Whole-Genome Sequencing for Predicting Clarithromycin Resistance in *Mycobacterium abscessus*

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ABSTRACT *Mycobacterium abscessus* is emerging as an important pathogen in chronic lung diseases, with concern regarding patient-to-patient transmission. The recent introduction of routine whole-genome sequencing (WGS) as a replacement for existing reference techniques in England provides an opportunity to characterize the genetic determinants of resistance. We conducted a systematic review to catalogue all known resistance-determining mutations. This knowledge was used to construct a predictive algorithm based on mutations in the *erm*(41) and *rrl* genes which was tested on a collection of 203 sequentially acquired clinical isolates for which there were paired genotype/phenotype data. A search for novel resistance-determining mutations was conducted using a heuristic algorithm. The sensitivity of existing knowledge for predicting resistance in clarithromycin was 95% (95% confidence interval [CI], 89 to 98%), and the specificity was 66% (95% CI, 54 to 76%). The subspecies alone was a poor predictor of resistance to clarithromycin. Eight potential new resistance-conferring single nucleotide polymorphisms (SNPs) were identified. WGS demonstrated probable resistance-determining SNPs in regions that the NTM-DR line probe cannot detect. These mutations are potentially clinically important, as they all occurred in samples that were predicted to be inducibly resistant and for which a macrolide would therefore currently be indicated. We were unable to explain all resistance, raising the possibility of the involvement of other as yet unidentified genes.

KEYWORDS macrolides, nontuberculous mycobacteria, whole-genome sequencing

Members of the *Mycobacterium abscessus* complex (*M. abscessus*) are rapidly growing nontuberculous mycobacteria (NTM) of increasing clinical concern because of a rising burden of associated pulmonary disease (1). *M. abscessus* poses a significant problem, particularly in patients with cystic fibrosis (CF), where infection is associated with a more rapid decline in lung function and can be a barrier to transplantation (2). Of particular concern are the findings from recent work that have suggested person-to-person transmission of virulent clones among the CF population within a health care setting (3, 4), although not all studies have supported this (5, 6).

The taxonomy of *M. abscessus* is contentious. It is currently divided into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (7). The organism has intrinsic resistance to multiple antibiotics, including β-lactams, rifampin, and aminoglycosides, due to the synergistic action of the cell envelope and genetic factors (8). Treatment requires prolonged courses of multiple antibiotics, but outcomes are thought to vary across the different subspecies. *M.*

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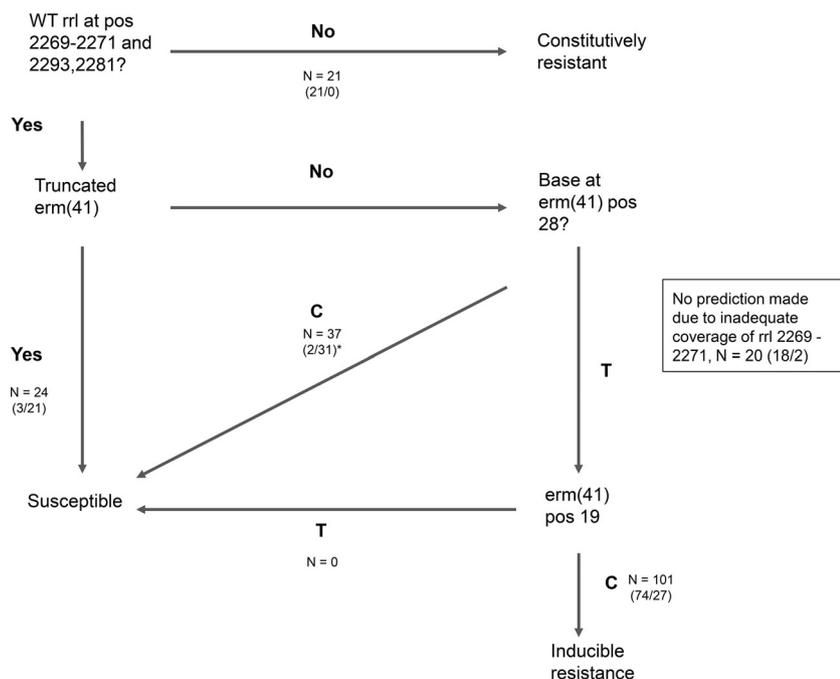S.L. and N.H. contributed equally to this article.

FIG 1 Decision algorithm for predicting drug resistance in *M. abscessus* based on the literature search, with the numbers of isolates meeting each predictive criterion shown. Numbers in parentheses represent the number resistant/the number sensitive. *, 4 isolates had intermediate susceptibility; pos, position.

abscessus subsp. *massiliense* has been associated with clarithromycin susceptibility and favorable treatment outcomes, whereas *M. abscessus* subsp. *abscessus* has been associated with inducible macrolide resistance and poorer treatment outcomes (9).

Whole-genome sequencing (WGS) has been implemented in stages across England since December 2016, replacing existing reference techniques for mycobacterial identification. As a consequence, there is now the opportunity to explore the molecular determinants of drug resistance for all clinical NTM isolates. Macrolides are important agents in the management of NTM infection. The American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) and British Thoracic Society (BTS) guidelines recommend including a macrolide in treatment regimens when samples either are susceptible or demonstrate inducible resistance (10, 11). They act by binding to the 50S ribosomal subunit, and resistance in mycobacteria primarily occurs through target site modification, for example, by *erm* methylases and point mutations (12). As there is a particularly strong correlation between *in vitro* susceptibility and the clinical response to macrolide treatment of *M. abscessus* infections (13, 14), we have undertaken a study to assess the feasibility of predicting clarithromycin susceptibility from whole-genome sequencing data for all three subspecies of *M. abscessus*.

RESULTS

We studied 143 *M. abscessus* subsp. *abscessus*, 20 *M. abscessus* subsp. *bolletii*, and 40 *M. abscessus* subsp. *massiliense* genomes. Genotypic predictions were made on the basis of mutations identified by the literature search. All relevant mutations identified were contained in the genes *rrl* and *erm*(41) (Fig. 1 and Table 1). The genes *rplV*, *whiB7*, and *rpld* were also considered of potential interest and were additionally searched for variants.

Genotypic predictions. Inducible resistance was predicted in 101 isolates, of which 74 (73%) were reported to be phenotypically resistant. After excluding isolates for which no prediction could be made due to missing data in key genomic loci ($n = 20$) as well as those with an intermediate phenotype ($n = 4$), the sensitivity was 95/100 (95%; 95% confidence interval [CI], 89 to 98%) and the specificity was 52/79 (66%; 95%

TABLE 1 Resistance-determining mutations for clarithromycin identified in the literature search[a]

erm(41) length	Nucleotide at:				Phenotype [reference(s)]
	erm(41) position 28	rrl position 2270 (2058)	rrl position 2271 (2059)	Other	
Full	T	A	A		Inducible resistance (3, 15, 16, 20, 21, 27–37), sensitive (33)
Truncated		A	A		Sensitive (9, 15, 20, 21, 29, 32–38)
Full	C	A	A		Sensitive (15, 16, 20, 21, 28–33, 35–37, 39)
Full or truncated	C or T	G	A		Resistant (3, 15, 16, 18, 20, 21, 27, 28, 36–41)
Full or truncated	C or T	C	A		Resistant (3, 15, 20, 21, 28, 38, 41)
Full or truncated	T	T	A		Resistant (15, 20, 27)
Full		A	C		Resistant (20, 27, 40, 41)
Full or truncated	T or C	A	G		Resistant (15, 16, 18, 20, 21, 28, 37, 40)
Truncated		A	T		Resistant (27)
Full	T	A	A	C19T in erm(41)	Sensitive (42)
Truncated		A	A	A2269G in rrl (2057)	Resistant (16)
Full	Unknown	Unknown	Unknown	A2293C in rrl (2082) + G2281C in rrl (2069)	Resistant (41)

[a]*M. abscessus* numbering is used, with *E. coli* numbering provided in parentheses.

CI, 54 to 76%). The very major error rate (phenotype resistant, WGS prediction sensitive) was 5/100 (5%; 95% CI, 1 to 9%), and the major error rate (phenotype susceptible, WGS prediction resistant) was 27/79 (34%; 95% CI, 24 to 44%). The positive predictive value was 95/122 (78%; 95% CI, 69 to 85%), and the negative predictive value was 52/57 (91%; 95% CI, 81 to 97%) (Table 2). The F score for WGS predictions was 0.86. When isolates with a prediction of inducible resistance were further excluded, the specificity of a resistance prediction was 21/21 (100%; 95% CI, 93 to 100%) and the sensitivity was 21/26 (81%; 95% CI, 61 to 93%).

Clarithromycin resistance in the subspecies. Of 143 *M. abscessus* subsp. *abscessus* isolates, 81 were resistant, 58 were sensitive, and 4 were intermediate. For *M. abscessus* subsp. *bolletii*, 18/20 were resistant, and for *M. abscessus* subsp. *massiliense*, 19/40 were resistant (Table 3). There was one *M. abscessus* subsp. *massiliense* isolate carrying a full-length *erm*(41) gene which was phenotypically resistant to clarithromycin. This was not unexpected from a genotypic perspective, as it harbored a wild-type thymine nucleotide at position 28 of *erm*(41), associated with inducible resistance.

Mechanisms of resistance. The negative predictive value of a truncated *erm*(41) gene for clarithromycin susceptibility was 53% [21/39; there was one *M. abscessus* subsp. *massiliense* isolate with a full-length *erm*(41)]. In 11/18 instances, resistance in the presence of a truncated *erm*(41) could be explained by a mutation in position 2270 or 2271 in *rrl*. No coverage at all was seen at these positions for 4/18 isolates. No genomic explanation could be identified for the remaining three discordant isolates (Table 3).

All isolates which had any mutation of position 2269, 2270, or 2271 (*Escherichia coli* numbering, positions 2057, 2058, and 2059, respectively) in *rrl* were resistant to clarithromycin (21/203 [10%]). Such a mutation was found in 3 *M. abscessus* subsp. *bolletii*, 11 *M. abscessus* subsp. *massiliense*, and 7 *M. abscessus* subsp. *abscessus* isolates.

TABLE 2 WGS predictions versus DST phenotype for clarithromycin[a]

Genomic prediction	No. of isolates with the following *in vitro* phenotype:		
	Sensitive	Resistant	Intermediate
No prediction	2	18	0
Inducible resistance	27	74	0
Resistant	0	21	0
Sensitive	52	5	4

[a]The sensitivity (95%; 95% CI, 89 to 98%), specificity (66%; 95% CI, 54 to 76%), positive predictive value (78%; 95% CI, 69 to 85%), and negative predictive value (91%; 95% CI, 81.0 to 97%) were calculated by excluding isolates with an intermediate phenotype and those for which no prediction was made due to inadequate coverage at key positions.

TABLE 3 Summary of genotypes and corresponding clarithromycin phenotypes for the 203 isolates[a]

| Organism | Nucleotide at erm(41) pos. 28 | erm(41) length | Nucleotide at erm(41) pos. 19 | Nucleotide at rrl pos.: | | | N | Phenotype[b] | Prediction |
				2269	2270	2271			
M. abscessus subsp. *abscessus*	T	Full	C	A	C	A	5	5R	R
	T	Full	C	A	T	A	2	2R	R
	C	Full	C	A	A	A	37	4I, 2R, 31S	S
	T	Full	C	A	A	A	87	62R, 25S	R
							12	10R, 2S[c]	
M. abscessus subsp. *bolletii*	T	Full	C	G	A	A	1	1R	R
	T	Full	C	A	G	A	2	2R	R
	T	Full	C	A	A	A	13	11R, 2S	R
							4	4R[c]	
M. abscessus subsp. *massiliense*	T	Truncated	C	A	C	A	3	3R	R
	T	Truncated	C	A	G	A	3	3R	R
	T	Truncated	C	A	A	G	5	5R	R
	T	Truncated	C	A	A	A	24	21S, 3R	S
	T	Full	C	A	A	A	1	1R	R
							4	4R[c]	

[a]pos., *M. abscessus* numbering position in the indicated gene; prediction, genotypic prediction using the algorithm shown in Fig. 1; *N*, total number of isolates with the genotype.
[b]The phenotype indicates the number of isolates that are sensitive (S), resistant (R), or inducibly resistant (I).
[c]These isolates were excluded due to inadequate coverage over *rrl* positions 2270 and 2271.

We did not observe any isolates with an *rrl* mutation which also harbored a T28C mutation in *erm*(41). Where this occurred in isolates reported in the literature, they were always resistant (15, 16).

Of 37 isolates with a T28C mutation in *erm*(41) and no other relevant mutations, 84% (31/37) were susceptible to clarithromycin, 11% (4/31) had intermediate susceptibility, and 5% (2/31) were resistant. This mutation was exclusively found in *M. abscessus* subsp. *abscessus* isolates. We did not identify any drug resistance-associated mutations in any of these intermediate or resistant isolates. Across all three subspecies, of 101 isolates with the *erm*(41)_T28 call associated with inducible resistance (and no other relevant mutation), 73% (74/101) were resistant and 27% (27/101) were susceptible at the final day 21 reading.

De novo search for resistance-determining mutations. The search for potential novel resistance-determining mutations for clarithromycin revealed 13 single nucleotide polymorphisms (SNPs) of interest (Table 4). Of these, five have previously been described in the literature. There were, additionally, four SNPs (*rrl*_A2746T, *rrl*_G836A, *rrl*_T2674G, and *rrl*_T636C) which were only ever seen in resistant isolates but which always co-occurred with known resistance-determining SNPs. There was one phenotypically resistant isolate which harbored 18 novel SNPs. On performing a BLAST analysis of the nucleotide sequence of a 120-base region encompassing all of these SNPs, there was a 99% match (E value, 2×10^{-53}) with *Streptococcus* species. This therefore likely represents sample contamination with flora from the nasopharynx. No new resistance-associated variants were discovered in *rplV*, *rpld*, or *whiB7*.

DISCUSSION

We conducted a systematic review of drug resistance-determining mutations for clarithromycin in *M. abscessus* and used the results to make genotypic predictions. The sensitivity of this approach was 95% (95% CI, 89 to 98%), and the positive predictive value was 78% (95% CI, 69 to 85%). The prevalence of resistance among our collection of isolates was high compared to that which has been reported elsewhere (9, 17–19).

These results show that for clarithromycin, drug resistance can be predicted from WGS data as it has been previously through targeted PCR and line probe assays, such as the Hain GenoType NTM-DR assay. Assessment of the genotype of *erm*(41) with molecular diagnostics allows prediction of its functional status, which has been thought

TABLE 4 Mutations (both novel and previously described) detected during the *de novo* search for resistance-determining SNPs[c]

Position	Nucleotide/amino acid change	Rule met[d]
rrl 2039	A > G	1
rrl 1401	T > C	2
rrl 371	T > C	2
rrl 795	G > A	1
rrl 2270[a]	A > C	1
rrl 2270[a]	A > G	2
rrl 2271[a]	A > G	2
rrl 2270[a]	A > T	2
erm(41) 131	A > V	2
rrl 2279	G > A	2
rrl 2269[a]	A > G	2
erm(41) −31[b]	A > T	2
rrl 1932	A > G	2

[a]The mutation is already described in the literature. *M. abscessus rrl* numbering 2270 and 2271 is *E. coli* numbering 2058 and 2059, respectively.
[b]Mutation in the *erm*(41) promoter region 31 bases upstream of start of the coding region.
[c]All numbering is relative to that for *M. abscessus*.
[d]Rule 1, occurs as the only SNP in relevant regions in resistant isolates; rule 2, all samples are resistant when SNP occurs, and the SNP is never seen in sensitive isolates.

to correlate with the treatment outcome (10). Similarly, as the absence of a functional *erm*(41) gene has been associated with good therapeutic outcomes, its molecular detection ought to be beneficial to patients (9), although in our study this alone was not an adequate predictor of *in vitro* resistance. A genotypic prediction of inducible resistance produced a variable phenotype in our study (27/101 sensitive isolates). Discriminating such isolates that are predicted to be inducibly resistant but that are unexpectedly sensitive after prolonged incubation with clarithromycin or that show high-level resistance at early time points may help to identify additional genotypic markers to better identify patients more likely to benefit from the use of macrolides.

In addition to the mutations identified in the literature search, we also managed to identify variants that may plausibly be new resistance-determining mutations. However, these will require validation against an independent data set. The use of routinely collected diagnostic data to improve our understanding of the molecular determinants of drug resistance is a key advantage that WGS has over line probe assays or PCR. The eight previously undescribed mutations that we report on in this work could be of clinical importance because they all occurred in samples which the existing literature predicts to be inducibly resistant. As BTS guidelines recommend that patients with such isolates be given a macrolide, it is important to determine further whether these SNPs are true resistance determinants and whether macrolide therapy should be avoided in their presence.

Previous authors have suggested that it is clinically useful to discriminate between subspecies (9), as *M. abscessus* subsp. *massiliense* is typically associated with durable susceptibility to clarithromycin and *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* are typically associated with inducible resistance (unless the T28C mutation is present). We found identifying subspecies alone to be an inadequate predictor of the *in vitro* clarithromycin susceptibility phenotype. There were three *M. abscessus* subsp. *massiliense* isolates in our collection that were resistant to clarithromycin and had no mutations known to be relevant. Mougari and colleagues found that in 39/40 *M. abscessus* subsp. *massiliense* isolates selected for clarithromycin resistance, this could be explained by an *rrl* mutation at positions 2270 and 2271, with a further sample containing an *rplV* insertion (20). All of our isolates contained this insertion (which was also present in the reference sequence with GenBank accession number NC_010397.1), which was associated with susceptibility to clarithromycin, except in the presence of a relevant *rrl* mutation.

In keeping with previous reports, we identified an isolate of *M. abscessus* subsp. *massiliense* with a full-length *erm*(41) and a thymine nucleotide at position 28 (21). This

likely represents recombination between the subspecies. A recent study showed that the Hain GenoType NTM-DR line probe assay incorrectly predicted subspecies in 8% of samples, presumably because it lacks the whole-genome resolution provided by sequencing and is vulnerable to between-species recombination (15).

Despite analyzing all mutations occurring in erm(41) and rrl for the full collection of genomes, we were unable to predict all clarithromycin resistance. This may be because there are other genes implicated or due to unreliable drug susceptibility testing (DST) results. Future work should aim to select discordant genotypes and identify additional infrequently occurring genetic loci implicated in clarithromycin resistance, for example, by using genome-wide association (GWAS) approaches. All of the new clarithromycin resistance-conferring mutations that we discovered occurred in isolates which we originally predicted to be inducibly resistant. Although M. abscessus is primarily thought to be an environmental organism, these patients may be colonized for long periods with subsequent potential exposure to multiple courses of macrolides. An alternative hypothesis may therefore be that some or all of these SNPs are compensatory mutations which act to reduce a fitness cost of the expression of erm, which has been experimentally demonstrated in other bacteria (22). There were four SNPs which occurred only in resistant samples but which were always seen with a known drug resistance-causing SNP; these four SNPs possibly also represent compensatory mutations.

Key weaknesses of our study include the fact that we were unable to establish a temporal relationship between antibiotic prescribing and inducible phenotypic resistance, as we did not have the relevant ethics approval to link to patient records. If, for example, any SNPs on our list of novel mutations were observed in isolates from patients who had never previously had macrolide therapy, it would be much more likely that they were genuine resistance-conferring mutations rather than compensatory mutations. In addition, it is possible that some of the genomes were the same patient replicates over a number of months or years, although this may have also diversified the range of mutations observed. We chose to include all available samples to maximize the detection of low-frequency resistance-determining SNPs, meaning that there was no validation set available. Our list of novel resistance-determining SNPs will therefore require validation on an independent data set before being applied to the clinical setting. We chose to target a select list of genes with known SNPs identified in the literature search; other approaches, such as GWAS, will likely be additive to the knowledge base that we present here.

In summary, WGS allows identification of known resistance-conferring mutations as well as demonstration of probable novel resistance-determining SNPs in regions that the Hain NTM-DR line probe assay cannot detect and that, if further validated, may change management. Identification of subspecies alone inadequately predicts macrolide resistance in M. abscessus. Our data do not support the replacement of phenotypic tests at this time; as more paired genome/DST data become available in the near future and we learn more about the molecular determinants of drug resistance, it is likely that the sensitivity and specificity of WGS resistance prediction will improve. Given that WGS data are already being produced in the United Kingdom for the purposes of molecular epidemiology, it would now be possible to phase out existing molecular tests and replicate their results in silico at no additional cost.

MATERIALS AND METHODS

Literature search. We first conducted a systematic review of the literature to search for known drug resistance-conferring mutations in M. abscessus. PubMed was searched with the terms "Mycobacterium abscessus" AND "clarithromycin" OR "macrolide" OR "drug resistance" OR "antibiotic resistance," looking for English-language articles published up to April 2018. To be included in the final list, articles had to contain the genotypes of coding regions relevant to clarithromycin resistance in M. abscessus, in addition to paired drug susceptibility data. Studies looking at both clinical and nonclinical samples were included. A total of 298 abstracts were screened for relevance, and 81 full-text articles were obtained; of these, 26 met the inclusion criteria (Fig. 2).

Sample selection and sequencing. We next sought all available clinical isolates (n = 180) which had undergone whole-genome sequencing by the Public Health England (PHE) laboratory in Birmingham, UK,
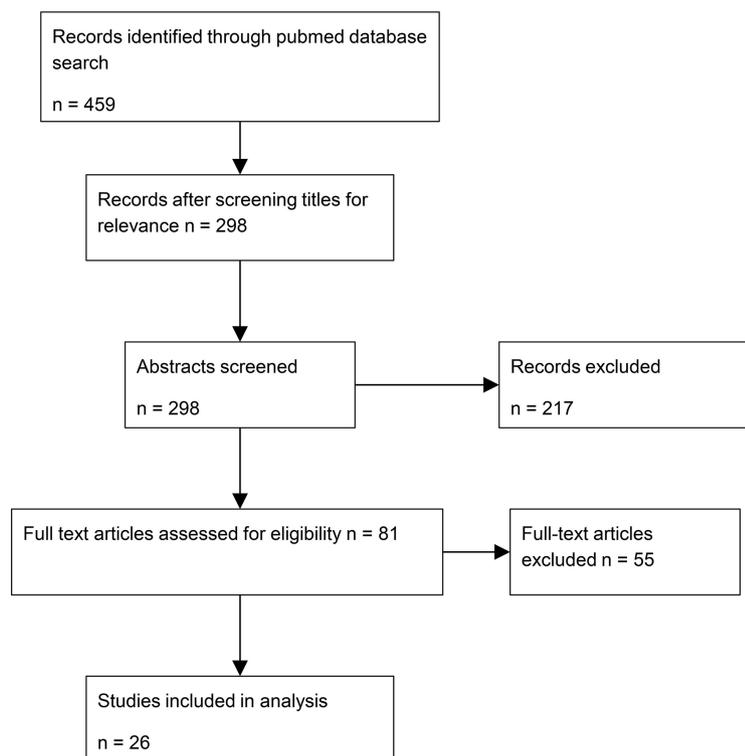
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│ Records identified through       │
│ pubmed database search           │
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│ eligibility n = 81               │        │ excluded n = 55      │
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FIG 2 Flow diagram showing the stages of the systematic literature search.

as part of the routine diagnostic work flow and for which paired phenotypic data were also available. We supplemented this with 23 isolates for which the same data were available from a WGS archive at the University of Oxford. Isolates were collected between May 2014 and January 2017, and no prior selection according to the site of isolation or whether *M. abscessus* complex disease confirmed by guidelines was made. Clinical samples were cultured in BD Bactec MGIT liquid mycobacterial growth indicator tubes, from which an aliquot was removed to be prepared for WGS as previously described (23).

Libraries for Illumina MiSeq sequencing were prepared using the Illumina Nextera XT protocol with manual library normalization. Samples were batched at 12 to 16 per flow cell, and paired-end sequencing was performed with a MiSeq reagent kit (v2). Bioinformatics was performed using the PHE bioinformatics pipeline as previously described (23, 24). Briefly, reads were mapped to the *M. abscessus* subsp. *abscessus* reference genome (GenBank accession number NC_010397.1) with Stampy (v1.22), and variants were called using Samtools (v0.1.18) (only variants with ≥5 high-quality reads, a mean quality per base of ≥25, and >90% high-quality bases were retained as variants; heterozygous variants with >10% minor variants were not retained). A self-self BLAST approach was used to mask repetitive regions. Subspecies were identified by computing maximum likelihood (ML) phylogenetic trees incorporating published representative isolates from each subspecies. A whole-genome SNP alignment was used as input to IQ-TREE OMP (v1.5.5) using a generalized time-reversible model. The *erm*(41) and *rplV* genes were manually inspected for insertions/deletions from aligned FASTA files using the Seaview (v4.6.2) program.

Drug susceptibility testing. Phenotypic drug susceptibility testing (DST) was performed at the PHE National Mycobacterial Reference Service in London, UK. DST was performed using the broth microdilution method with 96-well Rapmyco microtiter plates (Mueller-Hinton medium with TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer; Thermo Fisher). The plates were read at day 3 postinoculation, and if growth was poor, they were read again at day 5, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (25). Isolates deemed susceptible or intermediate were reincubated, and the plates were read again at days 7, 14, and 21. Those found to be resistant (R; MIC ≧ 8 μg/ml) at any of these time points were described as phenotypically resistant. A call of phenotypically sensitive (S; MIC ≦ 2 μg/ml) or intermediate (I; MIC >2 to <8 μg/ml) was made only after the full 21 days of incubation. This study was an opportunistic retrospective analysis of routinely collected clinical data, and as such, phenotypic testing was not repeated on discordant isolates.

Genotypic prediction of clarithromycin susceptibility. We used BioPython software to extract base calls from whole-genome sequence FASTA files, comparing these to a list of genomic loci which our literature search indicated were associated with clarithromycin resistance (Table 1). We then predicted phenotypes using a hierarchical algorithm (Fig. 1). A resistant phenotype was predicted where any mutations were present at *rrl* position 2270 or 2271 (*E. coli* numbering, positions 2058 and 2059, respectively) or where the less well characterized *rrl*_A2269G, *rrl*_A2293C, or *rrl*_G2281A mutation was seen. In the absence of these mutations, susceptibility was predicted where an isolate had a truncated

erm(41) gene or a C nucleotide at position 28 in *erm*(41). Inducible resistance was predicted where a wild-type call (T) was present at position 28 in *erm*(41). However, if an *erm*(41)_C19T mutation was also present, susceptibility instead of inducible resistance was predicted. In cases where there was a null call at *rrl* 2270/2271, we subsequently attempted local assembly of the *rrl* gene using the Ariba tool (26), followed by comparison by alignment against the reference sequence. Where this was not possible due to low coverage in this region, no prediction was made. The statistics quoted were calculated using R Studio (v1.1.383).

Search for novel resistance-conferring mutations. We attempted to characterize new resistance-conferring mutations within genes linked to drug resistance from the literature search. To maximize the power for discovering new potential resistance-conferring mutations, we included all genomes available to us. All variants in these genes or their promoter regions were extracted from variant call files using Python software. Phylogenetic SNPs, assumed to be benign, were identified by considering each subspecies in turn and excluded from further analysis.

We considered variants at the level of SNPs in promoter regions or rRNA and amino acid changes in coding regions. A mutation (a variant in an isolate with an observable phenotype) was characterized as causing resistance if it occurred as the only variant in the relevant region in a resistant isolate or if it was always associated with resistance when it was seen and did not co-occur with any other mutations known to cause resistance. Variants were characterized as consistent with susceptibility (benign) if all isolates were susceptible when a variant occurred alone or if it occurred only in susceptible isolates. We assumed no prior knowledge in this section of the analysis, and the identification of known resistance-conferring SNPs was used as an internal validation of our approach.

Accession number(s). All newly sequenced data have been uploaded to NCBI under BioProject accession number PRJNA420644.

ACKNOWLEDGMENTS

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