample using 16S rDNA sequencing was $47.91 irrespective of organism characteristics, less than one-third of the expense associated with phenotypic identification of biochemically inert, slow growers. Starting with a pure culture, the turnaround time to species identification is 1 to 2 days for 16S rDNA sequencing compared to 2 to 6 weeks for biochemical testing. The accuracy of results comparing both methodologies is briefly discussed. 16S rDNA sequencing provides a cost-effective alternative in the identification of clinically relevant forms of probe-negative NTM. This concept is not only useful in mycobacteriology but also is highly applicable in other areas of clinical microbiology.

Nontuberculous mycobacteria (NTM) have increased in incidence and in clinical significance over the past decade, which may be explained in part by advancements in culture isolation techniques, molecular identification, and clinical experience (2, 6, 21). Clinical disease caused by NTM is often indistinguishable from that caused by *Mycobacterium tuberculosis* complex (MTBC) (8, 21), and it is therefore important to rapidly and accurately identify NTM, as there remain differences with respect to public health and treatment (8). Diagnostic criteria and treatment guidelines have been established for the more common NTM (14, 21), but very few data exist on the association between clinical outcome and in vitro susceptibility testing for rare or newly identified species. These must be identified to the species level for an improved understanding of their epidemiology and pathogenesis.

The traditional method of NTM species identification relies upon the phenotypic characteristics of biochemical testing, pigment production, growth characteristics, and colonial morphology (11, 13, 22). The extent of biochemical testing depends on the ease of characterization and biochemical activity of the mycobacterium under investigation. Biochemical tests are simple to execute, require minimal equipment, and generally accurately differentiate between the more common species. However, they are time-consuming and present a delay to final identification due to long incubation times. They require experience in interpretation and are limited by subjectivity and low specificity. Biochemically unreactive (or inert) organisms can be formidable opponents. Interspecies homogeneity, intraspecies variability, and the existence of undescribed species often lead to phenotypic misidentification (17, 19). With almost 100 currently established species, a number that continues to rise, biochemical algorithms become too complex, which results in an inherent bias towards the identification of more familiar species of mycobacteria. Biochemical algorithms usually include on average 15 to 20 species only (11, 13). Phenotypic methods are still used in some laboratories to identify NTM despite their acknowledged difficulty.

More advanced techniques such as commercial nucleic acid probes (Accuprobe; Gen-Probe, Inc, San Diego, Calif.), high-performance liquid chromatography (HPLC) (3), PCR-restriction enzyme pattern analysis (PRA) (18), and sequencing have been developed in response to the need for a more rapid and accurate identification of clinically relevant NTM.

The most widespread of these newer techniques is the Accuprobe assay, specific for four species or groups: MTBC, *Mycobacterium avium* complex, *Mycobacterium gordonae*, and *Mycobacterium kansasii*. Accuprobe assays can be costly if several types are used simultaneously to identify an organism in one sample (from 14.60 Canadian dollars [CAD$] to 27.40 CAD$ per probe; CAD$ exchange rate, 0.63 against the U.S. dollar), but they are easy to implement, are simple to use, and rapidly identify these organisms within hours, dramatically de-

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creasing the labor and the turnaround time of results. In a clinical setting, Accuprobe assays can identify the majority of *Mycobacterium* strains isolated: an average of 93.5% (89 to 95%) of strains in our laboratory over the last 10 years.

In the case of probe-negative NTM, other methods of identification, as listed above, are necessary. The specificity of these methods is difficult to ascertain as it can only be determined accurately if one is certain of the species identification of strains tested, i.e., a type strain. Depending on the "gold standard" methodology used for comparison, identification of nontype strains may be inaccurate. This often depends as well on certain groups of organisms that may be less well defined than others. All methods have an advantage to conventional testing, mainly better accuracy and turnaround time. Factors that deter a laboratory from using certain methods are generally cost-related or feasibility of implementation.

Several genes common to all mycobacteria have been studied in sequence-based identification. The most well-known, the 16S rRNA (16) gene, is considered a gold standard for identification for all bacteria and is widely recognized as a rapid and accurate method of identifying known and novel mycobacteria (4, 12, 17, 20). Sequence identification can be completed as early as the next working day and is easily mastered by new staff that may not have a molecular background. Of 16S sequence databases available, such as RIDOM (7), MicroSeq (Applied Biosystems, Foster City, Calif.), GenBank, or those developed in-house (20), RIDOM is the only accurate, quality-controlled, and comprehensive 16S rRNA sequence database for mycobacteria species that is freely accessible on the Internet.

Sequence-based identification is the only approach that can provide useful information in the case of a new species (or a species not present in available databases) with the use of phylogenetic software. This is not accurately accomplished with HPLC and PRA and is not accomplished at all with Accuprobe-negative organisms.

The workflow chosen for our laboratory consists of identification of mycobacteria isolates by Accuprobe assays, followed by 16S rRNA gene sequencing of Accuprobe-negative isolates. Most large clinical microbiology laboratories would benefit from the acquisition of an automated sequencer, as its applicability can be found in all areas of microbiology and not just mycobacteriology. These laboratories often serve smaller laboratories as well, which would then also benefit from these services. There are many studies which compare 16S sequence-based identification with other methods, and this has been consistently described as an expensive technique requiring capital equipment best suited to specialized laboratories in developed countries. Excessive cost is often mentioned as a limitation to implementation. However, limited published data are available on cost associated to both conventional and molecular testing, which is in itself subject to interpretation.

The objective of this study is to compare the cost of identifying Accuprobe-negative NTM using traditional biochemical techniques versus 16S ribosomal DNA (rDNA) sequencing. We will also address the superiority of 16S rDNA sequence-based identification in the rapid and accurate identification of NTM by comparing laboratory turnaround time and agreement of identification. Our hypothesis is that in most cases and when clinically relevant, 16S rDNA sequencing is more cost-effective than conventional methodology in the identification of probe-negative NTM to the species level.

MATERIALS AND METHODS

Setting. This study was conducted through the National Reference Center for Mycobacteriology of the National Microbiology Laboratory, Health Canada, Winnipeg, Canada, and the mycobacteriology laboratory at the Health Sciences Center, University of Manitoba, Winnipeg, Canada, a reference laboratory for the province of Manitoba.

Study design. The primary objective of this study was to determine the cost-effectiveness of 16S rDNA sequencing versus conventional methodologies in the identification of Accuprobe-negative NTM. The study was designed as a cost comparison of the two distinct identification methodologies. Costs were determined by experienced technologists working within the field of mycobacteriology based upon the methodologies of each technique described as follows.

Conventional identification. Accuprobe-negative NTM samples are subject to phenotypic identification, i.e., biochemical tests, pigment production, and growth and colony characteristics. The number of biochemical tests performed depends upon the ease of characterization and biochemical activity of the organism isolated. Phenotypic testing at our provincial site can include the following: arylsulfatase (3 and 14 days), Tween 80 hydrolysis (5 and 10 days), urease, nitrate reduction, heat-stable (68°C) catalase, semiquantitative catalase, iron uptake, tolerance to 5% sodium chloride, growth in the presence of TCH (thiophene-2-carboxylic acid hydrazide), growth on MacConkey agar without crystal violet, niacin production, tellurite reduction, pyrazinamidase (4 and 7 days), beta-glucosidase, acid phosphatase, citrate test, polymyxin B and ciprofloxacin inhibition, amidases (nicotinamidase, acetamidase, allantoinase, benzamidase, and succinamidase), and carbohydrates (fructose, mannitol, inositol, and sorbitol). Growth at temperatures of 25, 28, 31, 37, and 42°C; pigmentation; growth rates; and colony characteristics were also assessed. The exact methodology followed at this laboratory has been described in detail elsewhere (11, 13, 22).

16S rDNA sequencing. Accuprobe-negative NTM isolates were referred for 16S rDNA sequence-based identification. Preparation of genomic DNA, amplification of the 16S rRNA gene by PCR, and detection and purification of PCR product has been previously described (20). Rapid identification of the strains was done with one sequencing reaction only per strain, using the forward primer 8FPL (5′ AGT TTG ATC CTG GCT CAG 3′). Sequencing reactions were performed using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, Calif.), the product was purified using the recommended Centricep columns (Princeton Separations, Adelphia, N.J.), and this was followed by preparation for running onto the ABI PRISM 310 genetic analyzer (PE Biosystems), according to the manufacturer's instructions. The sequencing output was analyzed using the accompanying DNA sequence analyzer computer software (Applied Biosystems). Resulting sequences were analyzed using Lasergene software (DNASTAR, Inc. Madison, Wis.). Analysis was performed by comparing the NTM strain sequences against our in-house database (20), RIDOM (7), and the Basic Local Alignment Search Tool (1).

Cost analysis. Complete cost analysis of conventional testing or 16S rDNA sequencing was based on the total variable costs of materials and labor associated with each methodology described. Fixed costs such as facility space, lighting, and heat were considered comparable for each method and were ignored. The costs associated with initial processing (includes stains, smears, solid and/or liquid culture, and DNA probe analysis) were eliminated from our cost assessment, as these steps were considered routine for processing of all samples regardless of eventual route of identification. The most cost-effective technique for identifying probe-negative NTM defined the start point of our cost comparison. All costs were calculated in Canadian dollars.

The cost associated with conventional identification included material costs and labor costs. Material costs were established from media manufacturing costs split into raw cost (media base, reagents, additives, and petri dishes/bottles), labor cost, processing overhead and reject rate cost. Media base, reagents, and media additive or supplement costs varied for each biochemical test performed. Total media costs included media cost for the biochemical tests as well as for quality control (QC) of the media (Table 1). Media costs for biochemical tests were based on the cost per media and the number of media used per test. Media costs for QC of the media were determined from the cost per medium × 2 (positive and negative control), and this result was then divided by 4 (assuming one set of control media per four tests). Media manufacturing labor costs were fixed at 0.29 CAD$ for each test performed per petri dish or bottle. Labor costs for test performance were determined separately. Processing overhead and reject rate cost was fixed at 0.02 CAD$ per test.

The direct labor cost for performing a given biochemical test was determined

TABLE 1. Cost determination of individual tests used in conventional identification of NTM, based on the simultaneous testing of four isolates and one set of test controls[a]

Biochemical test	Cost (CAD$)	Slow growers		Rapid growers
		Inert	Active	
Arylsulfatase (3 day)	5.35	√	√	√
Arylsulfatase (14 day)	5.35	√	√	√
Tween (5 day)	3.17	√	√	√
Urease	3.17	√	√	√
Nitrate	4.81	√	√	√
68°C catalase	4.49	√	√	√
Semiquantitative Catalase	5.70	√	√	√
Iron uptake	4.88	√	-	√
NaCl	4.89	√	-	√
Growth on MAC	4.58	√	-	√
Citrate	4.43	√	-	√
Tellurite reduction	6.04	√	√	√
Pyrazinamidase (4 day)	5.15	√	√	√
Pyrazinamidase (7 day)	5.15	√	√	√
Beta-glucosidase	4.44	√	-	-
Acid phosphatase	5.29	√	-	-
Amidases				
Nicotinamidase	7.35	√	-	-
Acetamidase	4.71	√	-	-
Allantoinase	7.35	√	-	-
Benzamidase	7.35	√	-	-
Succinamidase	7.35	√	-	-
Carbohydrates				
Fructose	4.71	√	-	√
Mannitol	4.71	√	-	√
Inositol	4.71	√	-	√
Sorbitol	4.71	√	-	√
Temperatures (°C)				
25	3.54	√	√	√
28	3.54	√	√	√
31	3.54	√	√	√
37	3.54	√	√	√
42	3.54	√	√	√
Pigment production				
Light at 37°C	3.54	√	√	√
Dark at 37°C	3.54	√	√	√
Purchased tests:				
Niacin	7.80	√	√	√
Polymyxin B	5.43	√	-	√
Ciprofloxacin	5.43	√	-	√
Total (CAD$)		173.23	80.93	129.40

[a] Tests performed within a particular group of organism are marked with a check.

TABLE 2. Cost report for one 16S DNA sequencing reaction[a]

Step	Total cost of materials[b]	Labor (min)
Lysate preparation and DNA quantitation	12.84	30
PCR[c]	9.00	30
Electrophoresis	17.86	30
Sequencing reaction with pGEM	46.35	30
Sequence analysis and reporting[d]		120
Total	86.05	340

[a] Costs and times were calculated based on a run batch of four samples. Given the totals reported here, the cost for materials is $21.51/sample and the labor cost is $26.40/sample, for a total cost of $47.91/sample (labor costs were calculated at the rate of $0.44/min of labor).

[b] Values are given in Canadian dollars (value of Canadian dollar is approximately 1.6 times that of the U.S. dollar).

[c] Values calculated for PCR step include processing of positive and negative controls.

[d] No cost for materials was assigned to this step. Given a run batch of four samples, the processing time per sample would be 30 min.

from the yearly salary and benefits of a senior technologist. In the year 2000, this rate was identified as 3,816.16 CAD$/month × 12 months × 15.15% benefits, equaling 52,731.69 CAD$ per year. Assuming this technologist worked 2,015 h per year (37.5-h work week), the labor cost was defined as 0.44 CAD$ per min of technologist time.

Calculation of direct labor cost required an estimation of the minutes required for test performance. Test performance was divided into preanalytical, analytical, and postanalytical activities. Preanalytical activities were defined as work list and label preparation, reagent preparation, and biosafety cabinet set up. Analytical activities were defined as the actual test performance and interpretation of results. Postanalytical activities involved initial manual reading of results, entering these results into a computer database, and the subsequent sorting and filing of results to hard copy records. The number of labor minutes required for the performance of each test was multiplied by the derived cost per minute of technologist time (0.44 CAD$) to determine the exact labor cost. The total labor costs for biochemical testing included the labor costs for the biochemical tests added to, when applicable, labor costs for QC. Labor costs for QC were derived by multiplying the cost per minute of technologist time (0.44 CAD$) by the number of minutes for test performance × 2 (positive and negative controls) divided by 4 (once per batch of four).

The total cost of biochemical testing per sample was determined from medium manufacturing, labor costs, and QC for both media and labor based upon a run batch of four (Table 1). Of note, almost all medium manufacturing occurred in-house at the provincial laboratory. Purchased biochemical tests included niacin test strips, polymyxin B disks, and ciprofloxacin disks. The medium manufacturing costs of purchased tests were based upon the raw cost for the purchased media and QC for the media.

As with conventional methodology, the costs associated with 16S rDNA sequencing included material costs and labor costs per sample based upon a run batch of four with controls (Table 2). The steps required for the performance of 16S rDNA sequencing included crude DNA lysate preparation by mechanical lysis using the Mini-Beadbeater (Biospec Products, Bartlesville, Okla.), DNA quantitation, PCR, electrophoresis, and sequencing. The costs associated with each step were determined from the materials used and estimates of labor minutes required. Subculture costs were not included in this cost analysis as the specimens received for 16S rDNA sequencing were already of pure culture. The materials required for crude lysate preparation included consumables associated with the Mini-Beadbeater as well as the PicoGreen dsDNA quantitation kit (Molecular Probes, Inc., Eugene, Oreg.) for quantitation of the lysate. The materials required for the PCR included *Taq* DNA polymerase and deoxynucleoside triphosphates (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), primers, $MgCl_2$, and associated consumables, including setup for positive and negative controls. The materials required for electrophoresis included the DNA marker, agarose gel, and Microcon 100 (Millipore Corporation, Nepean, Ontario, Canada) used for subsequent PCR product purification for sequencing. The actual sequencing reaction performed for each sample required Sequencing Polymer (POP-6), ABI Prism BigDye Terminator cycle sequencing ready reaction kit, and consumables as recommended from Applied Biosystems. Centricep columns (Princeton Separations, Adelphia, N.J.) were used for purification of sequencing product. Consumables generally consisted of variable amounts of materials such as latex gloves, loops and syringes, disposable transfer pipettes, pipette tips, and microcentrifuge tubes, among other things. A cost estimation for these consumables was included in the final cost analysis based upon average facility use.

Labor costs for 16S rDNA sequencing were determined in the same manner as for biochemicals. The technologist labor cost was defined as 0.44 CAD$ per minute of technologist time. The number of labor minutes required for the performance of each step in 16S DNA sequencing was determined by an experienced technologist working within the field. The total labor minutes were added together and divided by four to determine time per sample accounting for grouped reactions. The final minute count was multiplied by the derived cost per minute of technologists' time to determine the labor cost per sample. The labor

total was added to the materials total to give a grand total per sample upon a run batch of four with controls (Table 2).

One must note that the costs of capital equipment required for 16S rDNA sequencing were not directly included in this analysis and are stated separately. The laboratory equipment required for sequencing depends upon methodology. Equipment used at our facility included a Mini-Beadbeater, microcentrifuge, TD 700 fluorometer (Turner Designs, Sunnyvale, Calif.), thermal cycler, spectrophotometer, SpeedVac SC110, and sequencer (ABI PRISM 310 Genetic Analyzer). Other than the Mini-Beadbeater, fluorometer, SpeedVac, and sequencer, this equipment can be found in most clinical laboratories. Facilities can choose alternate DNA lysate preparation methods, such as by sonication or various kits at their disposal. Sequencing product can be purified by ethanol precipitation should a SpeedVac be unavailable. Alternatively, sequencing can be performed by an increasing number of companies or core laboratories that provide a fee for service.

RESULTS

Our direct cost comparison of conventional methodology versus 16S rDNA sequencing for the identification of probe-negative NTM revealed a higher cost per sample using the conventional algorithm. The total costs associated with conventional methodology varied by the actual biochemical test performed and by the total number of tests needed to identify probe-negative NTM (Table 1). In addition, overall cost depended upon the specific organism characteristics: biochemically inert, slow growers; biochemically active, slow growers; or rapid growers. The costs per sample using conventional methodology totaled 80.93 CAD$ for biochemically active, slow growers; 173.23 CAD$ for biochemically inert, slow growers; and 129.40 CAD$ for all rapid growers (Table 1). The proportional cost of conventional testing was determined by calculating the number of Accuprobe-negative NTM isolates per annum (average from 1991 to 1999: 47.5) at the Health Sciences Center Mycobacteriology Laboratory: 12 (25%) biochemically active, slow growers; 2.5 (5%) biochemically inert, slow growers; and 33 (70%) rapid growers. The proportional cost of conventional testing was calculated to be 119.47 CAD$ per isolate.

The total costs per sample using 16S rDNA sequencing totaled 47.91 CAD$ (Table 2). The final cost determined for sequencing of probe-negative NTM species was fixed regardless of growth characteristics or biochemical activity of the individual species isolated. NTM identification by 16S rDNA sequencing was less expensive than conventional methodology irrespective of growth characteristics or biochemical activity.

Costs not included directly in the sequence analysis were the cost of capital equipment, which varies depending on the choice of instrument. Automated capillary systems by Applied Biosystems are ideal in a clinical setting, as they are very easy to use and maintain, labor involved in running the instrument is very minimal, and data can be obtained as early as 1 h after a run has begun. A single-capillary instrument like the ABI PRISM 310 Genetic Analyzer costs approximately 83,000 CAD$, with a throughput of approximately 24 samples per day. The ABI PRISM 3100-*Avant* Genetic Analyzer has recently been made available, which holds four capillaries, increasing throughput to 100 samples per day and costing approximately 121,000 CAD$. Models with 16 and 96 capillaries are also available but would not normally be of value in a clinical laboratory. These purchasing costs include one software application, such as sequencing. Our yearly service contract for the ABI PRISM 310 is approximately 7,000 CAD$, which includes training and a yearly on-site visit for preventative maintenance.

Breakeven analysis was performed to determine the number of Accuprobe-negative NTM isolates required to justify the cost of sequence-based identification taking into consideration the cost of capital equipment and was calculated to be 1160 isolates, with the assumption that staffing is a variable cost.

DISCUSSION

This study compared the costs associated with 16S rDNA sequencing and conventional phenotypic identification of probe-negative NTM. Probe-negative NTM were chosen for this cost analysis, as they remain the most challenging mycobacteria to identify and continue to gain clinical significance. This study determined that the material and labor costs associated with the 16S rDNA sequencing are significantly less than those associated with traditional phenotypic identification. This in part may be explained by the fact that the extent of biochemical testing is determined by NTM growth characteristics and biochemical activity, which in turn directly affects cost.

DNA sequencing has been described as an expensive technique available only to large, specialized laboratories in developed countries. A major stumbling block in discussing cost analysis with newer laboratory techniques is the cost of capital equipment. With 16S rDNA sequencing, the capital cost associated with the purchase and maintenance of an automated sequencer might appear excessive. This opinion would seem reasonable if the sequencer was used for mycobacterial identification alone, in a laboratory setting with infrequent handling of probe-negative isolates. For example, it was determined in this study that 1,160 isolates are required in justifying the cost of capital equipment, a number reasonably achievable only the largest reference mycobacteria laboratories. However, sequencing technology is not limited to the identification of mycobacteria. As experience grows in the field of mycobacteriology as well as in other groups of organisms, both bacterial and fungal, interest should spread to other sections of the laboratory. Capital costs of a sequencer could then be minimized with the shared use of this technology to identify clinically relevant organisms other than mycobacteria. With the performance, accuracy and utility of these sequencers documented, they are now common equipment in many research and clinical laboratories worldwide. All provinces in Canada have access to sequencing technology through provincial and/or referral laboratories. The cost-effectiveness of sequencing technology is directly related to the number of specimens referred. Laboratories with small numbers of probe-negative or difficult to identify mycobacteria isolated may choose to use the services provided by a major referral laboratory. Concern regarding the initial capital equipment cost should not preclude the rapid, accurate and cost-effective identification of NTM to the species level by 16S rDNA sequencing. Broad applicability and an increased sample volume can work towards a reduction in capital costs.

With this cost-analysis we provide evidence that 16S rDNA sequencing is significantly less expensive than conventional techniques when identifying probe-negative NTM. Cost-effective methodologies are without purpose in the face of inaccu-

rate results. The accuracy of 16S rDNA sequencing in the identification of NTM to the species level is well supported in the literature (4, 9, 12, 15, 17, 20). Multiple studies have confirmed the speed and accuracy of 16S rDNA sequencing in the identification of mycobacteria by directly comparing traditional phenotypic methodology with 16S rDNA sequencing in the species determination of difficult to identify mycobacteria. In a clinical setting, values of 16% were obtained in two studies (4, 15), for discordance between original phenotypic identification and 16S rDNA sequencing of all clinical isolates, with this discordance on the basis of biochemical misidentification or unusual and difficult to identify mycobacteria by traditional techniques (5, 15). Selection bias towards unusual or difficult to identify organisms may lead to significant discordance of results (19).

Our own experience with phenotypic versus sequence-based identification of mycobacterium species revealed a similar scenario. Out of 4,996 analyzable positive mycobacterium cultures, the percentage of these requiring full phenotypic analysis ranged from 4.8% to 10.9% over the years sampled (from 1991 to 1999). A total of 82 slow-growing mycobacteria were retrospectively analyzed by 16S rDNA sequencing. In all, only 29 strains (35%) were correctly identified using conventional identification, while the remaining were either new species or subspecies or incorrectly identified by biochemical tests (data not shown). There were multiple reasons for unmatched identification, including frank misidentification or error of biochemical technique, new species or species that did not "exist" at the time of initial biochemical identification, subspecies identification, and those mycobacterial species known to be unidentifiable by biochemicals. This simply reflects the known limitations of phenotypic identification: intraspecies variability, interspecies similarity and bias towards previously identified species. These data will be submitted as a separate study, which will in addition evaluate the clinical significance of isolates found to have unique 16S sequences not corresponding to any established species.

These preliminary results confirm the fact that species identification of probe-negative NTM by phenotypic identification is limited to the most well-known established species, encompassing up to perhaps 30 of the nearly 100 established species and subspecies to date. Acceptance of new laboratory techniques is influenced by accurate results and rapid turn-around times. Expense is often disregarded for accuracy. Cost can also be influenced by rapid laboratory turn-around times, which improve clinical decision-making and overall patient management. Turnaround times for phenotypic identification range from 2 to 6 weeks, depending on the biochemical activity of the NTM isolated. Turnaround times for 16S rDNA sequencing range from 24 to 48 h, regardless of growth characteristics or biochemical activity. These values are widely accepted throughout the literature.

Biochemical testing continues to play a role in the identification of mycobacteria. Most molecular methods and HPLC cannot distinguish between all members of the MTBC, a task normally undertaken using biochemical tests. Biochemical testing also aides in the differentiation of *M. kansasii* and *Mycobacterium gastri* with identical 16S rDNA sequences, although most secondary gene targets can make the distinction. Biochemical testing should also be performed on subspecies or unique species of mycobacteria identified by 16S rDNA gene sequencing for characterization of the isolates.

Unlike MTBC, there is no consensus method for the identification of probe-negative NTM. In general, laboratories choose phenotypic, sequence-based methods (including PRA) or HPLC as a follow-up to Accuprobe. The last two of these offer better accuracy and turnaround time. If laboratories choose to identify NTM species using molecular methods or HPLC alone, basic characteristics such as pigment production, growth characteristics, and colonial morphology remain a very important component of the identification process, if only to confirm results and to ensure that a mix-up of specimens, at any step, has not occurred.

As other groups have shown for HPLC (10), we propose that 16S rDNA sequencing provides another cost-effective alternative to conventional biochemical methodology for identification of clinically relevant, probe-negative NTM, in addition to its already established higher level of accuracy compared to phenotypic methods. For common NTM, probe identification should remain the first step in species identification, as >90% of the species would be identified immediately, including the most clinically significant. With a probe-positive result and matching culture characteristics (pigmentation, growth rate, and colony morphology), in many cases no further testing is warranted. For probe-negative NTM, a suggested identification scheme would promote 16S rDNA sequencing as a rapid, accurate, and cost-effective step towards species identification in place of conventional biochemical testing.

Species identification remains of vast importance given an increased awareness of clinically significant NTM. Species identification provides an opportunity to further develop the clinical and epidemiologic database surrounding NTM that may eventually produce treatment trials and accurate outcome studies, expanding the scope of NTM disease. 16S rDNA sequencing provides a cost-effective alternative method of identification of probe-negative NTM and can be used alongside HPLC as an advanced technique of NTM identification. Cost analysis of other molecular-based identification techniques are warranted. With rapid, accurate, and now cost-effective methods of identifying probe-negative NTM, research focus can concentrate on expanding our understanding of the epidemiology, clinical spectrum, and therapeutics of these notoriously difficult-to-identify organisms. The experience and success of 16S rDNA sequencing in the identification of probe-negative NTM may be expanded to other organisms within microbiology.

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