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Comparative analysis of phenotypic and genotypic antibiotic susceptibility patterns in *Mycobacterium avium* complex

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ABSTRACT

Objective: Phenotypic (Sensititre Myco, pDST) and genotypic drug susceptibility testing (GenoType NTM DR, gDST) in *M. avium* complex (MAC) have become available as standardized assays, but comparable data is needed. This study aimed to investigate the phenotypic and genotypic drug susceptibility patterns in MAC clinical isolates.
Methods: Overall, 98 isolates from 85 patients were included. pDST and gDST were performed on all isolates and results compared regarding specificity and sensitivity using pDST as a reference method. The impact of drug instability on pDST results was studied using a biological assay over 14 days. In addition, the evolution of antimicrobial resistance was investigated in sequential isolates of 13 patients.
Results: Macrolide resistance was rare, 1.2% (95% CI 0.7–7.3) of isolates in the base cohort. No aminoglycoside resistances were found, but 14.1% of the studied isolates (95% CI 7.8–23.8) showed intermediate susceptibility. The GenoType NTM DR identified two out of four macrolide-resistant isolates. Antibiotic stability was demonstrated to be poor in rifampicin, rifabutin, and doxycycylin.
Conclusions: pDST results in NTM for unstable antibiotics must be interpreted with care. A combination of pDST and gDST will be useful for the guidance of antimicrobial therapy in MAC-disease.

Background

Non-tuberculous mycobacteria (NTM) comprise all mycobacteria except *M. tuberculosis complex* and *M. leprae* (currently more than 200 species) (Griffith et al., 2007). Within this large group of bacteria, *Mycobacterium avium* complex (MAC) has been identified in 47% of all cases in a Europe-wide NTM-NET collaborative study with more than 20,000 NTM-isolates (Hoefsloot et al., 2013). It includes twelve species; *M. avium*, *M. intracellulare*, and *M. chimaera* are the most frequent ones (van Ingen et al., 2018). While *M. avium* and *M. intracellulare* are known to be responsible for lung and disseminated infections in immunocompromised

Abbreviations: AMI, amikacin; CAMHB, cation adjusted Mueller-Hinton broth; CLA, clarithromycin; CIP, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; CTAB, Cetrimonium bromide; DOX, doxycycline; ECOFF, Epidemiological Cut Off Value; EMB, ethambutol; ETH, ethionamide; EUCAST, European Committee on Antimicrobial Susceptibility Testing; INH, isoniazid; IQR, interquartile range; gDST, genotypic drug susceptibility testing; pDST, phenotypic drug susceptibility testing; MAbsC, *Mycobacterium abscessus* complex; MAC, *Mycobacterium avium* complex; MXF, moxifloxacin; NTM, non-tuberculous mycobacteria; RIF, rifampin; RFB, rifabutin; SNP, single nucleotide polymorphism; SXT, trimethoprim/sulfamethoxazole; WT, wild type.

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hosts such as HIV patients or patients with underlying lung diseases, as bronchiectasis or cystic fibrosis (CF) (Griffith et al., 2007), *M. chimaera* has additionally been involved in a worldwide nosocomial outbreak of disseminated infections in patients having undergone cardiosurgical procedures (van Ingen et al., 2017). Here, heater-cooler units have been the source for infection with unfavorable outcomes in a variety of patients (Sommerstein et al., 2016). But *M. chimaera* has also been shown to be responsible for lung disease (Larcher, 2019; Rosero and Shams, 2019).

As NTM are increasingly recognized as clinically important pathogens, and treatment recommendations are mainly based on expert opinions, there is a need for the broad application of drug susceptibility testing (DST) in these bacteria and its correlation with clinical data. The Clinical and Laboratory Standards Institute (CLSI) is so far the only organization that has published guidelines on DST in NTM (Clinical Laboratory Standards Institute, 2018). Nevertheless, as data is scarce, there are no empirical breakpoints for many relevant antibiotics until now. The availability of a standardized phenotypic DST (pDST) testing system (Sensititre Myco SLOMYCO, Thermo Fisher Scientific, Waltham, USA), offers the opportunity to produce comparable MIC data for NTM on a large scale.

The use of pDST, however, poses several problems: First, in slow-growing mycobacteria (SGM), it is very time-consuming. Second, interpretation of results remains highly subjective, as a recent comparison between MIC reading of different European reference centers has shown (Nikolayevskyy et al., 2019). Automated analysis is not ready yet for standard utilization in the clinical context (Rockland et al., 2018). Third, as incubation times are long, the stability of the tested antibiotics remains a technical problem, as does the interpretation of results (Schoutrop et al., 2018).

To address these problems, researchers have identified several genetic mutations predicting resistance (Lipworth et al., 2018; Maurer et al., 2012; Jamal et al., 2000). Genotypic susceptibility for macrolides and aminoglycosides can be rapidly assessed by using the Genotype NTM-DR VER 1.0 strip test (Hain Lifescience GmbH, Nehren, Germany), being made available within a day. The assay allows us to differentiate between 7 NTM species/subspecies (*M. avium, M. chimaera, M. intracellulare, M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii, M. abscessus* subsp. *massiliense* and *M. chelonae*) and to identify the following mutations conferring macrolide resistances: *erm:* C28T (inducible resistance, only for *M. abscessus* complex- MAbsC) and *rrl*: A2058C, A2058G, A2058T, A2059C, A2059G, and A2059T (inherent macrolide resistance, MAbsC and MAC). Aminoglycoside resistances can be detected within the *rrs*-gene (A1408G, T1406A, C1409T, MAbsC, and MAC) ().

The objective of this study was to contribute to the growing need for comparable pDST data in MAC and to test the accuracy of the commercially available Genotype NTM-DR in different isolates. Also, the *in vitro* stability of antibiotic agents used in the pDST assay was evaluated. Finally, we investigated whether, in single patients, there were significant changes in antibiotic susceptibility in sequential isolates and addressed the question of whether those were linked to reinfection or mutations within the same strain.

Materials and methods

Clinical isolates

In total, 98 MAC isolates from our previously published cohort for the period 2006–2016 were available for further processing. Patient data were retrieved from the local patient management system as previously described (Wetzstein et al., 2019). The patients were subgrouped into different clinical categories (NTM pulmonary disease or colonization – NTM-PD/C, disseminated mycobacteriosis, dermatological manifestations, lymphadenitis, and others), as well as into different underlying dispositions (CF, HIV, structural lung disease – SLD, malignoma, and others).

Follow-up isolates were included if they were cultured at least nine months after the first isolate. Antibiotic treatment of patients with serial isolates was retrieved retrospectively by chart review and subgrouped into macrolides, rifamycins, ethambutol, and aminoglycosides. The duration of treatment with the associated antibiotic was recorded in weeks.

Bacterial culture

Clinical isolates were cultured at 37 °C on solid Middlebrook 7H10 Agar with OADC (Becton Dickinson, Heidelberg, Germany). Agar plates were controlled for visible growth at days 7, 10, and 14 and processed further if sufficient bacterial growth could be observed.

Species identification and genotypic drug susceptibility testing (gDST)

DNA extraction was performed using fresh bacterial cultures, processing them with the GenoLyse Kit (Hain Lifesciences, Nehren, Germany) (). PCR and DNA strip tests were conducted as recommended by the manufacturer with the Genotype NTM-DR (Hain Lifesciences, Nehren, Germany) (). In case of discordant results between gDST and pDST, the isolates underwent a PCR assay for the *rrl*-gene with the following primers: 23SFI 5'-TTTAAGCCCCAGTAAACGGC-3' and 23SRI 5'-CCAAACCATCCCGTC-GATAT-3' and cycling conditions: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, for 35 cycles and subsequent Sanger sequencing (Jamal et al., 2000).

Phenotypic drug susceptibility testing (pDST)

pDST was conducted using the Sensititre Myco SLOMYCOI assay (Thermo Fisher Scientific, Waltham, USA) as recommended by the manufacturer and following CLSI guidelines (Clinical Laboratory Standards Institute, 2018). Time points for plate reading were days 7, 10, and 14. *M. avium* ATCC 700898 was used as quality control and inoculated with each run of pDST. Plates were read by two investigators independently, and in case of disagreement, a consensus MIC was obtained.

The assay allows MIC testing for the following antibiotics (abbreviations and MIC ranges in brackets): clarithromycin (CLA 0.06–8 μg/ml), amikacin (AMI 1–64 μg/ml), rifabutin (RFB 0.25–8 μg/ml), rifampicin (RIF 0.12–8 μg/ml), ethambutol (EMB 0.5–16 μg/ml), trimethoprim/sulfamethoxazolee (SXT 0.12/2.38–8/152 μg/ml), ciprofloxacin (CIP 0.12–16 μg/ml), moxifloxacin (MXF 0.12–8 μg/ml), ethionamide (ETH 0.3–20 μg/ml), isoniazid (INH 0.25–8 μg/ml), doxycycline (DOX linezolid (LZD 1–64 μg/ml) and streptomycin (STR 0.5–64 μg/ml).

For clarithromycin, amikacin, moxifloxacin, and linezolid, CLSI breakpoints have been used to interpret MIC values (CLA: S ≤ 8 μg/ml, I = 16 μg/ml, R ≥ 32 μg/ml; AMI S ≤ 16 μg/ml, I = 32 μg/ml, R ≥ 64 μg/ml, MXF: S ≤ 1 μg/ml, I = 2 μg/ml, R ≥ 4, μg/ml LZD: S ≤ 8 μg/ml, I = 16 μg/ml, R ≥ 32 μg/ml) (Clinical Laboratory Standards Institute, 2018).

Antibiotic stability

MXF, RFB, RIF, STR, AMI, SXT, CIP, DOX, and EMB were purchased from Sigma Aldrich/Merck, Darmstadt, Germany. CLA was obtained from Hikma Pharmaceuticals, Sintrem, Portugal, and LZD from Rotexmedica, Trittau, Germany.

The antibiotic agents were dissolved in 100 μl cation-adjusted Mueller-Hinton broth (CAMHB, Thermo Fisher Scientific, Waltham, USA) in six different concentrations by serial dilution

(CLA 0.5–16 μg/ml, AMI 32–1024 μg/ml, MXF 16–512 μg/ml, DOX 4–128 μg/ml, RIF 0.5–16 μg/ml, RFB 4–256 μg/ml, SXT 0.5/9.5–16/304 μg/ml, CIP 64–512 μg/ml, LZD 16–512 μg/ml, STR 128–4096 μg/ml and EMB 0.3125 mg/ml to 10 mg/ml). This solution was incubated at 37 °C for 14 days, mimicking the incubation time of the pDST system.

Mueller-Hinton agar (Oxoid, Wesel, Germany) was inoculated with a 0.5 McFarland solution of *M. luteus* ATCC 9341 as an indicator organism for CLA, AMI, MXF, DOX, RIF, RFB, SXT, CIP, LZD, STR, and *Corynebacterium striatum* ATCC 1293 for EMB. On days 0, 7, 10, and 14, 10 μl of the antibiotic solution were loaded onto diffusion disks with a diameter of 6 mm (Macherey-Nagel, Düren, Germany) and placed on the inoculated agar plates. After overnight incubation at 37 °C, the inhibition zone was measured. Measurements from day 0 were considered the baseline and used to calculate a standard curve, from which subsequent concentrations were deduced. The experiment was performed at least in triplicate for every antibiotic agent.

To assess the effect of antibiotic stability on MICs measured by the SLOMYCO system, we read all MAC-isolates at days 7 and 14. MIC_{50} and MIC_{90} were compared for these time points. Due to the lack of an adequate biological assay for the assessment of biological activity in ethionamide and isoniazid, those two antibiotics were not analyzed.

Whole-genome sequencing of isolates and bioinformatical analysis

For whole-genome sequencing, Cetrimonium bromide-(CTAB)-chloroform DNA extraction was performed as previously described (de Almeida et al., 2013). From extracted genomic DNA, next-generation sequencing libraries were generated using a modified Illumina Nextera library kit protocol (Baym et al., 2015), and libraries sequenced in a 2 × 150 bp paired-end run on the Illumina NextSeq 500 instrument (Illumina, San Diego, CA, USA).

For phylogenetic analysis, all sequences were applied to the *nullarbor* pipeline (Seemann et al., 2019). The genomic sequence of *M. avium DSM 44156* was included as a reference. The resulting core genome alignment and core SNP distance matrix were utilized to build a phylogenetic tree with the *ggtree* package (Yu et al., 2017). Additionally, all pairs of first and copy isolates were compared using *snippy,* whereas a *de novo* assembly of the first isolate was used as a reference for SNP calling (Seemann, 2015). SNP distances above 100 were considered indicative of reinfection.

Statistical analysis

Statistical analyses were performed using LibreOffice (The Document Foundation) and R. For categorical variables, frequencies were calculated; continuous variables with non-normal distributions were presented as median and range or interquartile range (IQR). Minimal inhibitory concentrations for 50% of isolates (MIC_{50}) and 90% of isolates (MIC_{90}) were calculated. Linear regression for calculation of antibiotic concentrations was done by using R's base package (R Core Team, 2018). Graphs were drawn with R's ggplot2 package (Wickham, 2016).

In patients with follow-up isolates that met the inclusion criteria, comparison of antibiotic susceptibility between first and follow-up isolates was conducted using the Wilcoxon-Signed-Rank test at a significance level p = 0.05 (two-sided). Concerning pDST, isolates were grouped into S, I, and R according to CLSI breakpoints, as mentioned above. For sensitivity and specificity analysis, all isolates were included (first and follow-up isolates). S and I were regarded as one group.

For comparison of antibiotic concentrations in the *M. luteus* biological assay, a one-sided Student's t-test was conducted between values measured at d0 and d14.

Results

Patient's characteristics and MAC species identification

A total of 98 isolates from 85 patients were included (85 isolates comprising the base cohort, 13 follow-up isolates). Overall, 60.0% percent were male (n = 51). Median age was 45.5 years at the time of first isolation (range 1.4–87.7 years). The most important clinical manifestation was NTM PD/C (63.5%, n = 54), followed by disseminated mycobacteriosis (28.2%, n = 24). The most frequent underlying dispositions were HIV (47.1%, n = 40), structural lung disease (11.8%, n = 10), CF (10.6%, n = 9) and malignomas (10.6%, n = 9). Excluding copy isolates (base cohort, n = 85), 72.9% were *M. avium* (n = 62), 5.9% *M. intracellulare* (n = 5) and 21.2% *M. chimaera* (n = 18).

A total of 13 patients had serial isolates that met the inclusion criteria (patients with first and follow-up isolates). Of those, 53.8% were suffering from CF (n = 7), 15.4% were HIV positive (n = 2), 15.4% suffered from structural lung disease (SLD) (n = 2), one patient from rheumatic disease and one patient from a malignoma. The median time span between first and follow-up isolates was 1.97 years (IQR 1.11–5.07, range 0.88–9.23). In total, 69.2% (n = 9) received clarithromycin treatment with a median treatment duration of 52.0 weeks (IQR 23–54), 38.4% (n = 5) ethambutol treatment with a median treatment duration of 52.0 weeks (IQR 23–54), 38.4% (n = 5) either rifabutin or rifampin treatment with a median treatment duration of 37.5 weeks (17.5–52.0), and 53.8% (n = 7) aminoglycoside treatment with a median treatment duration of 6 weeks (3.5–7.5), between the two cultured isolates.

Genotypic drug susceptibility (gDST)

In isolates from the base cohort, no mutation in the *rrl* gene conferring a macrolide resistance could be detected using the Genotype NTM-DR. The same was the case for aminoglycosides, as no mutations in the *rrs* gene were found. Therefore, regarding genotype, 100% of isolates in this group were predicted to be susceptible to macrolides and aminoglycosides.

In the group of follow-up isolates, 15.4% showed an *rrl* mutation (n = 2), thus indicating macrolide resistance (A2058G and A2059C). Mutations conferring resistance to aminoglycosides could not be identified, either.

Phenotypic drug susceptibility testing (pDST) in the base cohort

In the base cohort, 98.8% of MAC isolates were phenotypically susceptible to clarithromycin (n = 84), while one *M. avium* isolate (1.2%) showed phenotypic resistance (Tables 1 and 2). Overall, 85.9% of isolates were susceptible to amikacin (n = 73), 14.1% showed intermediate susceptibility (n = 12), while no isolate had a MIC in the resistant range. For moxifloxacin and linezolid, 12.9% and 8.2% were susceptible, 38.8% and 24.7% intermediate, 44.7% and 67.1% resistant, respectively. MIC distributions were unimodal for all antibiotics except clarithromycin, where a bimodal distribution could be observed.

A total of 730 reads were compared between the two examiners: 75.6% were completely congruent (n = 552 reads), 21.6% deviated by one dilution (n = 158), and 6.4% deviated by more than one dilution (n = 47 reads). In those two situations, the two readers agreed on a consensus MIC.

Table 1
MIC_{50}, MIC_{90}, and MIC ranges in all MAC isolates in the base cohort (n = 85). For clarithromycin, amikacin, moxifloxacin, and linezolid relative frequency of susceptible (S), intermediate (I), and resistant (R) isolates are shown with 95% confidence intervals in brackets according to CLSI breakpoints (Clinical Laboratory Standards Institute, 2018).

Antibiotic	MIC_{50} [μg/ml]	MIC_{90} [μg/ml]	Range [μg/ml]	S [%]	I [%]	R [%]
CLA	2	4	0.12–>64	98.8 (92.7–99.9)	0.0 (0–5.4)	1.2 (0.1–7.3)
AMI	16	32	1–32	85.9 (76.2–92.1)	14.1 (7.8–23.8)	0 (0–5.4)
RFB	0.25	1	0.25–>8			
RIF	8	>8	1–>8			
EMB	8	16	1–>16			
INH	>8	>8	4–>8			
MXF	2	8	0.25–>8	12.9 (6.9–22.4)	38.8 (28.6–50.0)	44.7 (34.0–55.9)
SXT	2/38	8/152	0.5/9.5–>8/152			
LZD	32	64	4–>64	8.2 (3.7–16.8)	24.7 (16.3–35.4)	67.1 (55.9–76.6)
CIP	16	>16	2–>16			
STR	32	64	2–>64			
DOX	>16	>16	4–>16			
ETH	5	20	0.3–>20			

CLA, clarithromycin, AMI, amikacin, RFB, rifabutin, RIF, rifampicin, EMB, ethambuthol, INH, isoniazid, MXF, moxifloxacin, SXT, trimethoprim/sulfamethoxazole, LZD, linezolid, CIP, ciprofloxacin, STR, streptomycin, DOX, doxycycline, ETH, ethionamide.

Table 2
MIC_{50}, MIC90, and MIC ranges in *M. avium*, *M. chimaera*, and *M. intracellulare*. For clarithromycin, amikacin, moxifloxacin, and linezolid relative frequency of susceptible (S), intermediate (I), and resistant (R) isolates are shown. As only 5 *M. intracellulare* isolates were included, MIC_{50}, MIC_{90} are not shown.

Antibiotic	MIC_{50} [μg/ml]	MIC_{90} [μg/ml]	Range [μg/ml]	S [%]	I [%]	R [%]
M. avium n = 62						
CLA	4	4	1–>64	**98.4**	**0**	**1.6**
AMI	16	32	2–32	**85.5**	**14.5**	**0**
RFB	0.25	1	0.25–>8			
RIF	8	>8	1–>8			
EMB	8	16	1–>16			
INH	>8	>8	8–>8			
MXF	2	4	0.25–>8	**12.9**	**43.5**	**43.5**
SXT	2/38	8/152	0.5/9.5–>8/152			
LZD	32	64	4–>64	**3.2**	**19.4**	**77.4**
CIP	16	>16	2–>16			
STR	32	64	8–>64			
DOX	>16	>16	8–>16			
ETH	5	20	0.3–>20			
M. chimaera n = 18						
CLA	2	4	0.12–8	**100**	**0**	**0**
AMI	16	16	2–32	**94.4**	**5.6**	**0**
RFB	0.5	1	0.25–2			
RIF	8	>8	1–>8			
EMB	4	>16	2–>16			
INH	>8	>8	4–>8			
MXF	4	8	1–8	**16.7**	**22.2**	**61.1**
SXT	2/38	8/152	1/19–>8/152			
LZD	16	32	8–32	**16.7**	**38.9**	**44.4**
CIP	16	>16	4–>16			
STR	32	64	2–64			
DOX	>16	>16	16–>16			
ETH	5	20	0.6–20			
M. intracellulare n = 5						
CLA	NA	NA	0.25–1	**100**	**0**	**0**
AMI	NA	NA	1–32	**80**	**20**	**0**
RFB	NA	NA	0.25–0.5			
RIF	NA	NA	2–>8			
EMB	NA	NA	4–>16			
INH	NA	NA	4–>8			
MXF	NA	NA	2–4	**0**	**40**	**60**
SXT	NA	NA	1/19–>8/152			
LZD	NA	NA	2–32	**40**	**40**	**20**
CIP	NA	NA	2–>16			
STR	NA	NA	32–>64			
DOX	NA	NA	8–>16			
ETH	NA	NA	1–>20			

CLA, clarithromycin, AMI, amikacin, RFB, rifabutin, RIF, rifampicin, EMB, ethambuthol, INH, isoniazid, MXF, moxifloxacin, SXT, trimethoprim/sulfamethoxazole, LZD, linezolid, CIP, ciprofloxacin, STR, streptomycin, DOX, doxycycline, ETH, ethionamide.

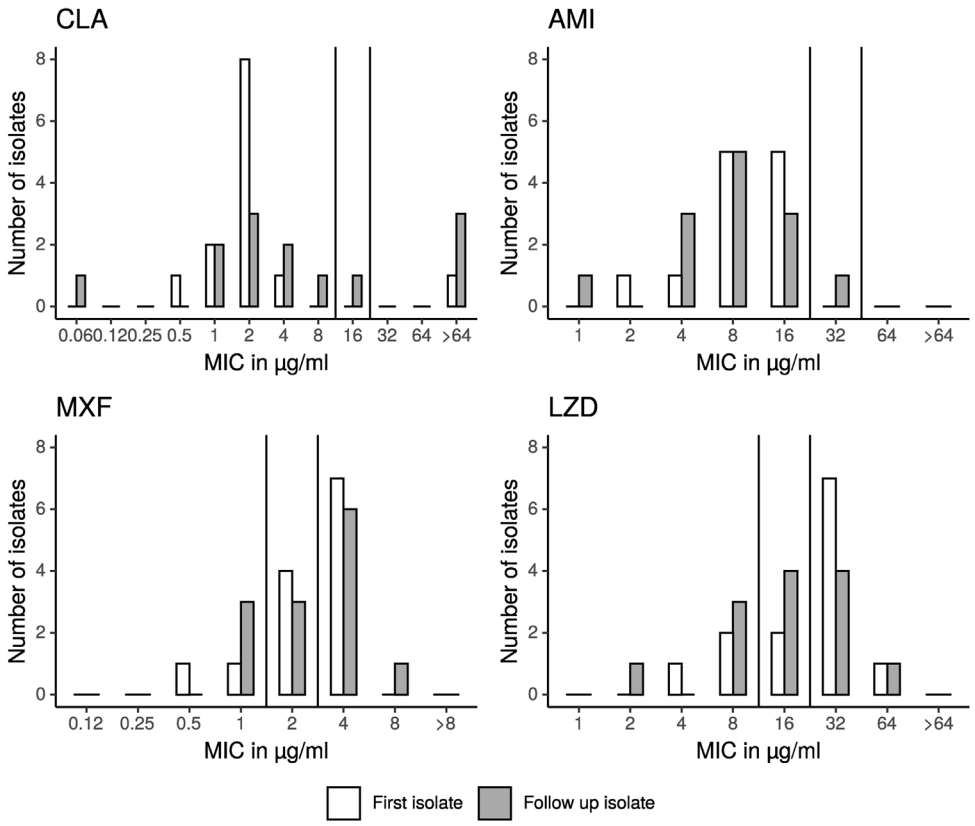

Figure 1. MIC distribution for CLA, clarithromycin, AMI, amikacin, MXF, moxifloxacin and LZD, linezolid in first, and follow-up isolates within 13 patients. Vertical lines depict clinical breakpoints for susceptible (S), intermediate (I), and resistant (R) MICs. In clarithromycin, the distribution for follow-up isolates is slightly skewed to the intermediate and resistant range. Three isolates in the follow-up isolates group were resistant, whereas one isolate was resistant at the beginning of the observation period. This trend was statistically non-significant.

Temporal drug susceptibility patterns and the effect of antibiotic treatment

Phenotypic macrolide resistance was more frequent in the group of follow-up isolates with 23.1% (n = 3, one *M. avium,* one *M. intracellulare,* one *M. chimaera*); one isolate showed intermediate susceptibility (7.4%) and 69.5% were fully susceptible (n = 9) (Figure 1). One patient kept an isolate with clarithromycin resistance, two others developed clarithromycin resistant isolates during the observation period, after having received clarithromycin treatment as part of a combination therapy (one of them received a 52 w course of rifabutin and clarithromycin/azithromycin, with eight weeks of liposomal amikacin, the other patient received a treatment regimen consisting of rifabutin, ethambutol, and clarithromycin for an entire year).

According to the exact Wilcoxon signed-rank test, categorical differences in antibiotic susceptibility between the group of first and follow-up isolates were non-significant (CLA p = 0.25, AMI p = 1, LZD p = 0.16, MXF p = 0.56, 26 isolates from 13 patients). The logistic regression showed that neither macrolide treatment, nor treatment with aminoglycosides were associated with a change in susceptibility category (p = 0.997 and p = 0.998).

Sensitivity and specificity of gDST

In total, two clinical isolates were considered genotypically resistant to macrolides with a mutation in the *rrl* gene (A2058G and A2059C); however, four isolates showed phenotypical resistance. Thus, for the prediction of clarithromycin resistance in our cohort, the sensitivity was 50%, specificity 100%, the negative predictive value 98.3% and the positive predictive value 100% (Table 3).

Table 3
Sensitivity and Specificity of the GenoType NTM-DR within the cohort. In the prediction of clarithromycin susceptibility, specificity was 100%, whereas sensitivity was only 50%. In the prediction of amikacin, susceptibility specificity was 100%.

		pDST	
gDST	Susceptible	Intermediate	Resistant
Clarithromycin			
Mutation	0	0	2
WT	93	1	2
	Specificity = 100%		Sensitivity = 50%
Amikacin			
Mutation	0	0	0
WT	86	12	0
	Specificity = 100%		

WT — wild type, gDST — genotypic drug susceptibility testing, pDST — phenotypic drug susceptibility testing.

For aminoglycosides, the genotypical prediction was confirmed in all isolates. Nevertheless, 12.2% of bacterial strains had intermediate susceptibility (n = 12) according to CLSI breakpoints. Sensitivity could not be calculated because no resistant isolates were identified.

For the two isolates with discordant results regarding macrolide susceptibility, an additional PCR and Sanger sequencing were performed. Here, in both cases, the WT-sequence of the *rrl*-gene (A2058) was detected, genotypically confirming the results of the GenoType NTM-DR.

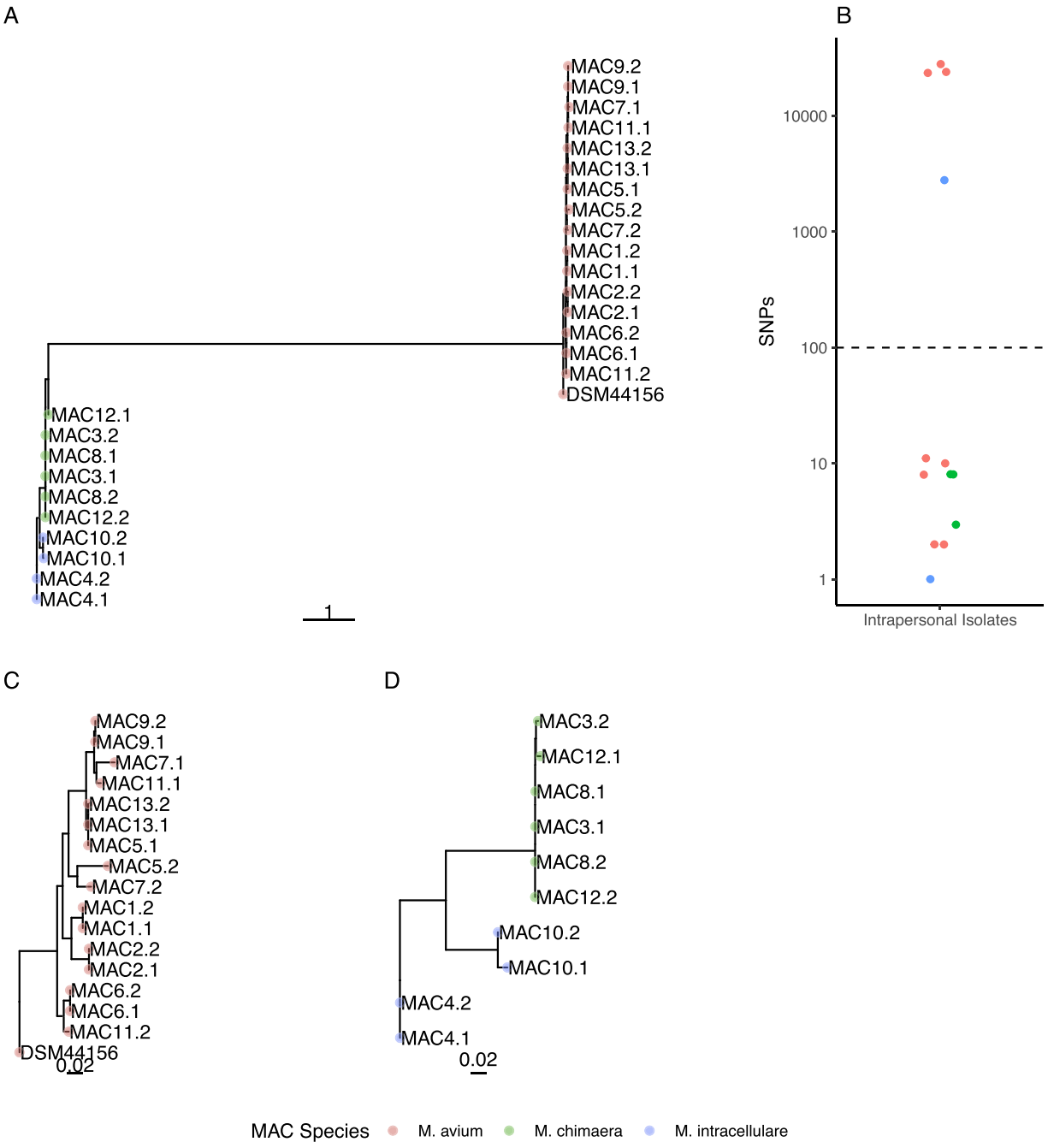

Figure 2. Genetic distances between first and copy isolates 13 patients (total number of isolates sequenced, n = 26). According to our definition, 9/13 follow-up isolates were probably the same strain or very closely related. In 4/13, reinfection seems to have taken place. The two patients that had a change in clarithromycin susceptibility kept the same isolate or very closely related ones. (A) Phylogenetic tree of core genome alignment with all isolates using DSM44156 as a reference, (B) whole-genome SNP distances in intrapersonal isolate comparisons (first vs. follow-up isolate), horizontal line marks 100 SNPs, (C) Core SNP tree for *M. avium* isolates, (D) core SNP tree for *M. intracellulare* and *M. chimaera*.

Whole-genome sequencing and phylogenetic analysis of first and copy isolates

First and copy isolates were sequenced in 13 patients (total number of isolates, n = 26). Of those, 16 isolates were *M. avium,* six *M. chimaera,* and four *M. intracellulare.* In nine patients, whole-genome SNP distances between first and follow-up isolates were below 100 SNPs (range 1–11 SNPs), indicating a close genetic relationship. In the other four patients, copy isolates showed more than 100 SNPs distance to the first isolate (range 2787–28141 SNPs), indicating reinfection with the same MAC species in those patients (Figure 2).

Of the patients whose isolates evolved clarithromycin resistance, both kept very closely-related isolates: In one patient first and copy isolates differed by eight SNPs (*M. chimaera*), in the other patient by only one SNP (*M. intracellulare*), thus rendering reinfection unlikely.

Antibiotic stability

Antibiotic activity was determined to be constant over the whole incubation period of 14 days for clarithromycin, amikacin, ciprofloxacin, moxifloxacin, sulfamethoxazole, streptomycin, linezolid, and ethambutol.

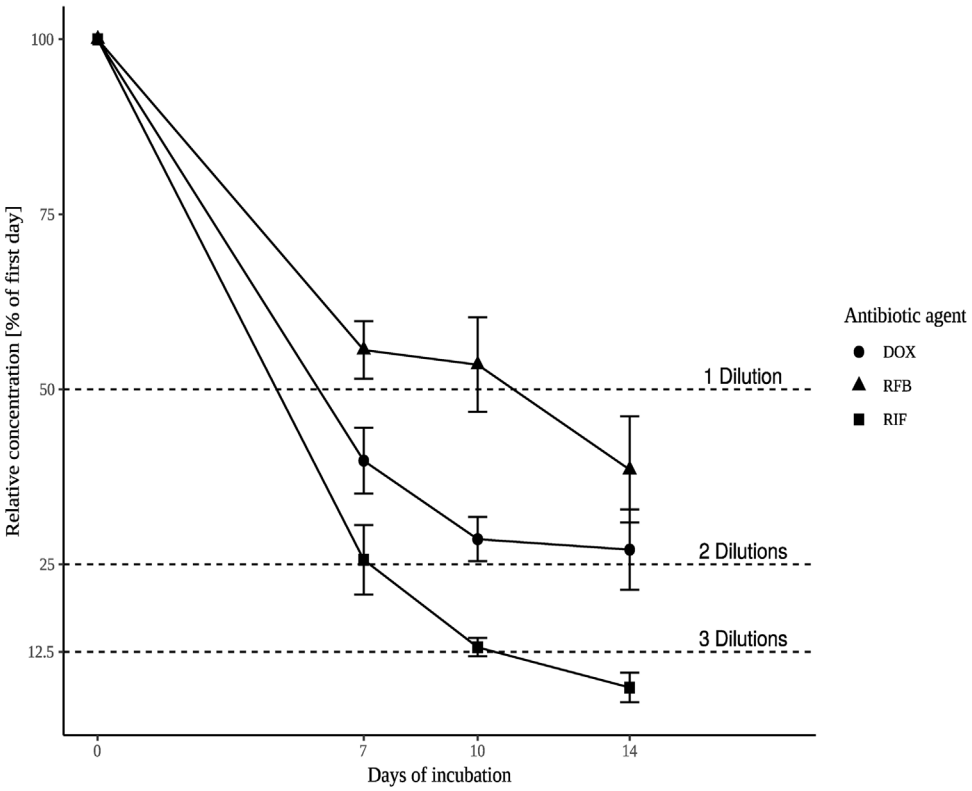

Figure 3. Relative antibiotic concentration for three unstable antibiotics: DOX, doxycycline, RFB, rifabutin, and RIF, rifampicin. Error bars mark the standard deviation.

Three antibiotics showed significant reductions of biological activity and therefore of their calculated concentration, over the course of 14 days: Rifampicin with 25.6% residual concentration after seven days, 13.1% after ten days and 7.4% after 14 days ($p < 0.01$), equaling more than three dilution steps in the broth microdilution method. For rifabutin relative concentrations of 55.6% at d7, 53.5% at d10 and 38.5% at d14 were measured ($p < 0.001$). Doxycycline concentrations were 39.8% at d7, 28.6% at d10, and 27.1% at d14 equaling one dilution in the broth microdilution method each($p < 0.01$) (Figure 3).

For the unstable antibiotics, MICs in all isolates were compared at readout days 7 (n = 70) and 14 (n = 98): MIC_{50} for DOX at d7 was >16 μg/ml, at d14 > 16 μg/ml, for RFB at d7 0.25 μg/ml and 0.25 μg/ml at d14, for RIF 4 μg/ml at d7 and >8 μg/ml at d14.

Discussion

MAC is the most frequent NTM species isolated from respiratory and other specimens. Currently, recommended therapy includes macrolides, rifamycins, and ethambutol, whereas aminoglycosides are added in severe cases. This study showed that macrolide resistances are rarely found in a cohort of 85 different patients, but seem to be more frequent in follow-up isolates, supporting the development of drug-resistance upon prolonged and insufficient antibiotic exposure. No aminoglycoside resistance could be identified. Thus, the isolates from our base cohort should have been predominantly susceptible to the standard treatment of MAC as recommended by the ATS guidelines. For all antibiotics except clarithromycin, unimodal distributions were observed, indicating that the measured MICs seem to represent only the wildtype population in all antibiotics tested except macrolides.

For *M. avium* and *M. intracellulare,* MIC-distributions for four antibiotics are available from the EUCAST database: clarithromycin, ethambutol, amikacin, and rifampicin (EUCAST, 2020). In the pooled data, macrolide and aminoglycoside resistance are also rare with 3.1% and 3.0% in *M. avium* isolates (*n* = 870) and 3.2% and 5.2% in *M. intracellulare* isolates (n = 309). Maurer et al. found that clarithromycin wild-type populations were mostly susceptible (MIC_{90} 4–8 mg/l), and *rrl*-mutations were rare in 683 MAC-isolates from Germany (Maurer et al., 2019). On the other hand, Litvinov et al. showed slightly higher macrolide resistance rates with 4.3% resistant and 9.3% intermediate isolates in isolates from the Russian Federation. Amikacin susceptibility was also distributed differently, with 9.3% resistant and 19.25% intermediate isolates for *M. avium* (Litvinov et al., 2018). This discrepancy might be due to inter-geographical variation or to different patient collectives.

Among follow-up isolates, there were more phenotypic clarithromycin resistances; two mutations within the *rrl* gene could be identified that were not present before treatment. This trend was statistically non-significant. Also, antibiotic treatment with either clarithromycin, ethambutol, a rifamycin, or an aminoglycoside, had no significant effect on the MICs within the same patients. Selective pressure via antibiotic exposure has been shown to trigger the advent of mutation in the *rrl* gene in MAC isolates *in vitro* (Jamal et al., 2000). On the other hand, Jhun et al. demonstrated that in 73% of patients with serial isolates, reinfection took place (Jhun et al., 2018). In our study, 4/13 patients were reinfected with another isolate of the same MAC-species. The two patients that evolved phenotypic CLA resistance during treatment retained very closely related isolates, rendering reinfection improbable.

Regarding the performance of the GenoType NTM DR, other publications showed discrepant results: Recently, Huh et al. determined a sensitivity of 96.3% for the detection of clarithromycin resistance in 54 resistant isolates (Huh et al., 2019). The French National Reference Center for Mycobacteria reached a 94.1%

concordance of the strip test with phenotypic testing results (Mougari et al., 2017). Nevertheless, a sensitivity of 100%, as described by the manufacturer, could not be reproduced by experimental data from clinical microbiology laboratories (GenoType NTM-DR: Detection of NTM resistances, 2020). In the isolates with discordant results regarding macrolide susceptibility, PCR and Sanger sequencing also detected the WT sequence. A different locus with a mutation conferring macrolide resistance in those isolates cannot be excluded. Also, other resistance mechanisms such as cell wall impermeability and increased efflux have to be considered (van Ingen et al., 2012).

In total, the GenoType NTM-DR still delivers very fast and mostly accurate results. With a much lower turnover time, it allows faster changes of therapeutic regimes than pDST. Thus, we conclude that the combination of pDST and gDST guiding macrolide and aminoglycoside therapy is probably the best practice (Huh et al., 2019).

Of the compounds used within the Sensititre Myco SLOMYCO test, three antibiotics showed impaired drug stability. The relative concentration of rifampicin was determined to be only 7.4% after 14 days of incubation. This loss of concentration would correspond to 3 dilution steps in the broth microdilution method. In rifampicin, this effect was strongest. We, therefore, propose that rifampicin MICs cannot sufficiently be measured *in vitro* after 10 and 14 days and that pDST results have to be considered with care for this compound. This confirms data from Schoutrop et al. (2018) and Griffith and Bodily (1992), where a significant reduction of concentration in rifampicin could be demonstrated. To a lesser extent, this applies to doxycycline and rifabutin: a loss of biological activity equaling only one dilution step could be observed. As the visual reading of MICs in the broth microdilution method is usually subjective and somewhat difficult to reproduce between different readers and centers (Nikolayevskyy et al., 2019), this loss of concentration may be of low practical importance but should be kept in mind.

This study has several limitations. First, the pDST is error-prone due to the subjective nature of visual reading, and a variation of one dilution step might already result in a different susceptibility category according to CLSI criteria (Nikolayevskyy et al., 2019). We tried to minimize this factor by independent plate reading by two different observers.

Second, all isolates were read at d7 as recommended by CLSI and been incubated longer, if growth was insufficient for visual reading. Taking into account the instability of the three antibiotics above (RIF, RFB, DOX), results might not be comparable between d7 and d14 for those agents.

Third, only 13 additional isolates could be recruited for longitudinal testing. No statistically significant effects could be observed regarding the influence of antibiotic treatment on the advent of resistance. Still, statistical power remains limited due to the low sample size, which reduces the chance of detecting an actual effect.

Fourth, in our cohort, the prevalence of macrolide resistance was comparatively low and aminoglycoside resistance was nonexistent, a fact to be considered in the interpretation of sensitivity of the genotypic assay.

Conclusion

Macrolide resistance was a rare event in our cohort of MAC isolates, and no aminoglycoside resistance could be detected. The GenoType NTM-DR is a convenient method for identification of the MAC species, identifying macrolide or aminoglycoside resistance, and should be combined with pDST to guide antimicrobial therapy. Changes in susceptibility can be linked to mutations within the same strain, but reinfection is frequent.

Ethics approval

Our local ethics committee approved this study under file number 386/18. No experiments on animals were involved.

Consent for publication

As data concerning individual patients is only presented in a retrospective and anonymous manner, no consent for publication was needed.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

NW, AG, EK, TB, MH, AL, TW, TAK, and TAW declare that they have nothing to disclose. CH reported lecture fees from Boehringer Ingelheim, refunds of congress fees and travel expenses from Gilead and Novartis, and was an organizer of a training course by the University Hospital Frankfurt am Main, sponsored by PulmonX, all outside the submitted work. MJGTV has served at the speakers' bureau Astellas Pharma, Basilea, Gilead Sciences, Merck/MSD, Organobalance, and Pfizer, received research funding from 3M, Astellas Pharma, DaVolterra, Evonik, Gilead Sciences, MaaT Pharma, Merck/MSD, Morphochem, Organobalance, and Seres Therapeutics, and is a consultant to Alb-Fils Kliniken GmbH, Ardeypharm, Astellas Pharma, Berlin Chemie, DaVolterra, Ferring, MaaT Pharma, and Merck/MSD, all outside the submitted work.

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Author contributions

NW conducted experiments, statistical and bioinformatical analysis, contributed to the conception of the study, and wrote the manuscript. TAW: contributed to the conception of the study, data analysis, manuscript draft, and supervision. FPM: data analysis, manuscript draft, and revision. TAK, SA, SN, FPM performed WGS. TGS was involved in the bioinformatical analysis. TB, AG, EK collected clinical data of the patients. All co-authors participated in drafting the manuscript and critically revising it.

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