Whole genome sequencing for predicting *Mycobacterium abscessus* drug susceptibility

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Abstract

Objectives:
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 characterise the genetic determinants of resistance.

Methods:
A systematic review was performed to catalogue all known resistance determining mutations. This knowledge was used to construct a predictive algorithm which was tested on a collection of 209 sequentially acquired clinical isolates for which there was paired genotype/phenotype data. Predictions were made for those drugs for which genetic loci involved in drug resistance were identified in the literature search. A search for novel resistance determining mutations was conducted using an heuristic algorithm.

Results:
The literature search identified two genes of interest for Clarithromycin (rrl and erm(41)) and Ciprofloxacin (gyrA and gyrB) and one for Amikacin (rrs). After excluding isolates predicted to be inducibly resistant and those for which there were null calls in key positions, the sensitivity of existing knowledge for clarithromycin was 76.19% (95% CI 52.8 - 91.8%) and the specificity was 100%. Subspecies alone was a poor predictor of resistance to macrolides. For Ciprofloxacin the sensitivity was 0% and for Amikacin it was 5.0% (95% CI 2.0 - 10.0). Seven potential new resistance conferring SNPs were identified for clarithromycin, four for ciprofloxacin and three for amikacin.

Conclusion:
We demonstrate that WGS demonstrates probable resistance determining SNPs in regions the NTM-DR line probe cannot detect. These mutations are potentially clinically important as they all occurred in samples 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.

Introduction

Mycobacterium abscessus is a rapidly growing non-tuberculous Mycobacterium (NTM) of increasing clinical concern with a rising burden of associated pulmonary disease[1]. It 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 amongst the CF population within a healthcare setting.[3,4]

The taxonomy of *M. abscessus* is contentious. It is currently divided into three subspecies: *M. abscessus* subspecies *abscessus (Maba), M. abscessus* \subspecies *massiliense (Mabm)*, and *M. abscessus* subspecies *bolletii (Mabb)*.[5] The organism is inherently resistant to multiple antibiotics including β-lactams, rifampicin and aminoglycosides due to the synergistic action of the cell envelope and genetic factors termed the 'intrinsic resistome'.[6] Treatment requires prolonged courses of multiple antibiotics, but outcomes vary across the different subspecies. *Mabm* has been associated with clarithromycin susceptibility and favourable treatment outcomes, whereas *Maba* has been associated with inducible macrolide resistance and poorer treatment outcomes.[7] However, the American Thoracic Society/Infectious Diseases Society of America and British Thoracic Society (ATS/IDSA and BTS) guidelines recommend including a macrolide in treatment regimens where samples are either susceptible, or demonstrate inducible resistance.[8,9]

Whole genome sequencing 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, as has been successfully achieved in a range of different bacteria.[10–12] As there is a particularly strong correlation between *in vitro* susceptibility and clinical response to macrolide treatment of *M. abscessus*,[13,14] we have undertaken a pilot study to assess the feasibility of predicting clarithromycin susceptibility from whole genome sequencing data for all three subspecies of *M. abscessus*. We also explore how well WGS is able to predict susceptibility to other drugs.

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 'drug resistance' OR 'antibiotic resistance', looking for English language

articles published up to August 2017. To be included in the final list, articles had to contain genotyping of coding regions relevant to clarithromycin resistance in *M. abscessus* in addition to paired drug susceptibility data. Studies looking at both clinical and non-clinical samples were included. 230 abstracts were screened for relevance and 52 full text articles were obtained of which 22 met the inclusion criteria (Figure 1).

Sample selection and sequencing

We next sought all available clinical isolates (N = 181) which had undergone whole genome sequencing by the Public Health laboratory in Birmingham (UK) as part of the routine diagnostic workflow and for which paired phenotypic data were also available. We supplemented this with 28 isolates for which the same data was available from a WGS archive at the University of Oxford. Isolates were collected between May 2014 and January 2017. All sequencing was performed using Illumina technology and reads were mapped to the *M. abscessus* subspecies *abscessus* reference genome (Genbank NC_010397.1) using the previously described Public Health England/University of Oxford bioinformatics pipeline.[10] Sub-species 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 version 1.5.5 using a generalised time reversible model. The integrity of the erm(41) gene was manually inspected from aligned fasta files using Seaview version 4.6.2. All sequence data has been uploaded to NCBI under project accession number PRJNA420644.

Drug susceptibility testing

Phenotypic drug susceptibility testing (DST) for clarithromycin was performed at the two laboratories of the Public Health England (PHE) National Mycobacterial reference service in Birmingham and London. In Birmingham, DST was performed using an in house preparation of Lowenstein-Jenson (LJ) Media and was read at 7, 14 and 21 days post-inoculation according to British Society of Antimicrobial Chemotherapy (BSAC) methodology. [15] Isolates were deemed either Susceptible (S) or Resistant (R). DST for the Oxford isolates was performed in London using the Standard Broth Microdilution method for which 96-well microtitre plates (Sensititre) were used and prepared as previously described.[16] Plates were read at day three post-inoculation according to Clinical and Laboratory Standards Institute (CLSI) guidelines [17] and classed as either susceptible (S), resistant (R) or intermediate (I) (appendix).

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 2). We then predicted phenotypes algorithmically (Figure 2). A resistant phenotype was predicted where any mutations were present at *rrl* positions 2058 or 2059, or where the less well characterized *rrl*_A2057G 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 *erm41* (meaning that either susceptible or resistant could be correct calls depending on the temporal relationship between antibiotic administration and sampling time). However, if an *erm41*_C19T mutation was also present, susceptibility was predicted instead of inducible resistance. In cases where there was a null call at *rrl* 2058/2059, we did not make a prediction but the number of resistance and susceptible alleles at those gene positions were inspected and compared to the phenotype (Figure 2).

Search for novel resistance conferring mutations

For each of amikacin, ciprofloxacin and clarithromycin, we attempted to characterise new resistance mutations within genes linked to drug resistance in the literature search. All mutations in these genes or their promoter regions were extracted using Python software from variant call files. Phylogenetic SNPs 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 in a similar manner to a previously reported algorithm[10]. To maximise power for discovering new potential resistance mutations, we included all genomes available to us.

A mutation was characterised as causing resistance if it occurred as the only mutation 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. Mutations were characterised as consistent with susceptibility ('benign') if all isolates were susceptible when the mutation occurred alone or if it occurred only in susceptible isolates. For amikacin and ciprofloxacin, where the number of susceptible samples was low, we only applied the first rule for characterising resistant mutations since the vast majority of samples were resistant and therefore the second rule would likely produce too many false positives. We assumed no prior knowledge in this section of the analysis and the identification of known resistance SNPs was used as an internal validation of our approach.

Results

We studied 143 *Maba*, 20 *Mabb* and 40 *Mabm* genomes (figure 3). Collectively, these isolates exhibited high levels of resistance to both first line agents (Amikacin (89%), Tigecycline (59%), Imipenem (58%) and Clarithromycin (58%)), and the BTS recommended continuation phase antibiotics (Linezolid (97%), Minocycline (100% - Oxford only), Moxifloxacin (99%), Cotrimoxazole (99%)).

Genotypic predictions were made on the basis of mutations identified by the literature search. No relevant genes were identified for drugs other than amikacin, ciprofloxacin and clarithromycin and these drugs were therefore not included in any futher analysis. For macrolides, all relevant mutations were contained in the genes *rrl* and *erm(41)*. These are summarised in figure 2 and table 2. For amikacin, four mutations in *rrs* A1408G, T1406A,

C1409T and G1491T were reported as causing resistance.[18–20] One article described *rpsL* as being important for aminoglycoside resistance in *M. abscessus*, but no specific SNPs were identified by the authors.[21] For ciprofloxacin, mutations in *gyrA* amino acid positions 92 and 96 and *gyrB* position 492 (*M. abscessus* numbering) were associated with resistance.[22]

Genotypic predictions

Inducible resistance was predicted in 91 isolates, meaning that these could be either susceptible or resistant on phenotypic testing. Of these, 65/91 (71%) were reported as phenotypically resistant. After excluding isolates for which no prediction could be made due to missing data in key genes (n = 38) as well as those were we made a prediction of inducibly resistant (n = 91), sensitivity was 76% (95% CI 53 - 92 %) and specificity was 100%. The very major error rate (WGS prediction sensitive, phenotype resistant) was 3.0% (95% CI 0.4 - 5.6%) and the major error rate (phenotype susceptible, WGS prediction resistant) was 0%. Positive predictive value was 100% and negative predictive value was 90.7% (95% CI 82.0 - 95.5%) (Table 3).

The mutations derived from the literature did not predict any resistance to ciprofloxacin (0/190). The literature search only identified one mutation associated with amikacin resistance (*rrs* A1408G). No nucleotide call at position 1408 was made for 18/157 resistant isolates and these were excluded from the analysis. For this mutation, the sensitivity was 5.0% (95% CI 2.0 - 10.0) but the positive predictive value was 100%.

Resistance in the subspecies

81/143 *Maba* were resistant, 58 sensitive and 4 intermediate. For *Mabb* 18/20 were resistant and for *Mmas* 19/40 (Figure 3). There was one *Mmas* isolate carrying a full length *erm(41)* gene which was phenotypically resistant to clarithromycin. This was not unexpected from a genotypic perspective as it harboured a wild type thymine nucleotide at position 28 *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 - the was one *Mmas* isolate with a full length erm(41). In 9/18 instances, resistance in the presence of a truncated *erm(41)* could be explained by a mutation in position 2058 or 2059 in *rrl*. No coverage at all was seen at these positions for 4/18 isolates, whilst coverage was inadequate to determine a consensus nucleotide call in 2/18. In the later two cases, minor alleles were however present that could potentially explain the resistant phenotypes. No genomic explanation could be identified for the remaining three discordant isolates.

All isolates which had any mutation of positions 2057, 2058 or 2059 in *rrl* were resistant to clarithromycin (15/203 (7.4%)). Such a mutation was found in 2 *M. boletti*, 8 *M. massiliense* and

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

Of 36 isolates with a T28C mutation in *erm(41)* and no other relevant mutations, 83% (30) were susceptible to clarithromycin, 11% (4) had intermediate susceptibility and 6% (2) were resistant. This mutation was exclusively found in *Maba* isolates. We did not identify any drug resistance associated mutations in any of these intermediate or resistant isolates. Across all three subspecies, of 91 isolates with the T28_erm41 call associated with inducible resistance (and no other relevant mutation), 71% (65) were resistant and 29% (26) susceptible.

De novo search for resistance determining mutations

The search for potential novel resistance determining mutations for clarithromycin revealed 12 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 always co-occurred with known resistance determining SNPs. There was one phenotypically resistant isolate which harboured 18 novel SNPs. On performing a nucleotide BLAST of a 120 base region encompassing all of these SNPs, there was a 99% (E 2 $x10^{-53}$) match with *Streptococcal spp.* This therefore likely represents sample contamination with flora from the nasopharynx.

For the two other drugs in which the literature search identified resistance associated mutations, ciprofloxacin and amikacin, a list of mutations occurring as the only mutation in resistant and never in sensitive samples is shown in supplementary table 1. We were unable to conduct searches for novel mutations for any of the other drugs, either because there were insufficient numbers of sensitive isolates to act as controls, or the gene targets were not known in Mycobacteria.

Discussion

We conducted a systematic review of drug resistance determining mutations in *M. abscessus* and used the results to make genotypic predictions for clarithromycin, amikacin and ciprofloxacin. For clarithromycin the sensitivity was 76% and the positive predictive value 100%. For amikacin, only one mutation was identified, and although its PPV was also 100%, the sensitivity was 5.8%. None of the mutations relevant to ciprofloxacin were present in our data.

For clarithromycin, these results show that drug resistance can be predicted from WGS data as it has been previously through targeted PCR and line probe assays such as the GenoType NTM-DR. Assessment of the genotype of *erm(41)* with molecular diagnostics allows prediction of its functional status which has been thought to correlate to treatment outcome.[8] 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,[7] although in our study this alone was

not an adequate predictor of resistance. Furthermore both in our study and others,[23] there are samples where inducible resistance would be genotypically predicted that show early time-point phenotypic resistance. With DST alone, patients with such isolates would not be treated with a macrolide however it is unclear whether this is the optimal strategy. Discriminating such isolates from those that are constitutively resistant is an important step towards answering this question.

In addition to mutations identified in the literature search, we also managed to identify mutations that plausibly explain some of the drug resistance we failed to predict. However, these mutations will require validation against an independent data set. Using routinely collected diagnostic data to improve our understanding of the molecular determinants of drug resistance is a key advantage WGS has over line-probe assays or PCR. The seven previously undescribed mutations we report in this work could be of clinical importance because they all occur in samples which the existing literature predicts to be inducibly resistant. As BTS guidelines recommend that patients with such isolates should 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, [7] as *M. massiliense* is typically associated with durable susceptibility to clarithromycin and *M. bolletii* and *M. abscessus* with inducible resistance (unless the T28C mutation is present). We found identifying sub-species alone to be an inadequate predictor of *in vitro* clarithromycin phenotype as 48% (19/40) of *M. massiliense* isolates were resistant which was not explained by current knowledge in 3 isolates where there was adequate coverage of *rrl* 2058/2059. In keeping with previous reports, we identified an isolate of *M. massiliense* with a full length *erm(41)* and a thymine nucleotide at position 28.[24] This likely represents recombination between the subspecies. Interestingly a recent study showed 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.[18]

Despite analysing 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 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 approaches. All of the new clarithromycin resistance mutations we discovered occurred in isolates which we originally predicted to be inducibly resistant. It could 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.[25] There were four SNPs which only occurred in resistant samples but were always seen with a known drug resistance causing SNP, possibly also representing compensatory mutations.

Key weaknesses of our study include that we were unable 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 rather than compensatory mutations. In addition it is likely that some of the genomes were same patient replicates over a number of months/years, although this may have also diversified the range of mutations observed. We chose to include all available samples to maximise detection of low frequency resistance determining SNPs meaning there was no validation set available. Our list of novel resistance determining SNPs will therefore require validation on an independent dataset before being applied to the clinical setting.

In summary, WGS can detect all of the same mutations as the Hain NTM-DR line probe assay, allows more accurate identification of sub-species and demonstrates probable resistance determining SNPs in regions the line probe cannot detect which if further validated would change management. In addition, we find that the identification of subspecies alone inadequately predicts macrolide resistance in *M. abscessus*. As more paired genome/DST data becomes available in the near future, and we learn more about the molecular determinants of drug resistance, it is likely that sensitivity will improve. Given that WGS data is already being produced in the UK for the purposes of molecular epidemiology, it would now be possible to phase out existing molecular tests and replicating their results *in silico*, from WGS data at no additional cost.

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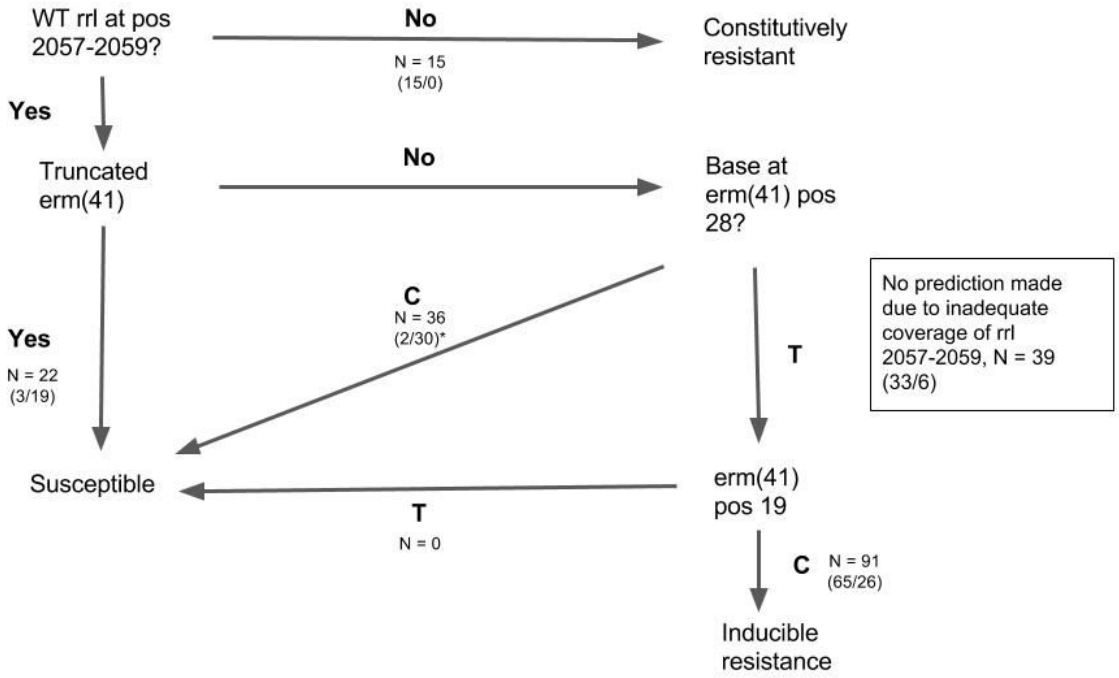

Figure 2: decision algorithm for predicting drug resistance in *M. abscessus* based on the literature search with numbers of isolates meeting each predictive criterion shown. Bracketed numbers represent (N resistant / N sensitive). * 4 isolates had intermediate susceptibility

	Resistant (%)	**Intermediate (%)**	**Sensitive (%)**
Tobramycin	187 (99)	1 (0.5)	1 (0.5)
Amikacin	157 (89)	4 (2)	16 (9)
Tigecycline	96 (59)	1 (1)	66 (40)
Linezolid	186 (97)	4 (2)	1 (1)
Cefoxitin	166 (89)	12 (6)	9 (5)
Doxycycline	191 (100)	0	0
Moxifloxacin	100 (99)	0	1 (1)
Cotrimoxazole	186 (99)	0	1 (1)
Imipenem	98 (58)	2 (1)	68 (40)
Clarithromycin	118 (58)	4 (2)	81 (40)

Ciprofloxacin	182 (99)	0	1 (1)

Table 1 - susceptibility for all antibiotics tested by the reference laboratories

erm(41) length	erm(41) position 28	rrl position 2058	rrl position 2059	Other	Phenotype
Full	T	A	A		Inducible resistance[3,18,23,24,26–35] Sensitive[32]
Truncated		A	A		Sensitive[7,18,24,27,30,32–36]
Full	C	A	A		Sensitive[18,24,26–32,34,35,37]
Full or Truncated	C or T	G	A		Resistant[3,18,23,24,26,31,34,36–39]
Full or truncated	C or T	C	A		Resistant[3,18,24,26,34,36]
Full or truncated	T	T	A		Resistant[18,23,34]
Full		A	C		Resistant[23,34,39]
Full or truncated	T or C	A	G		Resistant[18,24,26,31,34,38,39]
Truncated		A	T		Resistant[23]
Full	T	A	A	C19T erm(41)	Sensitive[40]
Truncated		A	A	A2057G rrl	Resistant[31]

Table 2: Resistance determining mutations for clarithromycin identified in the literature search

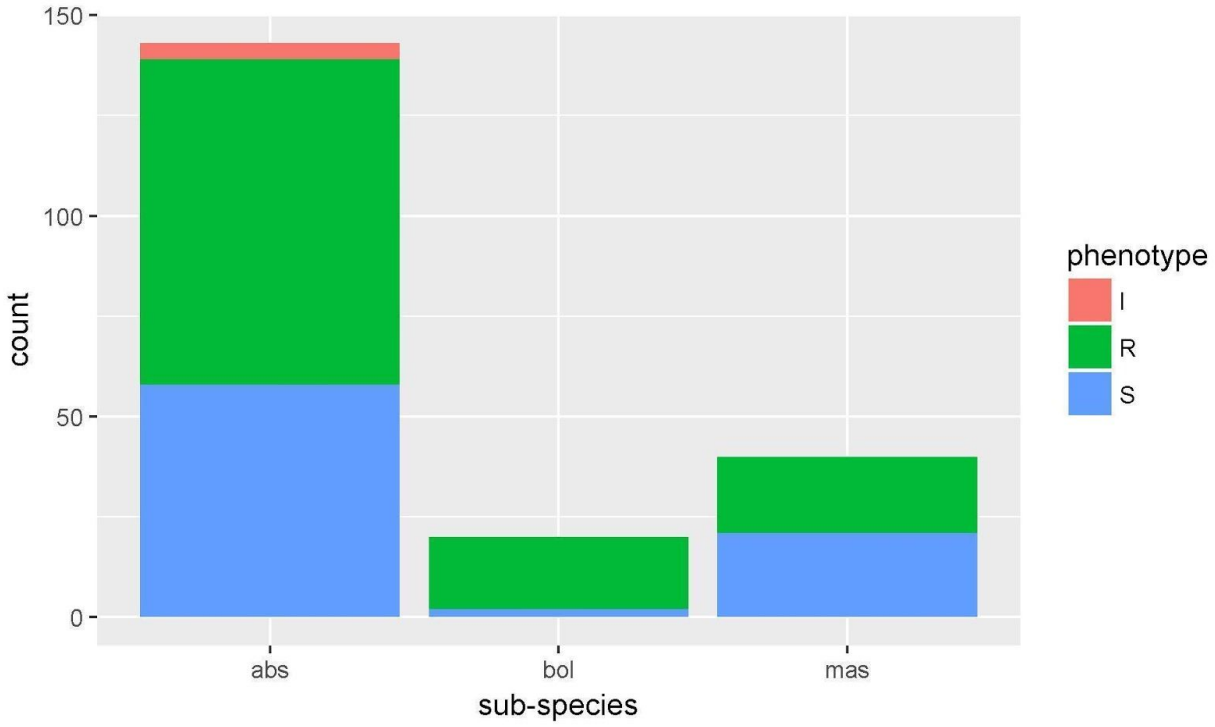

Figure 3: Resistance to clarithromycin by subspecies. I - intermediate, R - resistant, S - susceptible, abs - M. abscessus subsp. abcessus, bol - M. abscessus susbp. boletti, mas - M. abscessus subsp. massiliense

Prediction	**Phenotype**		
	Sensitive	Resistant	Intermediate
No Prediction	6	32	0
Inducible resistance	26	65	0
Resistant	0	16	0
Sensitive	49	5	4
Sensitivity	76.19% (95% CI 52.8 - 91.8%)		
Specificity	100%		
Positive predictive value	100%		
Negative predictive value	90.7% (95% CI 82.0 - 95.5%)		

Table 3 - WGS Predictions vs DST phenotype for clarithromycin. Sensitivity/Specificity/PPV/NPV are calculated excluding isolates predicted to be inducibly resistant and those where no prediction was made due to inadequate coverage.

Position	Nucleotide/Amino acid change	Rule met
rrl 2039	A > G	1
rrl 1401	T > C	2
rrl 371	T > C	2
rrl 795	G > A	1
rrl 2270*	A > C	1
rrl 2270*	A > G	2
rrl 2271*	A > G	2
rrl 2270 *	A > T	2
erm(41) 131	A > V	2
rrl 2279	G > A	2
rrl 2269*	A > G	2
erm (41) -31**	A > T	2
rrl 1932	A > G	2

Table 4: Mutations (both novel and previously described) detected during *de novo* search for resistance determining SNPs. Rule 1 = occurs as only SNP in relevant regions in resistant isolate, rule 2 = all samples resistant when SNP occurs, never seen in sensitive isolate. All numbering is relative to M. abscessus. * mutation already described in literature - *M. abscessus rrl* numbering 2270/2271 is *E. coli* numbering 2058/2059. ** mutation in erm(41) promoter region, 31 bases upstream of start of coding region.

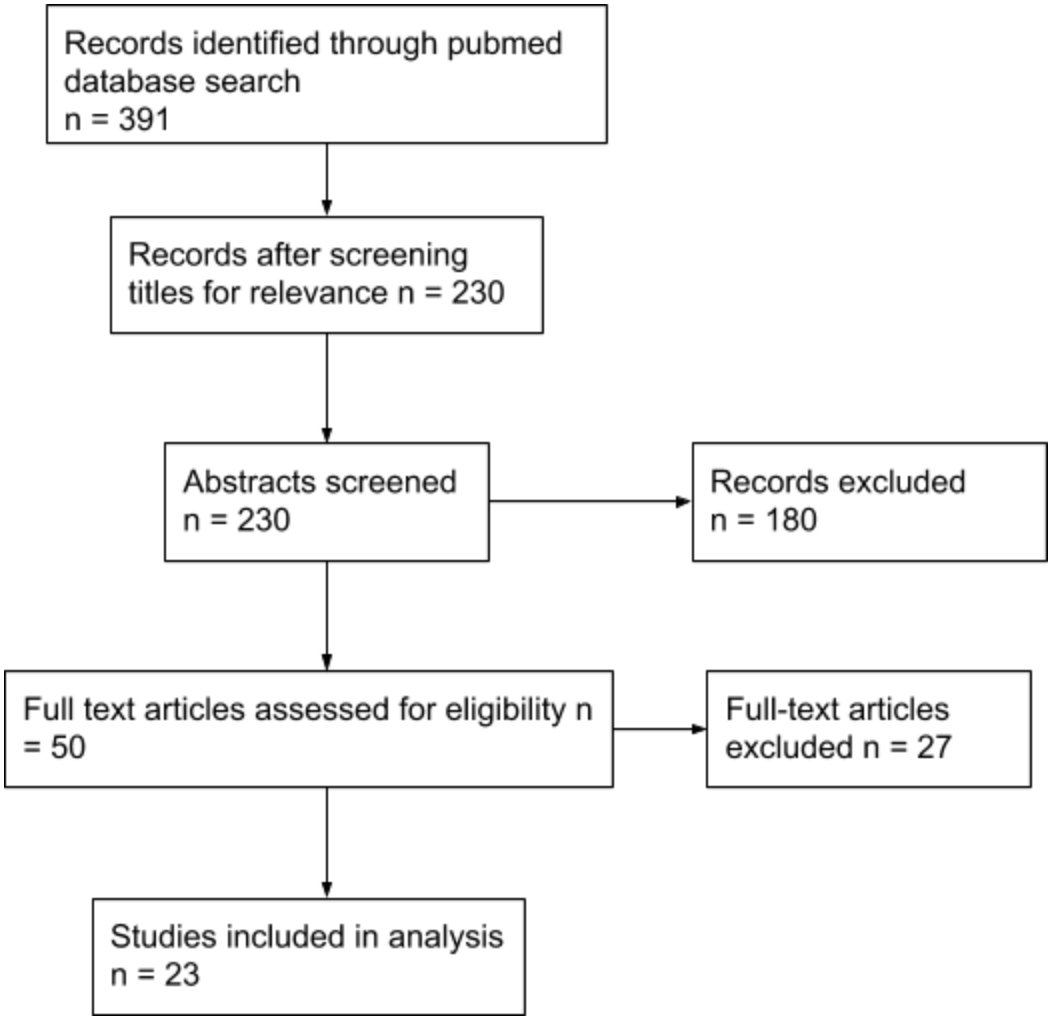

Figure 1: Flow diagram showing stages of the systematic literature search

Drug	Gene	Mutation
Ciprofloxacin	gyrA	S763P
Ciprofloxacin	gyrB	C-41G*
Ciprofloxacin	gyrB	A-78G*
Ciprofloxacin	gyrB	T576S
Amikacin	rpsL	G-95A*
Amikacin	rrs	A1375G (A1408G E. coli numbering)
Amikacin	rrs	A-33G*

Supplementary table 1: mutations occurring as the only mutation in drug resistance associated genes or their promoter regions for ciprofloxacin and amikacin. Due to the very low numbers of susceptible control isolates, other than A1375G which is described in the literature, these are low confidence loci. All mutations are given as *M. abscessus* numbering. Mutation in promoter region -(N) bases upstream of beginning of coding region.