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Clinical Whole Genome Sequencing for Clarithromycin and Amikacin Resistance Prediction and Subspecies Identification of *Mycobacterium abscessus*

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Mycobacterium abscessus infections are an emerging health care concern in patients with chronic pulmonary diseases, leading to high morbidity and mortality. One major challenge is resistance to clarithromycin, a cornerstone antibiotic with high efficacy. Therefore, treatment is primarily guided by phenotypic susceptibility results of clarithromycin, which requires extended incubation to assess for inducible resistance. Resistance mechanisms for clarithromycin include induction of *erm*(41) and mutations in the 23S rRNA gene (*rrl*). In addition, mutations in the 16S rRNA encoding gene (*rrs*) can confer high-level amikacin resistance, another essential drug in the treatment of *M. abscessus* infections. Herein, we developed a clinical whole genome sequencing (WGS) assay for clarithromycin resistance based on *rrl* and *erm*(41) gene sequences and amikacin resistance based on the *rrs* sequence in *M. abscessus*, as well as subspecies identification. Genotypic-based predictions were determined for 104 isolates from 68 patients. The overall accuracy of genotypic prediction for clarithromycin compared with phenotypic susceptibility results was 100% (95% CI, 96.45%—100%). For amikacin, we also obtained 100% accuracy (95% CI, 96.52%—100%). The high concordance between the genotypic and phenotypic results demonstrates that a WGS-based assay can be used in a clinical laboratory for determining resistance to clarithromycin and amikacin in *M. abscessus* isolates. WGS can also provide subspecies identification and high-definition phylogenetic information for more accurate *M. abscessus* strain typing. *(J Mol Diagn 2021, 23: 1460—1467; https://doi.org/10.1016/j.jmoldx.2021.07.023)*

Mycobacterium abscessus is a rapidly growing mycobacteria that is increasingly isolated in patients with predisposing lung conditions, such as cystic fibrosis.[1—4] Global transmission of dominant *M. abscesses* clones among the cystic fibrosis patient population has been reported and is potentially associated with worse clinical outcomes.[5,6] *Mycobacterium abscessus* treatment is particularly challenging because of the multidrug resistance nature of this organism. Therefore, treatment regimens often consist of a combination of three or more parenteral and oral drugs with a macrolide, such as clarithromycin or azithromycin, as the first-line treatment.[7] Other drug options include amikacin, imipenem or cefoxitin, tigecycline, clofazimine, and linezolid and depend on phenotypic susceptibility results.[7] Current treatment guidelines for *M. abscessus* pulmonary infections recommend antibiotic selection based on the phenotypic susceptibility data and detection of inducible or mutational resistance to macrolides, and using macrolides whenever possible.[7]

The most common mechanism of clarithromycin resistance is through the enzymatic modification of the drug target, 23S rRNA, by a ribosomal methyltransferase encoded by the *erm*(41) gene. The functional status of *erm*(41) gene is the primary factor for determining inducible resistance to

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clarithromycin.[8] The gold standard method for detecting inducible resistance is broth microdilution with an extended incubation of up to 14 days.[9] The *M. abscessus* complex currently consists of three subspecies, *M. abscessus* subspecies *abscessus*, subspecies *massiliense*, and subspecies *bolletii*. Identification to the subspecies level is not routinely reported, but intrinsic resistance patterns and clinical outcome between subspecies have been described.[10–12] *Mycobacterium abscessus* subspecies *abscessus* and subspecies *bolletii* are associated with a full-length *erm*(41) gene and therefore resistance to clarithromycin and potentially poor treatment outcomes.[8,13] In contrast, *M. abscessus* subspecies *massiliense* is associated with a truncated nonfunctional *erm*(41) gene and susceptibility to clarithromycin, leading to better treatment outcomes.[12,14] Alternatively, acquired resistance can be attributed to a single point mutation in the *rrl* gene encoding the drug target, 23S rRNA, regardless of the *erm*(41) gene functionality.[15,16] Resistance mechanisms to other drugs have also been well characterized, including amikacin. For instance, several point mutations (T1406A, A1408G, and C1409T) in *rrs* gene, encoding the drug target 16S rRNA, are shown to confer high-level resistance to aminoglycosides.[17,18]

Targeted molecular methods have focused on *erm*(41) and *rrl* for the detection of clarithromycin resistance and *rrs* for the detection of amikacin resistance.[19–24] Several studies using targeted sequencing of these genes of interest or whole genome sequencing (WGS) show a strong concordance with phenotypic susceptibility results.[6,20–22,25,26] Gene sequencing is therefore considered an acceptable alternative to determining clarithromycin and amikacin resistance.[9,27] Other molecular methods available include the NTM-DR line probe assay, a rapid method for species identification of clinically important nontuberculous mycobacteria, including *M. abscessus* subspecies as well as resistance prediction for clarithromycin and amikacin.[19,28,29] However, detection is based on a limited number of single-nucleotide targets in one or two genes.

Herein, we developed and validated a WGS-based workflow for predicting clarithromycin and amikacin resistance in *M. abscessus* clinical isolates. A previously published algorithm consisting of examining *rrl* variants and *erm*(41) functional gene status was used as the framework for the clarithromycin resistance prediction workflow.[25] Previously published variants in the *rrs* gene served as the basis for predicting high-level amikacin resistance.[15] In addition, with the availability of WGS data, we also implemented a phylogenetic analysis component that allowed for accurate subspecies identification.

Materials and Methods

Mycobacterium abscessus Isolates

A total of 104 *M. abscessus* isolates from 94 clinical specimens collected from 68 patients (2017 to 2019) and 1 reference strain (ATCC, Manassas, VA; 19977) were used in this study, including multiple specimens from a single patient and/or multiple isolates with different colony morphology from a single specimen (Supplemental Table S1). Species identification was determined by *rpoB* sequencing[30] or matrix-assisted laser desorption/ionization time-of-flight analysis using the Vitek MS (BioMerieux, Marcy l'Etoile, France). A total of 11 isolates were identified to the subspecies level by a reference laboratory by *rpoB* and *erm* gene sequence analysis[8,31–33] or by the GenoType NTM-DR line probe assay.[28,29] Most specimens were from lower respiratory sources, such as bronchoalveolar lavage, sputum, or tracheal suctions. Other specimens included wound, eye, or sinus. Antimicrobial susceptibility testing was performed using in-house broth microdilution trays, according to the Clinical and Laboratory Standards Institute guidelines[9] and performance standards for mycobacteria testing.[34] Isolates requiring subspeciation identification or confirmatory antimicrobial susceptibility testing were sent to a reference laboratory.

Whole Genome Sequencing

DNA was extracted using the EZ Tissue kit (Qiagen, Hilden, Germany), according to manufacturer's instructions after heat inactivation (95°C for 30 minutes) and a mechanical bead beating step for bacterial wall disruption. Sequencing libraries were prepared using the Nextera DNA Flex Library Prep kit (Illumina, San Diego, CA), according to manufacturer's instructions. Library quality was assessed using the High Sensitivity DNA Analysis kit (Agilent, Santa Clara, CA), and quantification was determined using Qubit dsDNA High Sensitivity Assay kit (ThermoFisher, Waltham, MA). Paired-end sequencing (250 bp × 2) was performed on the Illumina MiSeq platform. WGS was performed on a total of 104 isolates. Several isolates, including reference strain ATCC 19977, were repeated for precision studies or verification, leading to a total of 124 WGS results.

Bioinformatics Analysis

The bioinformatics pipeline workflow is depicted in Figure 1. Paired sequencing files were downloaded from BaseSpace (Illumina) and imported into CLC Genomics Workbench version 12.0.3 (Qiagen) for trimming of low-quality reads (quality limit, 0.05) and reference mapping. All sequence files were mapped to *rrl*, *erm*(41), and *rrs* genes from reference genome *Mycobacterium abscessus* ATCC 19977 chromosome, complete sequence (National Center for Biotechnology Information CU458896.1). Local realignment and variant detection were determined with the default minimum frequency set at 35%. Values for average coverage, percentage of coverage for target genes, coverage at individual nucleotide positions, and allele frequency generated by Genomics Workbench version 12.0.3 were

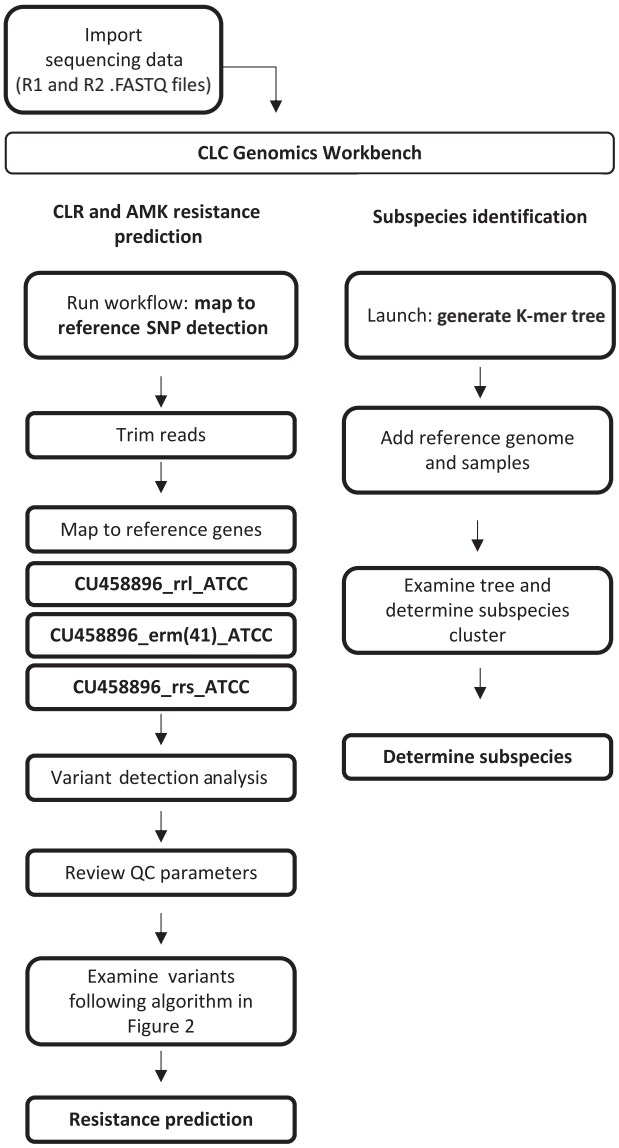

Figure 1 Bioinformatics pipeline for resistance prediction and subspecies identification. Individual workflow steps for sequence data analysis, quality control (QC) steps, and resistance prediction are shown. Analysis tools using CLC Genomics Workbench are shown for data processing, mapping, and variant calling. Subspecies identification tools and steps are also shown. Accession number for ATCC reference strain 19977 for each mapped gene is indicated. AMK, amikacin; CLR, clarithromycin; SNP, single-nucleotide polymorphism.

recorded per sequenced sample. The mean, SD, and range were then calculated to determine the minimum quality control requirements for each parameter.

Single-Nucleotide Polymorphism Analysis for Resistance Prediction

Clarithromycin resistance prediction algorithm was adapted on the basis of previously published findings.[25] Mapped reads were first examined for single-nucleotide variants at positions 2269 to 2271, 2293, and 2281 in the *rrl* gene. If the *rrl* gene was wild type, *erm*(41) was examined for truncation and, if full length, positions 28 and 19 were used to determine resistance prediction (Figure 2). Amikacin resistance prediction was based on examining variants in the *rrs* gene at positions T1373A, A1375G, and C1376T (*Escherichia coli* numbering: T1406A, A1408G, and C1409T, respectively).[17,18]

Subspecies Identification

A K-mer–based phylogenetic tree based on the Feature Frequency Profile via Jensen-Shannon divergences method was generated using the K-mer tree tool in CLC Genomics Workbench with raw sequence reads (both strands) and a K-mer length of 16. To identify the closest common subspecies, previously verified reference genomes obtained from National Center for Biotechnology Information were included for *M. abscessus* subspecies *abscessus* (CU458896.1), *M. abscessus* subspecies *massiliense*, and *M. abscessus* subspecies *bolletii* (AP018436.1) in the K-mer tree. In addition, the 11 clinical isolates verified to subspecies level by a reference laboratory were also included as reference for validation. The K-mer tree tool generated phylogenetic clustering for all unknown samples and reference genomes. Subspecies was determined by the common ancestor node shared by the unknown sample and a reference genome in the K-mer tree (Figure 3).

Results

WGS Quality Metrics for *M. abscessus* Sequencing Data

Sequencing quality control criteria were developed to provide consistency for genetic analysis of isolates for clinical testing. Quality metrics were established by evaluating sequencing data from 124 samples consisting of 104 patient isolates, the ATCC reference strain, and sample replicates. The average number of reads was approximately 2.4 million, with a range of 491,480 to 4.7 million; therefore, quality control criterion was set at 400,000 for minimum total reads per sample (Table 1). Sequencing data from each sample were mapped to the *M. abscessus* ATCC 19977 reference genome *rrl*, *erm*(41), and *rrs* genes, and parameters for each mapped gene were evaluated, including coverage and depth across the gene and the depth at specific allelic positions used to determine resistance prediction. All 124 samples had at least 5× depth spanning the entire full-length mapped genes. Most samples had at least 10× depth covering >99% of the gene and an average depth of 72× (Table 1). For samples with a truncated *erm*(41) gene, samples on average had a 271-bp gap between position 159 and 430, which was reflected in the percentage of the gene with at least 5× depth to be reliably <60% (Table 1). This was established as the interpretation criteria to report *erm*(41) gene truncation. In addition, depth and frequency of the individual target allele positions were evaluated. The average depth

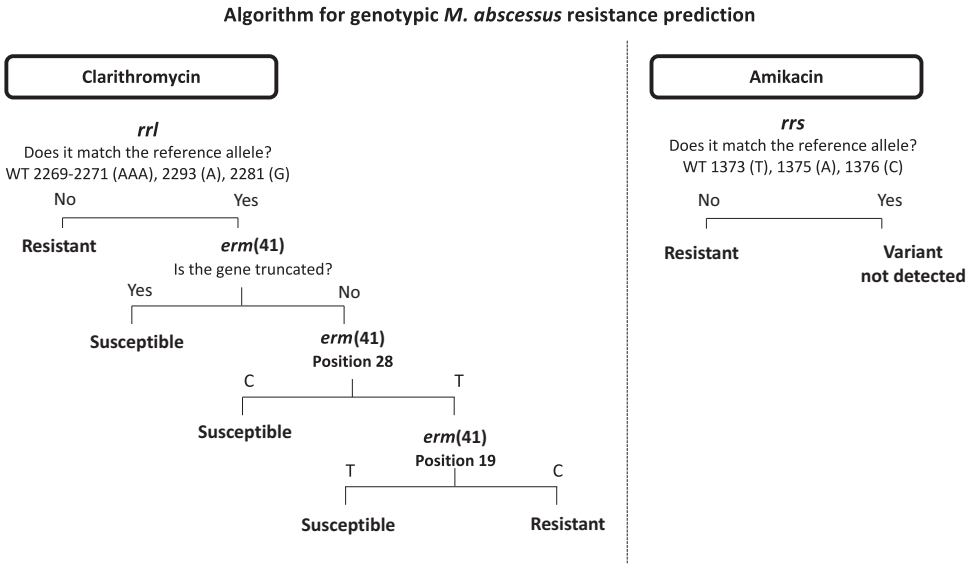

Figure 2 Algorithm for genotypic *Mycobacterium abscessus* resistance prediction. Decision tree for clarithromycin and amikacin based on variant detection at specified positions used to determine resistance in clinical testing. WT, wild type.

for target positions was 66.5×, and the lowest depth was at 11×. Therefore, the minimum cutoff value for acceptable depth at the target position was set at 10×. A total of 34 samples with non−wild-type alleles were examined and had an average allele frequency of 99.89% (Table 1). Data from these parameters were used to determine cutoff values for sequence analysis acceptability, demonstrating resistance prediction can be accurately obtained with at least 491,000 reads with consistent and sufficient depth at resistance determining regions.

Subspecies Identification

Subspecies identification for *M. abscessus* has been important clinically to predict treatment outcomes and for epidemiologic purposes.[5,6,11,12] However, *16S* and *rpoB* gene sequences alone are not sufficient to differentiate the three subspecies because of their high genetic similarity,[33,35,36] and evidence of lateral transfer of the *rpoB* between *M. abscessus* subspecies has been described.[37] Therefore, the workflow included phylogenetic analysis

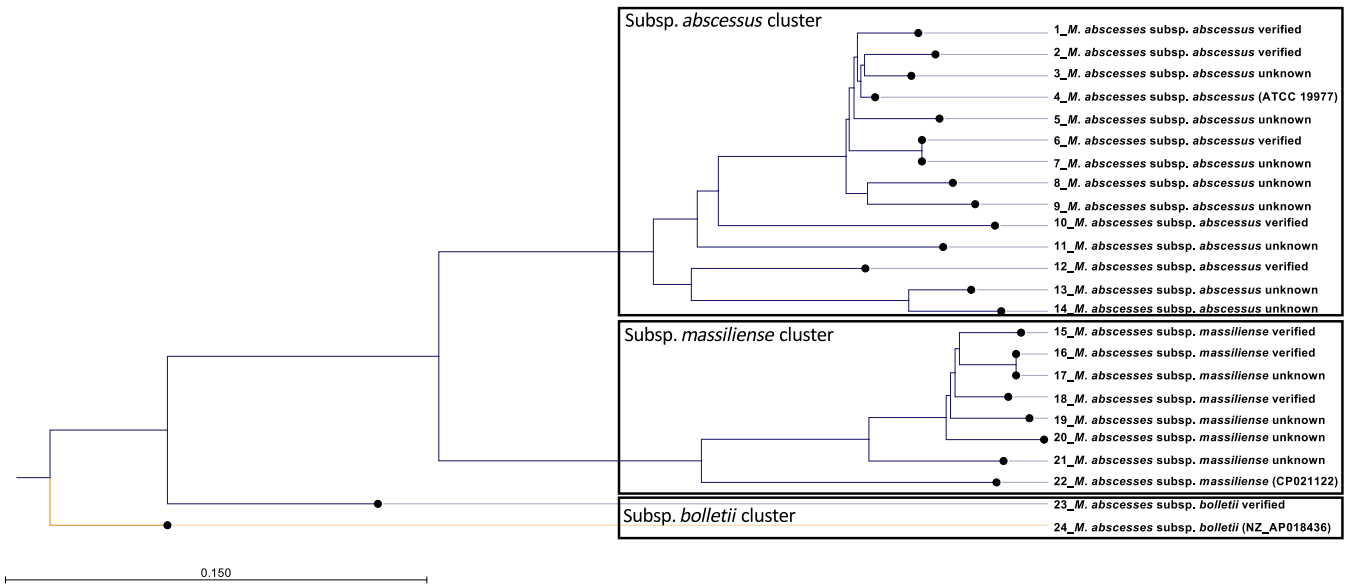

Figure 3 Representative phylogenetic analysis of grouping for subspecies (Subsp.) identification. Isolates that were verified to the subspecies level by a reference laboratory are indicated as well as isolates with unknown subspecies and identification determined by this method. Published genomes obtained from National Center for Biotechnology Information GenBank (*https://www.ncbi.nlm.nih.gov/genbank*, last accessed July 21, 2021) are indicated with the corresponding accession number. ATCC 19977 reference stain was sequenced and included as a reference genome. The subspecies groups are outlined. *M. abscessus*, *Mycobacterium abscessus*.

Table 1 WGS QC Metric Statistics and Cutoff QC Metric

Variable	Total target regions analyzed, *N*	Average	Range	SD	Cutoff value
Whole genome					
Total reads, *N*	124	2,455,772	491,480–4,708,966	669,510	400,000
rrl, full-length *erm*(41), and *rrs*					
Average depth, ×	341	72	11–137	21.94	≥10
Minimum coverage of region 5×, %	341	100	100	0	≥99
Minimum coverage of region 10×, %	341	99.77	76–100	2.2	≥70
Depth at specified position, ×	1178	66.5	11–159	23.03	≥10
Frequency of allele, %	34	99.89	99.67–100	0.19	≥90
Truncated *erm*(41)					
Minimum coverage of region 5×, %	31	41	30–48	8.7	≤60

QC, quality control; WGS, whole genome sequencing.

that determined subspecies based on closest relatedness to a known reference subspecies using the entire genome sequenced. In this approach, phylogenetic trees with unknown samples and reference genomes were generated, which consistently showed three distinct clusters corresponding to each subspecies (Figure 3). Using this approach, 73% (76/104) of the isolates were identified as *M. abscessus* subspecies *abscessus* from 47 patients and 26% (27/104) of the isolates were identified as *M. abscessus* subspecies *massiliense* from 20 patients (Table 2). All isolates identified as *M. abscessus* subspecies *massiliense* had a truncated *erm*(41) gene. *Mycobacterium abscessus* subspecies *bolletii* was identified in only one patient. No patients had more than one subspecies identified, despite having multiple isolates with different colony morphology. Results using the phylogenetic tree method were in 100% agreement with results obtained from a reference laboratory for a total of 11 isolates.

Clarithromycin Resistance Prediction

WGS was performed on 104 clinical *M. abscessus* isolates recovered from 94 specimens from 68 patients. Clarithromycin resistance was genotypically predicted in 56% (58/104) of the isolates (Table 3), with most (56/58) of the resistant isolates identified as *M. abscessus* subspecies *abscessus* (Table 2). Inducible resistance was identified in 96.5% of the genotypically resistant isolates (56/58) based on the full-length wild-type *erm*(41) (T at position 28) (Table 2). Acquired resistance was identified in two isolates based on mutations in the *rrl* gene at position 2270 (A2270T), both of which were identified as *M. abscessus* subspecies *massiliense*. These two isolates were recovered from two separate lower respiratory specimens from a single patient but collected 3 months apart. Phenotypic resistance for clarithromycin was reported in 57 isolates and sensitivity was reported in 1 isolate using broth microdilution, which included extended incubation for the detection of inducible resistance. One isolate of *M. abscessus* subspecies *abscessus* was predicted to be resistant because of a functional full-length *erm*(41) gene, but showed phenotypic sensitivity to clarithromycin. Repeated testing of the discrepant isolate at a reference laboratory confirmed the presence of inducible resistance. Therefore, there was 100% (58/58) agreement between the phenotypic and genotypic resistance results (Table 3).

A total of 45% (46/104) of the isolates were genotypically predicted to be susceptible to clarithromycin based on a nonfunctional *erm*(41) gene due to either truncation (25/46, all identified as *M. abscessus* subspecies *massiliense*) or

Table 2 Summary of *Mycobacterium abscessus* Genotypes and Subspecies Identification

Variable	Total isolates (total patients)	CLR	AMK	*rrl*			*erm*(41)				*rrs*		
				A2270T	A2293N	G2281N	T28	T28C	C19T	Truncated	T1373N	A1375G	C1376N
M. abscessus subspecies *abscessus*	76 (47)	S	S	0	0	0	0	21 (19)	0	0	0	0	0
		R	S	0	0	0	50 (26)	0	0	0	0	0	0
		R	R	0	0	0	5 (2)	0	0	0	0	5 (2)	0
M. abscessus subspecies *massiliense*	27 (20)	R	S	2 (1)	0	0	0	0	0	0	0	0	0
		S	S	0	0	0	0	0	0	25 (20)	0	0	0
M. abscessus subspecies *bolletii*	1 (1)	R	S	0	0	0	1 (1)	0	0	0	0	0	0

AMK, amikacin; CLR, clarithromycin; R, resistant; S, susceptible.

Table 3 Correlation of Clarithromycin and Amikacin Genotypic Prediction Versus Phenotypic Susceptibility Results

Genotypic prediction	Phenotypic susceptibility			
	Susceptible	Intermediate	Resistant	Total
Clarithromycin				
Susceptible	46	0	0	46
Resistant	0	0	58	58
Amikacin				
Variant not detected	84	15	0	99
Resistant	0	0	5	5

full-length *erm*(41) with a T-to-C substitution at position 28 (21/46, all identified as *M. abscessus* subspecies *abscessus*) (Table 2). No mutations in *rrl* at positions 2269 to 2271, 2293, and 2281 were identified in these isolates. Phenotypic susceptibility to clarithromycin was reported in 100% (46/46) of the isolates that were genotypically predicted to be susceptible (Table 3).

Overall, the test accuracy for genotypic prediction of clarithromycin was 100% (95% CI, 96.45%−100%) with a sensitivity of 100% (95% CI, 93.73%−100%), a specificity of 100% (95% CI, 92.29%−100%), and a positive and negative predictive value of 100%.

Amikacin Resistance Prediction

Variant analysis of the *rrs* gene was performed on the same 104 isolates to determine amikacin resistance. The sequences were evaluated for potential amikacin resistance by examining positions 1408, 1409, and 1491 (*E. coli* numbering). We identified four *M. abscessus* subspecies *abscessus* isolates with an A-to-G substitution at position 1408, all of which were resistant to amikacin (minimum inhibitory concentration, ≥64 μg/mL) by phenotypic methods. There were no variants identified in the remaining 99 isolates at the allelic positions of interest. Most of these isolates had susceptible minimum inhibitory concentrations of ≤16 μg/mL, 84% (83/99); and 15% (15/99) of the isolates had minimum inhibitory concentrations at 32 μg/mL, which are considered intermediate (Supplemental Table S1). Isolates that did not have a variant known to confer resistance were reported as variant not detected. Therefore, all 104 samples had 100% (95% CI, 96.52%−100%) agreement for amikacin resistance using genotypic prediction, with a sensitivity of 100% (95% CI, 47.82%−100%) and a specificity 100% (95% CI, 96.34%−100%).

Discussion

Herein, we present the performance characteristics of a WGS-based clinical test for the prediction of clarithromycin and amikacin resistance in *M. abscessus*. Patient isolates were used to validate the test, which demonstrated a high concordance compared with broth microdilution, the current gold standard for phenotypic susceptibility testing. WGS provides the ability to examine multiple genes with a single wet-laboratory process and therefore is advantageous for performing resistance prediction simultaneously for multiple drugs. In addition, WGS provides an opportunity to use sequencing data for subspecies identification, which is challenging using conventional methods. The technical workflow for WGS can be performed in 3 to 5 days, and the bioinformatics analysis can be completed using user-friendly software in 1 to 2 hours. Compared with phenotypic susceptibility testing, which requires a 14-day incubation to detect inducible resistance, WGS can potentially improve the turnaround time for a clarithromycin antimicrobial result by 10 days. Sequencing of *erm*(41) and *rrs* for clarithromycin and sequencing of *rrl* for amikacin resistance are endorsed by Clinical and Laboratory Standards Institute as alternatives to phenotypic testing.[9,27] Other methods, such as the line probe assay, are also available for clarithromycin and amikacin resistance prediction and subspecies identification.[28] However, the detection of resistance is limited to the specific mutations defined by the assay. Therefore, WGS has the advantage of identifying additional variants at allelic positions that line probe is unable to detect.

The workflow developed for clarithromycin resistance prediction was based on a previously published predicative algorithm with a reported sensitivity and specificity of 95% and 66%, respectively.[25] The improved sensitivity and specificity observed in this study might be due to the difference in geographic location, patient population, and/or prevalence of resistance. It appears that in our patient population, the prevalence of *M. abscessus* resistance to clarithromycin is high (56%), which may contribute to the higher specificity observed in this study. The number of isolates analyzed and the narrow geographic representation of these isolates are both limitations of this study. However, validation of this assay by additional laboratories would allow for a more thorough evaluation of representative patient populations.

Subspecies identification has been previously associated with treatment outcome and differing susceptibilities to clarithromycin,[12,14,38] which can be attributed to *erm*(41) gene functional status. *Mycobacterium abscessus* subspecies *abscessus* and *M. abscessus* subspecies *bolletii* typically have a functional *erm*(41), which confers inducible resistance to clarithromycin. Lengthy phenotypic susceptibility testing is the standard for detecting inducible resistance, which accounted for 54% (56/104) of the isolates sequenced. From our patient population, most clinical isolates were *M. abscessus* subspecies *abscessus*, followed by *M. abscessus* subspecies *massiliense*, which is consistent with other studies.[20] Because pathogenesis is similar across all three subspecies, treatment outcomes are largely due to *erm*(41) gene and clarithromycin resistance. In addition, some isolates of *M. abscessus* subspecies *massiliense* have been identified with a full-length *erm*(41) gene or display resistance that is not explained by known mutations.[23,25,39] In this study, two isolates of *M. abscessus* subspecies *massiliense* had variants

in the *rrl* gene that conferred high-level clarithromycin resistance, even though they displayed a truncated *erm*(41) gene. In addition, a substantial proportion (21/76, 27.6%) of *M. abscessus* subspecies *abscessus* in this study were actually susceptible to clarithromycin due to a T28C mutation in their *erm*(41) gene, which renders it nonfunctional. Therefore, the emphasis of clarithromycin susceptibility based on both *rrl* and *erm*(41) holds greater weight than subspecies identification alone or simply measuring the length of the *erm*(41) gene for predicting treatment outcomes.

Genetic variants of resistance have also been described for amikacin. Particularly, mutations in *rrs*, the gene encoding the drug target 16S rRNA, can confer high-level resistance. Because high-level resistance to amikacin is rare in *M. abscessus*, Clinical and Laboratory Standards Institute guidelines recommend confirmation of amikacin resistance by either confirmatory testing at a reference laboratory or *rrs* gene sequencing.[34] Consistent with the Clinical and Laboratory Standards Institute recommendation, there was a <5% (5/104) amikacin resistance rate in this study. Although a high level of accuracy was achieved between genotypic and phenotypic amikacin susceptibility data, one of the limitations is the low number of amikacin-resistant isolates. Additional studies to improve our confidence in the sensitivity and specificity are needed.

Another advantage of the WGS test is its capability to provide high-definition phylogenetic information to determine the genetic relatedness among different isolates for epidemiologic purposes or to help determine if a patient had acquired different strains of *M. abscessus* infection after treatment. However, the utility of such type of phylogenetic analysis of multiple *M. abscessus* isolates from the sample patient with chronic infections requires further investigation and clinical validation.

In summary, this study showed that WGS can serve as a reliable and more rapid method for determining clarithromycin and amikacin resistance compared with lengthy *in vitro* susceptibility testing in a clinical laboratory. This laboratory-developed test has been successfully implemented in our laboratory for routine clinical testing. The WGS test holds the promise for potentially improving patient care by reducing turnaround time, enhancing accuracy, and providing high-definition genetic information for drug resistance mechanism discovery and genomic epidemiologic investigation.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.07.023*.

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