UNIVERSITAT DE BARCELONA

Activity of antimicrobial peptides, antibiotic combinations and antibiofilm agents against *Mycobacterium avium-intracellulare* complex and *Mycobacteroides abscessus* clinical isolates causing nontuberculous mycobacterial lung-disease

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Facultat de Medicina i Ciències de la Salut
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Dissertation

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Under the supervision of

Dr. Julià González Martín and Dr. Griselda Tudó Vilanova

Servei de Microbiologia, CDB, Hospital Clínic de Barcelona-ISGlobal, Departament de Fonaments Clínics, Facultat de Medicina i Ciències de la Salut, Universitat de Barcelona

December 2020, Barcelona

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Certifiquen:

Que la tesi doctoral titulada "Activity of antimicrobial peptides, antibiotic combinations and antibiofilm agents against *Mycobacterium avium-intracellulare* complex and *Mycobacteroides abscessus* clinical isolates causing nontuberculous mycobacterial lung-disease", presentada per ELENA PORTELL BUJ ha estat realitzada al Servei de Microbiologia de l'Hospital Clínic de Barcelona-ISGlobal i al Departament de Fonaments Clínics de la Facultat de Medicina de la Universitat de Barcelona sota la seva direcció i compleix tots els requisits necessaris per la seva tramitació i posterior defensa davant del tribunal corresponent.

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1. LIST OF ABBREVIATIONS

AFB	Acid-Alcohol-Fast Bacilli
AG	Arabinogalactan
AMPs	Antimicrobial Peptides
APD	Antimicrobial Peptide Database
AUC	Area Under the Curve
BAL	Bronchoalveolar lavage
BMI	Body Mass Index
BTS	British Thoracic Society
CAMH	Cation Adjusted Mueller-Hinton
CBD	Calgary Biofilm Device
CF	Cystic Fibrosis
CFU	Colony Forming Unit
COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
DAMPD	Dragon Antimicrobial Peptide Database
DNA	Deoxyribonucleic acid
DST	Drug Susceptibility Testing
eDNA	Extracellular DNA
e.g.	From Latin, exempli gratia. Abbreviation for "for example"
EPS	Extracellular Polymeric Substances
FIC	Fractional Inhibitory Concentration
G+C	Guanine plus cytosine
GDP	Guanosine Diphosphate
GL	Glycolipid
HDT	Host-Directed Therapies
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
i.e.	From Latin, id est. Used to introduce specific information or examples
IFN-γ	Interferon-gamma

IV	Intravenous
LAM	Lipoarabinomannan
LAMP	Linking Antimicrobial Peptide
LD	Lung Disease
LJ	Lowenstein-Jensen
LP	Lipoprotein
M.	*Mycobacterium*
MA	Mycolic Acid
MABSC	*M. abscessus* complex
MAC	*Mycobacterium avium-intracellulare* complex
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry
MAP	Membrane-Associated Protein
MBC	Minimum Bactericidal Concentration
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequencing Typing
MSMD	Mendelian Susceptibility to Mycobacterial Disease
MTBC	*Mycobacterium tuberculosis* complex
NAA	Nucleic Acid Amplification
NTM	Nontuberculous Mycobacteria
OD	Optical Density
PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
PE	Proline-glutamic acid
PG	Peptidoglycan
PLG	Poly-a-L-glutamine
PPE	Proline-Proline-glutamic acid
PVC	Polyvinyl Chloride
RGM	Rapidly-Growing Mycobacteria
ROS	Reactive Oxygen Species
SGM	Slowly-Growing Mycobacteria
SUBSP.	Subspecies

TB	Tuberculosis
THP-1	Human monocytic cell line derived from an acute monocytic leukemia patient
VBNC	Viable But Non-Culturable
WGS	Whole Genome Sequencing
X-ray	X-radiation
ZN	Ziehl-Neelsen

2. LIST OF PAPERS

The present doctoral thesis is submitted as a compendium of articles. This work includes five objectives, 3 papers and 2 manuscripts.

Paper 1: **Portell-Buj E**, Vergara A, Alejo I, López-Gavín A, Monté MR, San Nicolás L, González-Martín J, Tudó G. *In vitro* activity of 12 antimicrobial peptides against *Mycobacterium tuberculosis* and *Mycobacterium avium* clinical isolates. J Med Microbiol. 2019;68(2):211-215. doi:10.1099/jmm.0.000912. Impact Factor: 2.156-JCR Microbiology Q3; Scimago Medicine Q1. (Objective 1).

Paper 2: **Portell-Buj E**, Bonet-Rossinyol Q, López-Gavín A, Roman A, Fernández Pittol M, Tudó G, González-Martín J. Comparison of two drug combinations, amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin against *Mycobacteroides abscessus* subsp. *abscessus* using the *in vitro* time-kill assay. J Antibiot (Tokyo). 2020. Accepted. Impact Factor: 2.668-Q2. (Objective 3).

Paper 3: **Portell-Buj E**, López-Gavín A, González-Martín J, Tudó G. *In vitro* biofilm formation in *Mycobacterium avium-intracellulare* complex. Arch Bronconeumol. 2020;S0300-2896(20)30263-5. doi:10.1016/j.arbres.2020.07.028. Impact Factor: 4.95-Q1. (Objective 4).

Manuscript 1: **Portell-Buj E**, Bonet-Rossinyol Q, López-Gavín A, Tudó G, González-Martín J. Activity of amikacin, ethambutol and clarithromycin against *Mycobacterium avium* and *Mycobacterium intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model. (Objective 2).

Manuscript 2: **Portell-Buj E**, González-Criollo C, López-Gavín A, Tudó G, Gonzalez-Martin J. Activity of antibiotics and antibiofilm agents against *Mycobacterium avium-intracellulare* complex biofilm. (Objective 5).

3. RESUME IN CATALAN

INTRODUCCIÓ

Família Mycobacteriaceae

Els micobacteris van ser descoberts l'any 1882 quan Robert Koch va observar per primera vegada bacils tuberculosos amb el microscopi. Els micobacteris pertanyen a una gran família de bacteris que inclou gairebé 200 espècies (www.bacterio.net/mycobacterium.html). Actualment es classifica en el fílum Actinobacteria, la família Mycobacteriaceae i els gèneres *Mycobacterium*, *Mycolicibacterium*, *Mycolicibacter*, *Mycolicibacillus* i *Mycobacteroides*. Aquests cinc gèneres corresponen, respectivament, als clades *Tuberculosis-Simiae*, *Fortuitum-Vaccae*, *Terrae*, *Triviale* i *Abscessus-Chelonae*.

Característiques generals dels micobacteris

Són aeròbics, amb forma de bastó (0.2-0.6 × 1.0-10 μm), immòbils, no formadors d'espores i tenen un alt contingut en guanina més citosina (57-73%) en el seu àcid desoxiribonucleic (ADN). Els micobacteris tenen un temps de generació que oscil·la entre les 2 a les 20 hores, en funció de l'espècie micobacteriana. Conseqüentment, es classifiquen en micobacteris de creixement lent i micobacteris de creixement ràpid. Els micobacteris de creixement lent creixen en més de set dies mentre que els micobacteris de creixement ràpid creixen en menys de set dies. La filogènia ha establert que els micobacteris de creixement ràpid van aparèixer abans en el llinatge micobacterià i que els micobacteris de creixement lent van evolucionar d'ells. Els micobacteris posseeixen una paret complexa que es rica en lípids i àcids grassos. Aquesta paret és responsable de la resistència als àcids-alcohols, creixement lent, antigenicitat i resistència als antibiòtics, detergents i el sistema immune.

Característiques generals dels micobacteris notuberculosos

Els altres membres de la família Mycobacteriaceae, a part de MTBC i *M. leprae*, són els anomenats micobacteris notuberculosos (MNT). Els MNT es troben àmpliament distribuïts a l'ambient i generalment no produeixen infeccions en humans. Es troben en ambients naturals (aigua i terra) i també en ambients artificials (capçals de dutxa i canonades).

Alguns MNT són pigmentats amb o sense estimulació de la llum degut a la producció de carotenoides grocs. Conseqüentment, es classifiquen en tres grups diferents: fotocromògens necessiten estimulació de la llum (*Mycobacterium kansasii*, *Mycobacterium marinum* i *Mycobacterium simiae*), escotocromògens presenten pigmentació independentment de la llum (*Mycobacterium scrofulaceum*, *Mycobacterium gordonae* i *Mycobacterium szulgai*) i els no cromògens no tenen pigmentació (*e.g.* complex *Mycobacterium avium-intracellulare*, *Mycobacterium bovis*, *Mycobacterium ulcerans*, *Mycobacterium xenopi*, *Mycobacterium fortuitum* i *Mycobacterium chelonae*).

Micobacteris de creixement lent i micobacteris de creixement ràpid

Els NTM van ser identificats per primera vegada a finals del segle 19. Malgrat això va ser l'any 1953 quan la seva rellevància clínica va esdevenir clara en ser descrits com a bacils grocs causants de malaltia pulmonar. Des del seu descobriment el MNT han rebut diversos noms: micobacteris anònims, micobacteris altres que tuberculosi, micobacteris ambientals i micobacteris atípics.

Actualment els MNT comprenen més de 160 espècies. Els micobacteris de creixement lent inclouen espècies com ara *M. avium*, *M. intracellulare*, *M. chimaera, M. kansasii, M. xenopi*, *Mycobacterium haemophilum, M. simiae, Mycobacterium malmoense, M. gordonae*, *M. bovis*, *M. ulcerans, M. szulgai, M. scrofulaceum*, *M. marinum* i el complex *Mycobacterium terrae*. Per altra banda, els micobacteris de creixement ràpid inclouen *Mycobacteroides abscesuss*, *M. chelonae*, *M. fortuitum*, *Mycobacterium smegmatis*, *Mycobacterium vaccae* i *Mycobacteirum mucogenicum*, entre altres.

El complex *Mycobacterium avium-intracellulare*

El complex *Mycobacterium avium-intracellulare* inclou diferents espècies de MNT, *M. avium*, *M. intracellulare*, *M. chimaera, M. arosiense*, *M. columbiense*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense* i *M. ituriense.* A més a més, *M. avium* compren quatre subespècies; *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis*, i *M. avium* subsp. *hominissuis*.

Mycobacteroides abscessus

Per altra banda, el complex *M. abscessus* es divideix en tres subespècies d'acord amb les diferències en el seu fenotip però sent alhora molt similars genèticament. *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* i *M. abscessus* subsp. *massiliense*. *M. abscessus* subsp. *abscessus* i *M. abscessus* subsp. *bolletii* presenten resistència induïble als macròlids (claritromicina) a causa del gen funcional *erm*(41) gen que codifica una metilasa 23S rRNA. Mentre que *M. abscessus* subsp. *massiliense* és sensible als macròlids degut a la presència de dues delecions que fan que el gen *erm*(41) no sigui funcional. A més a més, l'adquisició d'una mutació en el gen *rrl* gen resulta en la resistència constitutiva als macròlids, tenint lloc en *erm*(41) T28 i *erm*(41) C28.

Transmissió

Els MNT són molt resistents a l'ambient i sobreviuen en condicions dures (presència de desinfectants, antibiòtics i altes temperatures) gràcies a la seva membrana externa rica en lípids. A més a més, i degut a la seva hidrofobicitat poden ser aerosolitzats durant les activitats diàries com ara jardineria i dutxes. Aquests bioaerosols poden ser inhalats al sistema respiratori o ser ingerits. Un altra possible via de contagi és la inoculació directa a la pell. Els MNT aïllats del sistema d'aigua domèstica tenen el mateix fenotip que l'aïllat causant la infecció en el pacient, confirmant que l'aigua domèstica és una de les principals fonts d'infecció. Ambdues rutes poden causar malaltia en individus susceptibles.

Generalment els MNT no es transmeten de persona a persona però uns pocs casos de transmissió de *M. abscessus* entre pacients amb fibrosi quística s'han descrit.

Epidemiologia

En les últimes dècades, els casos de MNT han augmentat significativament a nivell mundial i es classifica com una malaltia emergent. Tot i tenir una distribució global s'observen diferències a nivell geogràfic. A nivell mundial, el micobacteri de creixement lent més abundant és MAC, seguit de *M. gordonae*, *M. xenopi* and *M. kansasii*. Pel que fa a micobacteris de creixement ràpid, MABSC és el més freqüent seguit de *M. fortuitum*.

Presentacions clíniques

Els MNT poden causar una àmplia varietat d'infeccions i que van d'infeccions de la pell a infeccions pulmonars. Tanmateix, la presentació clínica més habitual és la infecció pulmonar, en especial en persones amb malalties pulmonars de base, com ara la fibrosi quística o bronquièctasis.

La infecció pulmonar per MNT, en general té una major incidència en homes i en persones majors de 50 anys. Així com, les persones amb un sistema immune debilitat, com ara, persones amb VIH o que reben teràpies immunosupressores. A més a més, tendeixen a patir formes més severes de la malaltia, amb formes extrapulmonars i disseminades, inclús fatals.

Molt sovint, els símptomes i la presentació clínica són difícils de distingir de la TB. Entre els símptomes més freqüents trobem febre, pèrdua de pes, fatiga, anorèxia, tos, producció d'esput, dolor al pit, hemoptisi, dispnea, entre altres. I sent la tos el més habitual.

La incidència de la malaltia pulmonar per MNT ha augmentat en els últims anys. Aquest increment es pot explicar per la disminució de la incidència de la TB, les millores en els mètodes de detecció, un millor diagnòstic, l'envelliment de la població i l'increment de la

població immunosuprimida. Tot i això, les dades semblen indicar que hi ha una autèntic augment en la incidència dels MNT.

L'epidemiologia de la malaltia pulmonar per MNT és complicada perquè en molts països la notificació de casos no és obligatòria, el diagnòstic és complicat ja que la diferenciació entre infecció i malaltia és complexa i molts països manquen els recursos necessaris per a fer el diagnòstic. A més a més, en països amb pocs recursos econòmics la malaltia pulmonar causada per MNT és sovint diagnosticada com a TB, desestimant la situació real. Els principals micobacteris de creixement lent causant malaltia pulmonar per MNT són MAC, *M. kansasii*, *M. malmoense* i *M. xenopi*. Pel que fa a micobacteris de creixement ràpid trobem, MABSC, *M. chelonae* and *M. fortuitum*.

Patogènia de la malaltia

Quan els MNT són inhalats cap al tracte respiratori, es troben amb els macròfags alveolars i queden envoltats pels fagosomes primaris. Aleshores els macròfags processen els antígens micobacterians per a la presentació de superfície als limfòcits T, produint el reclutament i proliferació de limfòcits T específics d'antigen. A continuació, els limfòcits interactuen amb els macròfags infectats per induir la destrucció del micobacteris que es troben a l'interior dels macròfags o inclús destruir tot el macròfag infectat. Aquest fenomen condueix a la formació de granulomes on els macròfags infectats queden envoltats per cèl·lules inflamatòries mononuclears i histiòcits epitelioides.

La formació de granulomes és un mecanisme de defensa i de confinament micobacterià. No obstant això, aquests poden explotar, causant la disseminació dels micobacteris i el declivi de la funció respiratòria. Per tant, la eradicació de la infecció pot ser incompleta o inexistent, depenent de factors de l'hoste i de l'espècie de MNT implicada. Aquests MNT viables poden proliferar i produir malaltia. També és probable que en la malaltia pulmonar per MNT la producció de biofilm estigui involucrada, fent la eradicació més complicada.

Per altra banda, els pacients amb malalties respiratòries acostumen a tenir una major producció de mocs i de secrecions i un funcionament defectuós dels cilis i els mocs no poden ser eliminats dels pulmons. Els bacteris que prosperen en el moc escapen el sistema immune i sobreviuen en el moc adherit al sistema respiratori. A més a més, la presència de moc i d'altre secrecions dificulta l'entrada dels antibiòtics.

Diagnòstic de la malaltia

La colonització per MNT no sempre és indicatiu de malaltia. Per tant, cal tenir en compte altres paràmetres com, la presència de símptomes, radiografia o TAC anormals, presència de bacils àcid-alcohol resistents en el frotis o cultius positius i altres infeccions, com per exemple, la TB s'haurien d'excloure.

Tècniques de tinció

La tècnica del Ziehl-Neelsen s'utilitza per a la tinció de micobacteris pel seu baix cost, rapidesa i especificitat.

Susceptibilitat antibiòtica

La susceptibilitat antibiòtica és útil per a la identificació a nivell d'espècie i principalment informa sobre el perfil de susceptibilitat als antibiòtics, que indica el règim antibiòtic més adequat. No obstant, en els MNT no s'observa una correlació entre la susceptibilitat antibiòtica *in vitro* i els resultats del tractament *in vivo*.

Tractament antibiòtic

El tractament antibiòtic per la malaltia pulmonar causada per micobacteris no tuberculosos no sempre és necessari ni està recomanat. Actualment, no hi ha un tractament estandarditzat però la majoria de guies recomanen l'administració de règims de combinacions d'antibiòtics per tal d'evitar l'aparició i la propagació de la resistència als antibiòtics. Malgrat això, els tractaments disponibles són llargs, costosos i tenen molts efectes adversos. En alguns casos,

la cirurgia sola o en combinació amb el tractament antibiòtic, particularment en infeccions ben localitzades també és recomanable. La resecció pulmonar de les lesions és útil a l'hora d'eliminar una càrrega important de micobacteris. El tractament contra els MNT difereix significativament entre espècies, especialment entre micobacteris de creixement ràpid i micobacteris de creixement lent.

Tractament antibiòtic per a MAC

Actualment i d'acord amb les guies de la British Thoracic Society (BTS), es recomana el següent règim antibiòtic: administració de rifampicina, etambutol, azitromicina i claritromicina durant un mínim de dotze mesos després d'obtenir un cultiu negatiu. En aquelles soques resistents a claritromicina, isoniacida o moxiflocina s'haurien d'utilitzar en comptes de la claritromicina. A més a més , en pacients amb una malaltia severa o causada per soques resistents a la claritromicina, amikacina intravenosa durant un màxim de tres mesos o amikacina nebulitzada s'haurien de considerar.

Tractament MABSC

Per altra banda, la BTS recomana el següent tractament en el cas de soques sensibles o amb resistència induïble als macròlids, una fase inicial durant almenys un mes d'amikacina o tigeciclina intravenosa, quan sigui tolerat imipenem intravenós i quan sigui tolerat claritromicina o azitromicina oral. Seguida d'una fase de continuació amb amikacina nebulitzada, claritromicina o azitromicina oral, i d'un a tres dels antibiòtics següents d'acord amb el perfil de susceptibilitat antibiòtica i la tolerància del pacient: clofazimina oral, linezolid oral, minociclina oral, moxifloxacina oral i cotrimoxazol oral.

Però en casos de resistència constitutiva als macròlids s'aconsella aquest altre tractament: una fase inicial d'amikacina intravenosa i tigeciclina intravenosa i quan sigui tolerat imipenem intravenós durant com a mínim un mes. Aquesta fase hauria de ser continuada per una amb

amikacina nebulitzada i de dos a quatres dels següents antibiòtics en funció dels resultats de la susceptibilitat antibiòtica i la tolerància del pacient: clofazimina oral, linezolid o miniciclina oral, moxifloxacina oral i cotrimoxazol oral.

Aminoglicòsids

Els aminoglicòsids tenen un aminosucre en comú i un anell ciclohexà. Inhibeixen la síntesi de proteïnes unint-se a la subunitat 30S del ribosoma i, per tant, produint la lectura errònia del ARNm. Els aminoglicòsids presenten activitat bactericida. Tanmateix, poden provocar efectes secundaris com ara, ototoxicitat, hepatoxicitat, pèrdua de l'equilibri, nàusees i reaccions al·lèrgiques.

Amikacina

Es un antibiòtic aminoglicòsid semisintètic derivat de la kanamicina A. L'amikacina és refractària a les modificacions enzimàtiques per part d'enzims que modifiquen els aminoglicòsids. Conseqüentment, és l'aminoglicòsid més utilitzat, especialment enfront de bacils gramnegatius. L'amikacina també presenta molt bona activitat enfront de bacils aerobis grampositius de la família Enterobacteriaceae, incloent alguns Mycobacterium (MAC, *M. chelonae*, and *M. fortuitum*). Tanmateix, la resistència a l'amikacina en espècies micobacterianes és causada generalment per substitucions en el ARN 16S ribosomal.

Macròlids

Els macròlids tenen un anell lactona amb entre 12-22 àtoms de carboni als quals un o més sucres s'uneixen. Inhibeixen la síntesi de proteïnes unint-se a la subunitat 50S del ribosoma, impedint la traducció del RNAm. Els macròlids es classifiquen com a bacteriostàtics, però poden ser bactericides quan s'administren a altes dosis. Poden causar efectes secundaris a nivell gastrointestinal.

Claritromicina

La claritromicina és un macròlid semisintètic derivat de l'eritromicina. Inhibeix la síntesi de proteïnes unint-se al 23S ARNr de la subunitat 50S. La claritrimicina pot ser bacteriostàtica o bactericida depenent de l'organisme i la concentració.

Azitromicina

L'azitromicina és un macròlid d'ampli espectre amb una vida mitja llarga i una elevada acumulació intracel·lular i en els teixits. Estructuralment està relacionada amb l'eritromicina. L'azitromicina és efectiva enfront de bacteris com, *Chlamydia trachomatis*, *Legionella pneumophila*, *Mycoplasma pneumoniae* i mycobacteria (*M. avium*).

Carbapenems

Els cabapenems són antibiòtics betalactàmics i, per tant, s'uneixen a les proteïnes d'unió a penicil·lina i inhibeixen la síntesi de paret bacteriana. Però, exhibeixen una activitat més àmplia, un major espectre i resistència a les betalactamases en comparació amb les cefalosporines i penicil·lines. En conseqüència, s'acostumen a utilitzar com a antibiòtics d'últim recurs en infeccions causades per bacteris resistents.

Imipenem

L'imipenem és un carbapenem semisintètic amb un ampli espectre d'activitat antibacteriana. És efectiu enfront de bacteris gramnegatius i grampositius, com ara *Pseudomonas aeruginosa* i Enterococcus, incloent soques multiresistents. L'imipenem és estable a la majoria de les betalactamases. Habitualment s'utilitza en combinació amb cilastatina i està disponible en un format de tres fàrmacs amb cilastatina i relabactam. Els carbapenems tenen poca bioabilitat oral i no creuen les barreres gastrointestinals fàcilment. Tanmateix, l'imipenem, la cilastatina i el relabactam es poden administrar de forma intramuscular.

Rifamicines

Les rifamicines són un grup d'antibiòtics que actuen com a inhibidors transcripcionals unint-se específicament a la subunitat β de la ARN polimerasa i suprimir la síntesi de ARN. A més a més, no tenen o tenen molt poca activitat enfront de la ARN polimerasa humana.

Rifampicina

La rifampicina és un antibiòtic semisintètic derivat de la rifamicina. Té un àmpli espectre d'activitat antibacteriana enfront de cocs grampositius, micobacteris, *Clostridium difficle*, i limitats bacteris gramnegatius, *Neisseria meningitides*, *N. gonorrhoeae*, i *Hemophilus influenzae*. La rifampicina és bactericida i actua tant enfront d'organismes intracel·lulars i extracel·lulars. Està disponible de forma orla i intravenosa.

Tetraciclines

L'estructura comuna de les tetraciclines consisteix en un esquelet de quatre anells als quals vàries cadenes laterals s'uneixen. Les tetraciclines s'uneixen a la subunitat 30S del ribosoma i inhibeixen la síntesi de proteïnes aturant la unió de l'aminoacil-RNAt a la posició A del ribosoma. Són bacteriostàtiques.

Tigeciclina

La tigeciclina s'utilitza en el tractament d'infeccions complicades de pell i teixit. És efectiu enfront de micobacteris de creixement ràpid. Però es tolera malament i no té formulació oral. En combinació amb l'amikacina, l'imipenem i el linezolid mostra bons resultats en el tractament enfront de *M. abscessus*.

Fluoroquinolones

Les fluoroquinolones són antibiòtics sintètics que contenen l'anell de 4 quinolones. Inhibeixen la síntesi d'àcids nucleics aturant la ADN girasa i la topoisomerasa IV. La inhibició

de la ADN girasa interromp la replicació i la reparació de l'ADN i la separació del cromosoma bacterià durant la divisió. Les fluoroquinolones també inhibeixen la topoisomerasa IV, un altre enzim que desenrotlla el ADN durant la replicació. Amb tot, les fluoroquinolones són bactericides i s'administren per via oral.

Moxifloxacino

El moxifloxacino és d'origen sintètic pertany a la quarta generació de fluoroquinolones. Es bactericida amb activitat enfront de grampositius i gramnegatius incloent *M. tuberculosis*. La resistència al moxifloxacino i a les altres fluoroquinolones és conseqüència de mutacions puntuals a l'ADN dels gens girasa A (*gyrA*) i girasa B (*gyrB*).

Altres antibiòtics

Etambutol

L'etambutol és un fàrmac antituberculós que inhibeix l'arabinil transfersa, que transfereix els àcids micòlics durant la síntesi de paret. La seva activitat és bactericida i té un bon nivell de penetració de les membranes. L'etambutol és actiu enfront d'espècies de *Mycobacterium* en creixement actiu, incloent *M. tuberculosis*. Pràcticament totes les soques de *M. tuberculosis* i *M. kansasii* així com vàries soques de MAC són sensibles a l'etambutol. Tanmateix, la resistència a l'etambutol està causada per mutacions en els gens *emb*.

Isoniazida

La isoniazida és un profàrmac que no és actiu llevat que sigui activat per l'enzim KatG, una catalasa-peroxidasa. Aquest enzim produeix espècies reactives que produeixen la inhibició de la producció d'àcids micòlics. Per tant, la isoniazida presenta activitat bactericida. Continua sent dels fàrmacs de primera línia per el

tractament de la TB. La resistència a la isoniazida està mediada per mutacions en el gen *katG*.

Cotrimoxazol: trimetroprim-sulfametoxazol

Trimetropim

El trimetroprim es un antibiòtic sintètic que interfereix amb la síntesi d'àcid fòlic. S'uneix a la dihidrofolat reductasa, un enzim crític que catalitza la formació d'àcid tetrahidrofòlic, prevenint la síntesi d'ADN. El trimetroprim s'utilitza habitualment en combinació amb sulfametoxazol degut a la seva activitat sinèrgica.

Sulfametoxazol

El sulfametoxazol és una sulfamida bacteriostàtica que interfereix amb la síntesi d'àcid fòlic. Normalment s'administra en combinació amb trimetroprim. Mostren sinergisme bloquejant els dos passos de la síntesi d'àcids nucleics i proteïnes que són essencials pel creixement i divisió. A més a més, utilitzar-los en combinació prevé l'aparició de resistència.

Linezolid

El linezolid és un antibiòtic sintètic que s'utilitza per a tractar pneumònies i infeccions de pell. Inhibeix la síntesi de proteïnes unint-se a les dues subunitats del RNAr, 30S i 50S, estant l'útlima envoltada per nucleòtids del 23S RNAr. En conseqüència, un mecanisme comú de resistència al linezolid és la presència de mutacions en el 23S RNAr.

Clofazimina

La clofazimina és un colorant que s'ha utilitzat tradicionalment en el tractament de la lepra. Presenta una molt bona activitat inhibitòria enfront de MAC. Pel micobacteris

de creixement ràpid, estudis *in vitro* han demostrat una baixa CMI i sinèrgia en combinació amb claritromicina, amikacina i tigeciclina. A més a més, la clofazimina prevé el creixement de *M. abscessus* exposat prèviament a amikacina i claritromicina.

Antibiòtics inhalats

Nous sistemes d'administració d'antibiòtics com per exemple l'aerosolització poden millorar els resultats del tractament, permetent administrar concentracions més altes al lloc de la infecció i reduir els efectes adversos a nivell sistèmic. Per exemple, els antibiòtics inhalats són engolits pels macròfags, facilitant l'eradicació dels bacils donades les elevades concentracions que s'aconsegueixen on els MNT resideixen i proliferen. Malauradament, no tots els antibiòtics poden ser aerosolitzats degut a les seves propietats fisicoquímiques.

Un antibiòtic que actualment s'administra aerosolitzat i amb bons resultats és l'amikacina. A més a més, la tigeciclina inhalada ha mostrat resultats prometedors *in vitro* i *in vivo* enfront de *M. abscessus*. Els pacients que reben antibiòtics aerosolitzats necessiten un nebulitzador per convertir les partícules grans d'antibiòtic en d'altres més petites que poden ser inhalades fàcilment. Però aquests aparells són cars i requereixen d'una font d'energia. També, alguns efectes indesitjats dels antibiòtics aerosolitzats, com ara, irritació del tracte respiratori, tos i al·lèrgies.

Resistència antibiòtica

Un tret característic dels MNT són els seus elevats nivells de resistència natural i adquirida als antibiòtics, fent el tractament contra les MNT encara més complicat que el de la TB.

Resistència natural

Hi ha molts factors descrits que fan els MNT resistents de forma natural a la majoria de famílies d'antibiòtics. Factors com, sistemes d'exportació, porines, la biotransformació dels antibiòtics, la disminució de l'afinitat amb la diana de l'antibiòtic, la producció d'enzims,

modificació de la permeabilitat, hidrofobicitat i del contingut. La paret micobacteriana actua com una important barrera física i química que intervé directament amb l'entrada dels antibiòtics. A més a més, Whib7 és un factor de transcripció conservat que regula diferents gens involucrats en la resistència antibiòtica en MNT. Un increment en l'expressió de Whib7 resulta en la sobreexpressió de bombes de flux i de gens modificadors d'antibiòtics.

Resistència adquirida

El mecanisme més probable involucrat en la resistència adquirida és l'adquisició de mutacions en gens que actuen com a dianes dels antibiòtics. Aquestes mutacions es poden transmetre a la descendència, resultant en la propagació de la resistència antibiòtica entre els MNT. A més a més, la resistència adquirida ha estat afavorida pels llargs tractaments antibiòtics i especialment la monoteràpia, conduint a l'aparició de soques resistents.

Producció de biofilm

La majoria d'espècies de MNT tenen l'habilitat de produir biofilm com a resultat de la seva hidrofobicitat. Els biofilms són una comunitat de microorganismes adherits a una superfície biòtica o abiòtica i protegits per una matriu extracel·lular de substàncies polimèriques que confereix protecció de l'ambient, antibiòtics, resposta immune, radiació ultraviolada i desinfectants.

Les espècies de MNT que poden produir biofilm són més difícils d'eradicar ja que els biofilms compliquen l'entrada dels antibiòtics al lloc de la infecció. Per tant, fent el tractament encara més complicat i requerint concentracions d'antibiòtic molt superiors per a una completa eradicació a les que calen per inhibir les formes planctòniques. Com a resultat, el biofilm és un dels principals factors de virulència. A més a més, els MNT prosperant en una comunitat de biofilm mostren una major resistència antibiòtica degut a la transferència horitzontal de gens i al quòrum sensing.

Per altra banda, una subpoblació de cèl·lules persistents i que no mostren activitat metabòlica es poden distingir dins del biofilm. Aquesta major resistència en la comunitat del biofilm es pot explicar per la protecció que proporciona la matriu, el limitat transport a través de la matriu i els canvis en la regulació gènica. Tanmateix, l'absència d'0_2 promou la tolerància als antibiòtics i l'activitat de molts antibiòtics es veu incrementada amb la respiració aeròbica. El biofilms dels MNT difereix de la resta de biofilms tenint una matriu rica en lípids i no en polisacàrids. Amb tot, la susceptibilitat antibiòtica *in vitro* pot no ser útil en aquelles infeccions on hi ha producció de biofilm. No obstant, aquesta temporal i fenotípica resistència antibiòtica es perd quan els MNT deixen la comunitat del biofilm.

Mètodes per estudiar la producció de biofilm

Malgrat que no hi ha un mètode estandarditzat, hi ha alguns mètodes *in vitro* que poden ser utilitzats per avaluar la formació de biofilm en MNT. Aquests mètodes també es poden utilitzar per estudiar l'activitat de diferents antibiòtics i agents antibiofilm enfront del biofilm de MNT. Obtenint informació més propera a la situació de la malaltia pulmonar per MNT degut a que una gran proporció dels MNT que produeixen infecció pulmonar poden produir biofilm.

El mètode descrit per Christensen *et al.* 1982 permet determinar la producció de biofilm mitjançant la inoculació de tubs que contenen medi líquid. Aleshores els tubs són incubats i es tenyeixen amb cristall violeta, assecats i llegits visualment, observant la formació d'una línia a les partes dels tubs i que es indicativa de la producció de biofilm.

En altres mètodes, el cultiu de MNT es diposita en plaques de 96 pouets que contenen medi líquid. Les plaques s'incuben a diferents temperatures i períodes de temps en funció de l'espècie de MNT. Després, els elements que no estan adherits són eliminats, les plaques s'assequen completament i tenyits amb cristall violeta o colorants similars. Finalment, es determina la densitat òptica utilitzant un espectrofotòmetre.

Mètodes per estudiar antibiòtics

Corbes de letalitat

Les corbes de letalitat permeten establir la velocitat a la qual els MNT moren enfront d'un determinat antibiòtic i concentració al llarg d'un període de temps definit. Els MNT són exposats a una concentració antibiòtica establerta durant un temps i en els punts de tall determinats es fa la presa de mostres per realitzar la determinació de les CFUs. Aquest mètode avalua la farmacodinàmica dels antibiòtics.

A més, permet estudiar els antibiòtics de forma individual o en combinació, de gran interès donat que la majoria de tractaments actuals consisteixen en l'administració de diferents antibiòtics alhora. Els resultats obtinguts amb les corbes de letalitat s'interpreten de la següent manera: indiferència, sinèrgia i antagonisme. Aquests conceptes s'estableixen en base a l'activitat de l'antibiòtic individual més efectiu en comparació amb la combinació d'antibiòtics. Una diferència de menys de 2 $\log_{10}$ es considera indiferent. Sinergisme es determina quan l'activitat de la combinació antibiòtica és 2 $\log_{10}$ superior que l'antibiòtic individual més efectiu. Antagonisme quan l'activitat de la combinació és 2 $\log_{10}$ inferior comparat amb l'antibiòtic individual més efectiu.

El tauler d'escacs

El tauler d'escacs s'utilitza per estudiar possibles sinèrgies entre diferents antibiòtics. En aquest mètode, diversos antibiòtics són estudiats en una àmplia gamma de concentracions, incloent la CMI i concentracions per sobre i per sota la CMI. Després, la FIC es pot calcular utilitzant la següent fórmula:

$$\Sigma FIC = FIC\ A + FIC\ B$$

On FIC A és la CMI de l'antibiòtic A a la combinació/CMI de l'antibiòtic A sol, i FIC B és la CMI de l'antibiòtic B a la combinació/CMI de l'antibiòtic B sol. La combinació es

considera sinèrgica quan ΣFIC és ≤0.5, indiferent quan la ΣFIC és >0.5 to <4 i antagonista quan ΣFIC és ≥4.

Models d'infecció cel·lular

Aquesta tècnica *ex vivo* consisteix en el cultiu cel·lular (per exemple macròfags THP-1) i aleshores infectar-les amb espècies de MNT que es volen estudiar. Aquestes cèl·lules es poden obtenir fàcilment de manera comercial i tenen diferents orígens. Per exemple, els monòcits THP-1 s'obtenen de sang perifèrica de malalts amb leucèmia monocítica aguda. Quan creixen sota les condicions idònies de temperatura, nivells de $C0_2$, i temps, són capaces de transformar-se en una línia cel·lular immortal amb la capacitat de proliferar indefinidament i que es pot cultivar durant llargs períodes de temps. La línia cel·lular es pot tractat amb diferents antibiòtics i combinacions d'antibiòtics per determinar l'activitat intracel·lular. Aquest mètodes és més proper a l'ambient que es troba a la malaltia pulmonar per MNT degut a que els macròfags juguen un paper important en les infeccions micobacterianes fagocitant micobacteris i dificultant l'entrada dels antibiòtics al lloc de la infecció.

Nous tractaments

Actualment, el tractament de la malaltia pulmonar per MNT es basa en l'administració de múltiples antibiòtics durant llargs períodes de temps. Degut als pobres resultats i els elevats nivells de residència antibiòtica és necessari desenvolupar altres opcions de tractament que resultin en un millor maneig del pacient, una millor qualitat de vida i millors resultats. Reutilitzar antibiòtics existents, particularment aquells aprovats per al tractament de la TB bedaquilina, clofazimina, rifabutina i tedizolid) o el descobriment de nous antibiòtic amb una major eficàcia enfront de MNT milloraria el tractament.

Els nous antibiòtics haurien de ser preferiblement bactericides i tenir una activitat d'ampli espectre, administració oral, bona penetració, activitat intracel·lular i pocs efectes secundaris. A més a més, els MNT prosperen i proliferen a l'interior dels macròfags i una bona entrada i penetració a l'interior dels macròfags és clau per a una bona activitat antimicrobiana. Tanmateix la falta de nous compostos antibiòtics és preocupant.

Pèptids antimicrobians

Els pèptids antimicrobians són potents antibiòtics d'origen natural i que són produïts per totes les formes de vida, des de microorganismes a humans. Els pèptids antimicrobians tendeixen a ser relativament curts (20-60 residus d'aminoàcids), amfipàtics i amb càrrega positiva.

Els pèptids antimicrobians juguen un paper important en la resposta immune innata per regular les defenses de l'hoste. Promouen l'eliminació de patògens amb la seva activitat antimicrobiana d'ampli espectre enfront de bacteris, virus, paràsits i fongs. Diversos estudis han demostrat que els pèptids antimicrobians incrementen la permeabilitat de la paret dels micobacteris, fet que facilita la translocació dels pèptids antimicrobians a través de la membrana i fins al citoplasma. A més a més, l'adquisició de resistència als pèptids antimicrobians és molt poc habitual degut a que són estables estructuralment i només uns pocs bacteris presenten resistència. També, no presenten propietats hemolítiques, no presenten toxicitat a les cèl·lules hoste i poden ser fàcilment sintetitzats i modificats. Conseqüentment, els pèptids antimicrobians tenen aplicacions mèdiques prometedores i el potencial per combatre les infeccions micobacterianes.

JUSTIFICACIÓ DEL TREBALL I HIPÒTESIS

Els alts nivells de resistència als antibiòtics, l'habilitat per a formar biofilm, i també l'aparició de la malaltia pulmonar causada per micobacteris notuberculosos (MNT) del complex *Mycobacterium avium-intracellulare* (MAC) i *Mycobacterium abscessus* fan necessaris tractaments efectius contra la malaltia pulmonar per MNT. Actualment, els tractaments disponibles són llargs, consisteixen en l'administració d'un mínim de dos a tres antibiòtics, tenen nombrosos efectes secundaris i presenten resultats dolents. Un major coneixement de la resposta de MAC i *M. abscessus* a diferents antibiòtics és clau per a trobar noves opcions de tractament que resultin en una millora del maneig del pacient, menys efectes secundaris i millors resultats.

I. La resistència natural i adquirida als antibiòtics de MAC i *M. abscessus* és de gran preocupació. Per tant, una bona caracterització del perfil de susceptibilitat als antibiòtics és necessària. A més a més, els tractaments alternatius podrien ajudar en la lluita contra la malaltia pulmonar per MNT.
    a. Els pèptids antimicrobians (AMPs) podrien ser efectius enfront de MNT

II. El tractament de la malaltia pulmonar per MNT requereix l'administració de múltiples antibiòtics; no obstant això, pocs antibiòtics mostren una bona activitat. Per altra banda, encara que les proves de susceptibilitat als antibiòtics es realitzen individualment, durant el tractament els antibiòtics són administrats en combinació. A més a més, pocs estudis han testat combinacions d'antibiòtics i, particularment, combinacions triples d'antibiòtics enfront de MNT.
    a. L'estudi de combinacions triples d'antibiòtic podria avaluar l'activitat sinèrgica
    b. L'adaptació de les corbes de letalitat a MNT podria avaluar l'activitat sinèrgica i determinar la farmacodinàmica dels antibiòtics

c. L'adaptació del model d'infecció de macròfags a MNT podria avaluar l'activat sinèrgica i determinar l'activitat intracel·lular dels antibiòtics

III. La formació de biofilm juga un paper important en la malaltia pulmonar per MNT oferint protecció enfront dels antibiòtics i altres agents. No obstant, no hi ha un mètode estandarditzat per a la formació *in vitro* de biofilm en MNT. A més a més, els coneixements de l'activitat dels antibiòtics i agents antibiofilm enfront del biofilm dels MNT són escassos.

a. La estandardització d'un model *in vitro* de formació de biofilm podria avaluar la formació de biofilm, així com determinar l'activitat d'antibiòtics i agents antibiofilm enfront del biofilm de MNT

OBJECTIUS

1. Determinar les CMIs de 12 pèptids antimicrobians (AMPs) enfront de soques clíniques de *M. avium*.

2. Avaluar l'activitat *in vitro* i *ex vivo* d'una combinació triple d'antibiòtics (amikacina/etambutol/claritromicina) enfront de soques clíniques de MAC utilitzant les corbes de letalitat i el model d'infecció de macròfags.

3. Avaluar l'activitat *in vitro* de dues combinacions triples d'antibiòtics (amikacina/tigeciclina/imipenem i amikacina/tigeciclina/claritromicina) enfront de soques clíniques de *M. abscessus* utilitzant les corbes de letalitat.

4. Estandarditzar un mètode per avaluar la formació *in vitro* de biofilm en soques clíniques de MAC.

5. Avaluar l'activitat *in vitro* de 9 combinacions diferents, incloent 4 antibiòtics i 5 agents antibiofilm, enfront de soques clíniques de MAC formadores de biofilm.

CONCLUSIONS

1. Encara que els AMPs són alternatives prometedores en el tractament de les infeccions micobacterianes, dels 12 AMPs estudiats només 1 va presentar activitat moderada enfront de soques clíniques de *M. avium* i tres enfront de *M. tuberculosis*. Caldria realitzar més estudis amb l'objectiu de potenciar la seva activitat i determinar el seu lloc en el tractament micobacterià.

2. L'amikacina, l'etambutol, la claritriomicina i particularment la combinació d'amikacina/etambutol/claritromicina van presentar bona activitat i no es va observar antagonisme enfront de soques clíniques de *M. avium* i *M. intracellulare* utilitzant la técnica de les corbes de letalitat i el model d'infecció de macròfags THP-1.

3. La combinació d'amikacina/etambutol/claritromicina va mostrar activitat significativament superior enfront de soques clíniques de *M. intracellulare* que enfront de *M. avium* amb les corbes de letalitat, remarcant la importància de realitzar identificació específica per a cada espècies de MAC.

4. Les tres combinacions triples, amikacina/tigeciclina/imipenem i amikacina/tigeciclina/claritromicina, van mostrar un activitat *in vitro* similar enfront de soques clíniques de *M. abscessus* subsp. *abscessus* així com l'absència d'activitat antagònica.

5. La combinació d'amikacina/tigeciclina/imipenem pot ser una alternativa a la combinació d'amikacina/tigeciclina/claritromicina enfront de soques clíniques de *M. abscessus* subsp. *abscessus* resistents a la claritromicina.

6. Una important proporció de soques clíniques de *M. avium* i *M. intracellulare* tenen l'habilitat de produir biofilm.

7. S'ha estandarditzat un mètode senzill i reproduïble per a determinar la formació de biofilm en soques clíniques de *M. avium* i *M. intracellulare*.

8. S'ha descrit i implementat un mètode per analitzar l'activitat antibiòtica enfront de soques clíniques de *M. avium* i *M. intracellulare* productores de biofilm.

9. Les concentracions mínimes d'eradicació de biofilm (CMEBs) de quatre antibiòtics enfront de *M. avium* i *M. intracellulare* van incrementar fins a mil vegades en presencia de biofilm en comparació amb les CMIs de les formes planctòniques.

10. Tres combinacions d'antibiòtics (claritromicina/amikacina, claritromicina/etambutol i amikacina/moxifloxacino) van presentar sinèrgia enfront de soques clíniques de *M. avium* i *M. intracellulare* productores de biofilm en comparació amb els antibiòtics individuals.

11. L'addició de compostos químics amb potencial activitat antibiofilm a les combinacions d'antibiòtics, va presentar sinèrgia en soques clíniques de *M. avium* i *M. intracellulare* productores de biofilm en comparació als antibiòtics individuals i tindrien activitat lítica enfront de la matriu del biofilm.

4. INTRODUCTION

4.1 Characteristics of nontuberculous mycobacteria

4.1.1 Family Mycobacteriaceae

Mycobacteria were discovered by Robert Koch in 1882 when he observed for the first time tubercle bacilli under the microscope. They are a large family of bacteria that includes almost 200 species (www.bacterio.net/mycobacterium.html) and are currently classified into the phylum Actinobacteria, the family Mycobacteriaceae and the genera *Mycobacterium*, *Mycolicibacterium*, *Mycolicibacter*, *Mycolicibacillus* and *Mycobacteroides*. These five different genera respectively correspond to the clades *Tuberculosis-Simiae*, *Fortuitum-Vaccae*, *Terrae*, *Triviale* and *Abscessus-Chelonae*. The *Tuberculosis-Simiae* clade includes the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae* which are pathogenic mycobacteria and cause disease in humans and animals, tuberculosis (TB) and leprosy respectively (1).

4.1.2 General characteristics of Mycobacteria

They are aerobic, rod-shaped (0.2-0.6 × 1.0-10 μm in size), non-motile, nonspore-forming and have a high content (57-73%) in guanine plus cytosine (G+C) in their deoxyribonucleic acid (DNA). Mycobacteria have a generation time ranging from 2 to 20 hours, depending on the mycobacterial species. Consequently, they are classified into slowly growing mycobacteria (SGM) and rapidly growing mycobacteria (RGM) (Fig. 1). SGM grow in more than seven days, while RGM grow in less than seven days. Phylogeny established that RGM appeared earlier on the mycobacterial lineage and that SGM evolved from them (1).

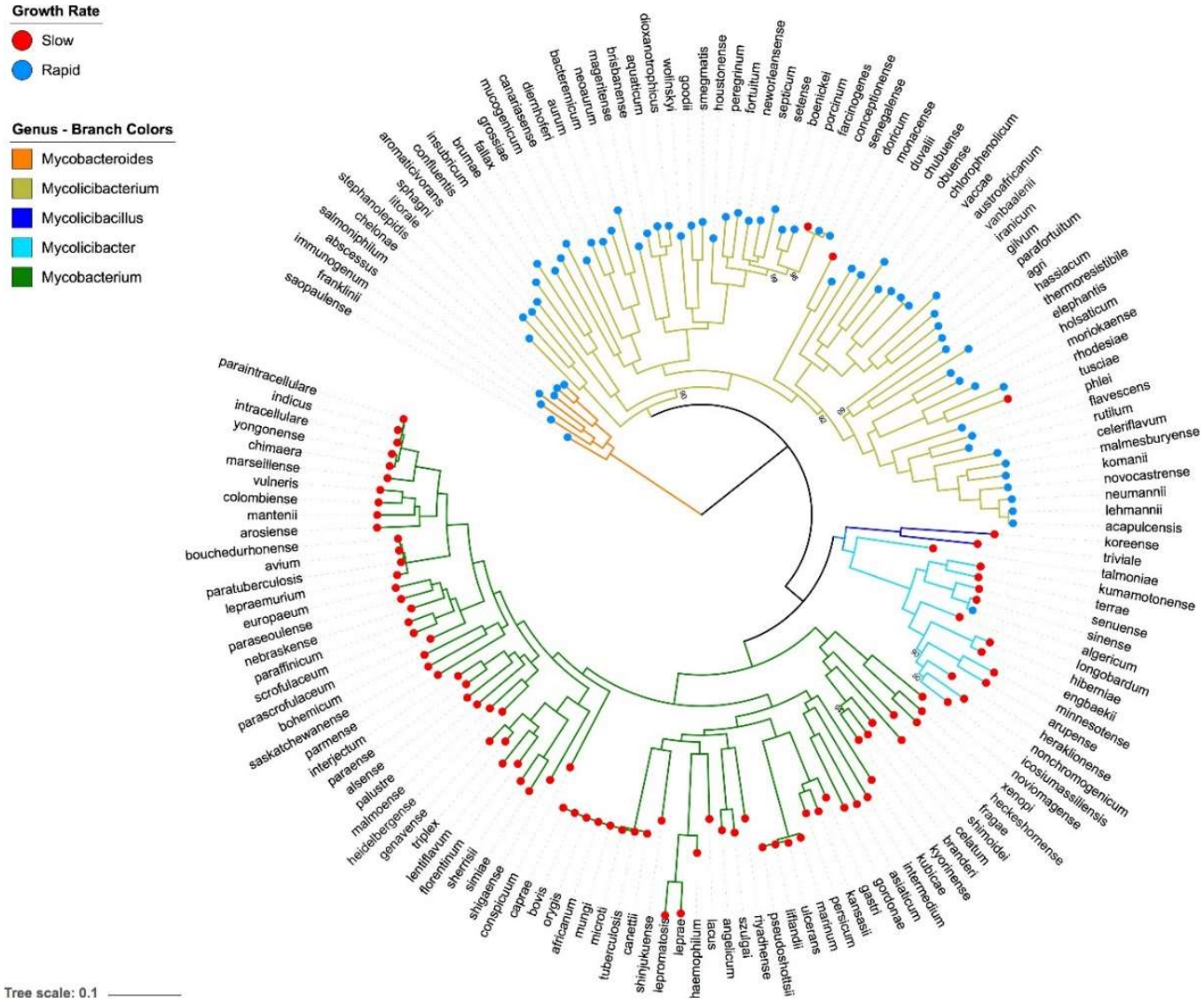

Figure 1. Phylogenetic tree of mycobacteria (2).

4.1.3 Mycobacterial cell wall

Mycobacteria possess a complex cell wall which is rich in lipids and fatty acids (Fig. 2). The cell wall is responsible for acid-fastness, slow growth, antigenicity and resistance to antibiotics, detergents and the host immune system (3,4).

Its main components are the peptidoglycan layer, the arabinogalactan and the mycolic acids. The thick peptidoglycan layer, located outside the inner plasma membrane, confers rigidity to the cell and resistance to the osmotic pressure by maintaining the cell shape and integrity. The arabinogalactan is a polysaccharide that constitutes about 35% of the mass of the cell

wall and provides permeability (5). Finally, mycolic acids containing 70 to 90 carbons are the hallmark of the mycobacterial cell wall and are involved in the cord factor properties (*i.e.* aggregation of mycobacteria in a characteristic and microscopic structure that resembles cords) (6). Characteristically, mycobacterial cell wall does not contain outer membrane (4).

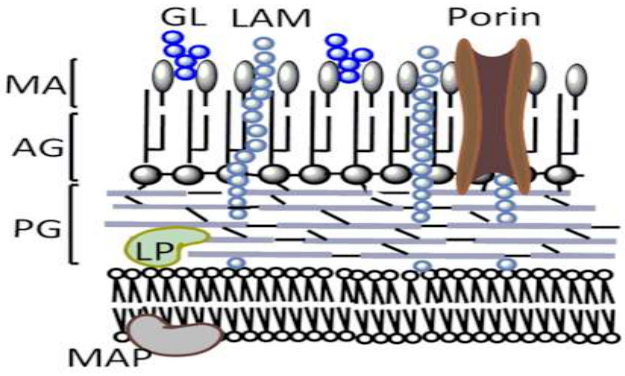

Figure 2. Mycobacterial cell wall (5).
AG, arabinogalactan; GL, glycolipid; LAM, lipoarabinomannan; LP, lipoprotein; MA, mycolic acid; MAP, membrane-associated protein; PG, peptidoglycan.

4.1.4 General characteristics of nontuberculous mycobacteria

The other members of the Mycobacteriaceae family, apart from MTBC and *M. leprae*, are denominated nontuberculous mycobacteria (NTM). NTM are ubiquitous in the environment and generally do not produce infections in humans. They are widely found in natural settings (*e.g.* water and soil) as well as human engineered environments (*e.g.* showerheads and pipelines). In addition, they can survive within lower eukaryotes, such as amoeba and aquatic insects. Higher eukaryotes (*e.g.* armadillo and deer) contribute to their zoonotic transmission. Ultimately, inanimate objects act as fomites and as substrates for biofilm formation (7). The isolation of NTM in hospital settings is of great concern given that by forming biofilms they persist in the environment and can be aerosolized; resulting in the contamination of hospital material and equipment which may lead to nosocomial infections.

On the other side, some NTM species are pigmented with light or without light stimulation due to the production of yellow carotenoids. Accordingly, they are classified into three

different groups: photochromogens require light stimulation (*e.g. Mycobacterium kansasii*, *Mycobacterium marinum* and *Mycobacterium simiae*), scotochromogens have pigmentation regardless of light (*e.g. Mycobacterium scrofulaceum*, *Mycobacterium gordonae* and *Mycobacterium szulgai*) and non-chromogens do not have pigmentation (*e.g. Mycobacterium avium-intracellulare* complex (MAC), *Mycobacterium bovis*, *Mycobacterium ulcerans*, *Mycobacterium xenopi*, *Mycobacterium fortuitum* and *Mycobacterium chelonae*).

4.1.5 Slowly growing mycobacteria and rapidly growing mycobacteria

NTM were first identified in the late 19th century. However, it was in 1953 when their clinical relevance became clear as they were described as "yellow bacilli" causing lung disease. The first review on NTM appeared in 1959 by Ernest H. Runyon. The review classified NTM into four different groups according to their phenotypic characteristics (*i.e.* growth rate and pigmentation traits): SGM photochromogens (*e.g. M. kansasii* and *M. marinum*), SGM scotochromogens (*e.g. M. gordonae*), SGM nonpigmented mycobacteria (*e.g. M. avium* and *M. intracellulare*) and RGM (*e.g. Mycobacteroides abscessus*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*). Nonetheless, the current techniques used for NTM identification have outdated this scheme of classification (4). Since their discovery NTM have received different names: anonymous mycobacteria, mycobacteria other than TB, environmental mycobacteria and atypical mycobacteria.

At the moment NTM include over 160 species (7). SGM consist of species such as *M. avium*, *M. intracellulare*, *M. chimaera, M. kansasii, M. xenopi*, *Mycobacterium haemophilum, M. simiae, M. malmoense, M. gordonae*, *M. bovis*, *M. ulcerans, M. szulgai, M. scrofulaceum*, *M. marinum* and *Mycobacterium terrae* complex (Table 1). On the other side, RGM comprise *Mycobacteroides abscesuss*, *M. chelonae*, *M. fortuitum*, *Mycobacterium smegmatis*, *Mycobacteirum vaccae* and *Mycobacterium mucogenicum* among others (Table 2).

Table 1. Several NTM species classified as SGM (7).

SGM		
M. avium	*M. haemophilum*	*M. ulcerans*
M. intracellulare	*M. simiae*	*M. szulgai*
M. chimaera	*M. malmoense*	*M. scrofulaceum*
M. kansasii	*M. gordonae*	*M. marinum*
M. xenopi	*M. bovis*	*M. terrae* complex

Table 2. Several NTM species classified as RGM (7).

RGM	
M. abscessus	*M. smegmatis*
M. chelonae	*M. vaccae*
M. fortuitum	*M. mucogenicum*

4.1.6 *Mycobacterium avium-intracellulare* complex

The MAC includes different NTM species; *M. avium*, *M. intracellulare*, *M. chimaera, M. arosiense*, *M. columbiense*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense* and *M. ituriense* (8). Additionally, *M. avium* includes four subspecies (subsp.); *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *hominissuis* (9). *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* infect birds and cause avian tuberculosis in both wild and domestic birds. Moreover, *M. avium* subsp. *avium* can also infect animals other than birds as well as humans. On the other side, *M. avium* subsp. *hominissuis* is isolated from swine, humans and domestic animals as well, suggesting their role as potential sources of infection or a shared environmental reservoir. *M. avium* subsp. *paratuberculosis*, also known as the Johne's disease agent, mainly produces disease in ruminants. However, other wild and domestic animals have also been infected with this subspecies. In addition, there are some evidences that it may be involved in Crohn's disease in humans (10).

Different morphologies can be distinguished within NTM species. In *M. avium*, smooth transparent and smooth opaque colonies, as well as rough morphologies can be distinguished (Fig. 3). Accordingly, the antibiotic susceptibility profile is related to the morphology and smooth opaque colonies are generally more susceptible to penicillin, clarithromycin and ciprofloxacin, resulting from a change into a stationary metabolic phase during biofilm production. However, the relation between morphology and virulence is less intense than in the case of MABSC.

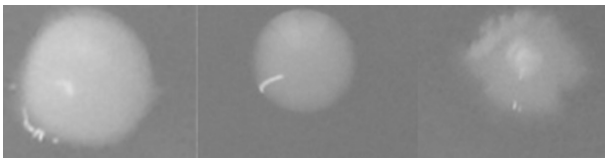

Figure 3. MAC morphologies (https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0045411)

4.1.7 *Mycobacteroides abscessus*

On the other side, the *M. abscessus* complex (MABSC) is divided into three subspecies according to the divergences in their phenotype but, being at the same time, genetically very similar; *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*.

M. abscessus subsp. *abscessus* and *M. abscessus* subsp. *bolletii* show inducible macrolide (*e.g.* clarithromycin) resistance due to a functional *erm*(41) gene that codes for a 23S rRNA methylase. Whereas *M. abscessus* subsp. *massiliense* is susceptible to macrolides by presenting two deletions that render the *erm*(41) gene non-functional (11). The complete *erm*(41) includes 10 sequevars, a group of strains which have a particular DNA sequence, in *M. abscessus* subsp. *abscessus*. The sequevars presenting the nucleotide T28 express the inducible macrolide resistance, while those with the nucleotide C28 are associated to macrolide susceptibility. Moreover, the acquisition of a mutation in the *rrl* gene results in constitutive macrolide resistance, occurring in both *erm*(41) T28 and *erm*(41) C28 (12).

In MABSC, rough and smooth morphologies can be clearly differentiated when growing on solid media (Fig. 4). The main difference is that in some colonies the cell wall content is poor in glycopeptidolipid, resulting in the rough morphology. This morphology is associated to the presence of abundant cording, which is a crucial virulence factor (13). In addition, this invasive and more virulent morphology has the ability to produce biofilm (7). On the other side, in smooth morphologies glycopeptidolipids are localized in the outer part of the mycobacterial cell wall (13). This morphology is usually associated with environmental isolates (14). However, there is evidence that glycopeptidolipid-producing colonies are also able to produce biofilm (7). Accordingly, the immune response against the different MABSC morphologies varies and results in poor killing. In the rough morphology is the intrinsic resistance to phagocytosis that reduces the killing. While in smooth colonies, the inhibition of phagocytosis and the presence of extracellular traps produced by neutrophils hamper MABSC killing (14).

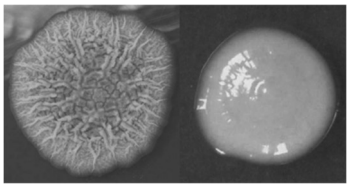

Figure 4. Rough and smooth MABSC morphologies (13).

4.1.8 Transmission of NTM

NTM are highly resistant in the environment and can thrive in harsh conditions (*e.g.* presence of disinfectants, antibiotics and high temperatures) thanks to their lipid-rich outer membrane. Moreover, and given their hydrophobic nature they can potentially be aerosolized into small particles during daily activities, such as gardening and showering. Then, these bioaerosols can be inhaled into the human respiratory tract or be ingested. Another possible route of infection is by direct inoculation into the skin. However, NTM isolated from household water systems frequently have the same phenotype than the isolate causing infection in the

patient; confirming that domestic water possibly is one of them the main sources of infection. Either way, both routes of infection can cause disease in susceptible individuals.

Generally, NTM are not transmitted from person-to-person but few cases of *M. abscessus* transmission between cystic fibrosis patients have been reported. The whole genome sequencing (WGS) determined that the isolates involved in the outbreaks were the same isolate, with only a difference of 10 base pairs. What is more, the origin of the outbreak was nosocomial infection (15).

In addition, several outbreaks of *M. chimaera* infections have been reported in patients who underwent open-heart surgery in different countries. The heater-cooler devices used during surgery were already contaminated with *M. chimaera* from the manufacturing facility. These devices contained *M. chimaera* in their water systems and the bioaerosols generated during surgery led to airborne infections in these patients. Unfortunately, the current methods of disinfection are not effective in the eradication of *M. chimaera* from these devices and measures such as physical separation from the device and the operating room are necessary to prevent *M. chimaera* infections (16).

Moreover, the current methods used in the disinfection of water systems do not eradicate NTM and exert a selective pressure on competitor microorganisms, thus selecting NTM. As a result, the possibility of environmental exposure to NTM is increased (17).

4.1.9 Epidemiology of NTM

In the last decades, cases of NTM infection have significantly increased worldwide and it is now classified as an emerging disease. Despite having a global distribution, significant geographical differences have been observed. Around the globe, the most abundant SGM is

MAC followed by *M. gordonae*, *M. xenopi* and *M. kansasii*. Regarding RGM, MABSC is the most frequently isolated followed by *M. fortuitum* (8).

In Europe, MAC is more abundantly isolated in Northern Europe (44% of all NTM) in comparison with the South of Europe (31%). Nevertheless, in both North and South European countries, *M. avium* is the most common species of the MAC (47% of all MAC) (Table 3). Furthermore, *M. avium* predominates in both North America and South America (64-78% of MAC isolates). On the other hand, *M. intracellulare* is more common in Australia (80% of MAC) and South Africa (78% of MAC) (17).

In Spain, the most abundant NTM is MAC, followed by other SGM, RGM, *M. gordonae*, *M. xenopi* and *M. kansasii*. Interestingly, *M. xenopi* is the most frequently isolated NTM in the Barcelona region but was third in frequency, after MAC and *M. fortuitum*, in the Madrid region (18, 19).

Table 3. NTM species distribution in Europe (19).

	NTM species (%)						
Country	MAC	*M. kansasii*	*M. xenopi*	*M. malmoense*	RGM	*M. gordonae*	Other SGM
Spain	39	2	8	-	17	10	24
The Netherlands	34	7	3	2	17	16	21
Germany	55	6	3	2	12	19	3
Denmark	54	1	3	4	10	18	10
Norway	55	1	2	5	15	15	7
Sweden	67	-	1	4	12	1	15
Finland	38	1	-	3	15	15	28
Poland	23	35	22	-	14	6	-
Slovakia	20	36	4	-	29	11	-
Hungary	16	5	49	-	13	13	4
Turkey	6	-	-	-	16	8	70
Croatia	10	-	17	-	25	31	17
Greece	36	2	2	2	46	9	3
Austria	40	4	7	2	22	21	4
Italy	33	2	22	-	13	24	6
Portugal	40	7	4	-	22	14	13
France	38	5	8	1	16	29	3

Belgium	38	9	26	1	6	15	5
Ireland	43	8	4	9	17	10	9
UK-London	22	11	10	1	44	10	2

These geographical differences can be explained for differences in, among others, climatic conditions, percentage of relative humidity, population health condition, population density and their lifestyle (*i.e.* higher incidence of NTM infections in urban areas compared to rural areas). Additionally, other factors that are also associated with a higher incidence are, higher soil evapotranspiration, bigger percentage of land covered by surface water, greater copper and sodium levels in the soil and high saturated vapour pressure (19).

4.1.10 Clinical manifestations of NTM

NTM can cause a wide range of infections, from skin to lung infections. However, the most frequent clinical manifestation is the lung infection or NTM lung disease (NTM-LD), particularly among individuals with an underlying lung condition, such as cystic fibrosis (CF), bronchiectasis, chronic obstructive pulmonary disease (COPD), pneumoconiosis, lung cancer and prior TB. About 20% of patients with CF have NTM isolated from respiratory specimens (20) and 9-50% of individuals with bronchiectasis may have active NTM-LD. However, it remains unclear if bronchiectasis leads to NTM-LD or NTM-LD induces bronchiectasis (21).

NTM-LD has a higher prevalence in men and in individuals older than 50 (22). Moreover, patients with a hampered immune system, such as human immunodeficiency virus (HIV) patients and patients receiving immunosuppressive therapies (*e.g.* interferon-gamma -IFN-γ- and corticoids) and transplant recipients are at a higher risk of NTM-LD. What is more, they tend to have more severe disease presentations with extrapulmonary and disseminated forms, even fatal NTM-LD.

The Lady Windermere Syndrome is also associated to NTM-LD. Women affected by this syndrome usually share certain features, such as being white, thin, tall, postmenopausal, having pectus excavatum and mitral valve prolapse, suffering from bronchiectasis and from a genetic predisposition as they display genes involved in immune function, cilial action and connective tissue. Moreover, they are non-smokers and are not affected by any underlying lung condition; nonetheless, they have a higher incidence of NTM-LD.

NTM infections in highly susceptible individuals are considered a mendelian susceptibility to mycobacterial disease (MSMD), defined by mycobacterial infections due to a deficiency in the type-1 cytokine response. MSMD commonly affect the paediatric population and implicate mutations in genes that result in deficient production of interferon-γ or response to cytokines (23).

Very often the symptoms and the clinical presentations are difficult to distinguish from TB and other pulmonary infections. However, among the most common clinical manifestations we find fever, loss of weight, fatigue, anorexia, cough, sputum production, chest pain, haemoptysis and dyspnoea, among others. And being the cough the most common symptom (Table 4) (24).

Table 4. Symptoms and clinical manifestations of NTM-LD (24).

Clinical manifestations of NTM-LD	
Fever	Cough
Loss of weight	Sputum production
Fatigue	Chest pain
Anorexia	Haemoptysis
Dyspnoea	

NTM-LD is diverse and the clinical manifestations go from mild bronchiectatic forms to tuberculosis-like fibrocavitary presentations and hypersensitivity pneumonitis (8, 25). Therefore, NTM-LD is associated to poor quality of life in many patients.

The prevalence of NTM-LD ranges from 0.2-9.8/100.000, depending on the country reporting cases (26). This increase can be explained by the decrease in the incidence of TB, the improvements of the methods of detection, the better diagnosis, the ageing of the population and the increment of immunocompromised individuals. However, reports show that there is a real increase in NTM incidence (17).

The epidemiology of NTM-LD is challenging because in most countries disease reporting is not mandatory, the diagnosis is complicated because the differentiation between infection and disease is difficult and some countries lack the resources to do the diagnosis. Furthermore, in low-income countries many times NTM-LD is misdiagnosed as TB, underestimating the real burden (26). The main SGM causing NTM-LD are MAC, *M. kansasii*, *M. malmoense* and *M. xenopi*. Regarding RGM, MABSC, *M. chelonae* and *M. fortuitum* are the most common species involved in this infectious disease.

NTM-LD patients showed mortality rates of 27% in Europe, 35% in the US and 33% in Asia in follow ups over a period of 5 years (8). As a result, NTM-LD mortality is higher than that of TB as well as its recurrence. Moreover, fibrocavitary disease, pulmonary hypertension, low body mass index (BMI), presence of other comorbidities and male gender are factors for increased mortality in NTM-LD (8, 17). Patients with chronic infection (*i.e.* they remain culture positive after 12 months of treatment) also have higher mortality in comparison with those that eradicated NTM burden in the sputum (8).

On the other side, patients with MAC-LD due to *M. intracellulare* have a more severe form of disease in comparison with those infected with *M. avium*. Nonetheless, they have less probabilities of relapse or reinfection after cure is achieved (17).

Finally, patients with NTM-LD have greater rates of hospitalization and of long-term treatment since they may suffer worsening of previous health conditions, thus resulting in

expensive medical costs. For instance, in the USA patients spend an average of 481 $ per month in their antibiotic treatment against NTM-LD (27).

4.1.11 Pathogenesis of NTM-LD

When NTM are inhaled into the respiratory tract, they come across alveolar macrophages and are engulfed into primary phagosomes. Then, macrophages process mycobacterial antigens for surface presentation to T lymphocytes, driving to the recruitment and proliferation of antigen-specific T lymphocyte clones. Afterwards, lymphocytes interact with infected macrophages to induce the destruction of the mycobacteria within macrophages or even destroy the whole infected macrophage. This leads to the production of granulomas where infected macrophages are surrounded by mononuclear inflammatory cells and epithelioid histiocytes. For instance, Fig. 5 illustrates the transmission and pathogenesis of MABSC (7).

The creation of granulomas is a mechanism of defence and of mycobacterial confinement (7). Nevertheless, they can burst resulting in the dissemination of mycobacteria and in the decline of the respiratory function (25). Therefore, the eradication of the infection may be incomplete or even missing, depending on host factors and the NTM species implicated. These viable NTM surviving in the host can thrive and produce disease. Moreover, it is probable than in most NTM-LD biofilm production is involved, making eradication even more difficult (7). On the other side, in the immune response against mycobacteria and its regulation, cytokines such as, IL-12, IFN-γ and TNF-α have a crucial role. Furthermore, and despite not playing a major role, antibodies contribute to protective immunity (21).

Besides, patients with chronic lung disease tend to have increased production of mucus and/or deficient cilia function; hence mucus cannot be removed from the lungs. Bacteria thriving in mucus escape the immune system and survive in the mucus adhered to the

respiratory system. Additionally, with the presence of mucus and other secretions the entry of antibiotics and other drugs is hampered (26).

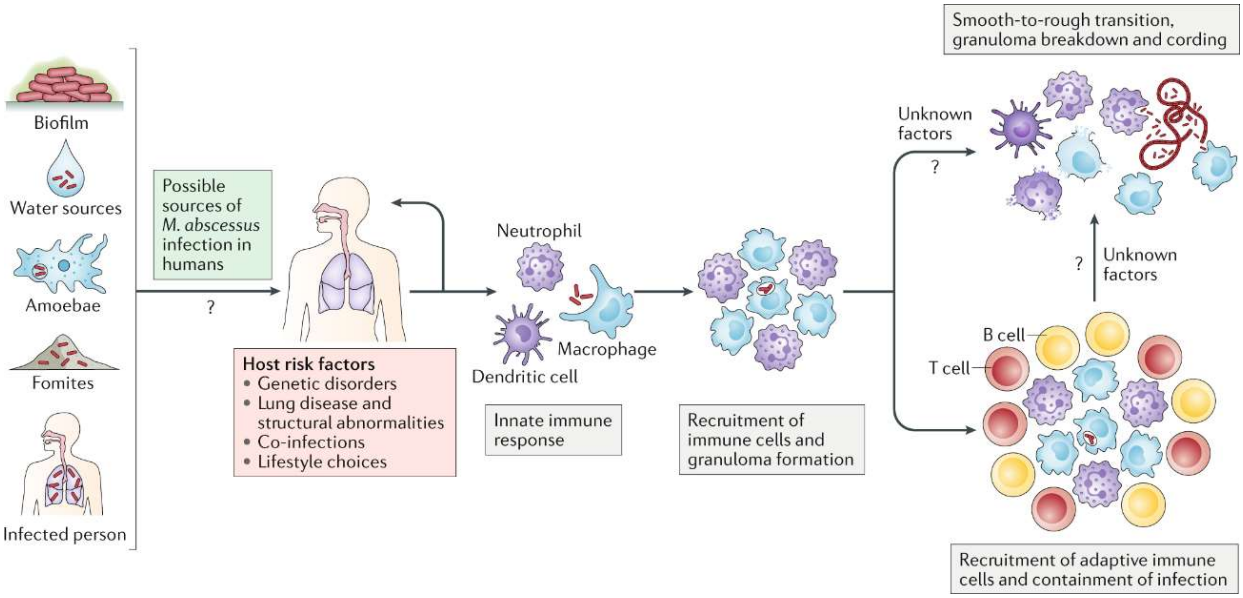

Figure 5. Transmission and pathogenesis of MABSC-LD (https://www.nature.com/articles/s41579-020-0331-1).

4.1.12 Prevention of NTM-LD

NTM-LD prevention is challenging since NTM are ubiquitous in the environment and there is no available vaccine or prophylactic treatment. Nevertheless, some preventive measures can reduce the risk of NTM-LD. Such measures include: reduce the burden of NTM in the household, periodically change showerheads, disinfection and cleaning of showerheads, increase the temperature of the hot water to ≥55 ºC, use of bacterial filters (<0.45 mm) on showerheads and taps, use of showerheads with big holes to reduce mist formation and avoidance of spas and hot tubs. On the other side, avoidance of certain activities with increased risk of aerosol formation such as gardening may be beneficial as well. It is also advised to wear a face mask and moisten the soil when gardening.

4.2 Diagnosis of NTM-LD

NTM colonization is not always an indicative of disease. Therefore, other parameters need to be considered, such as the presence of symptoms, abnormal chest X-ray or computed

tomography (CT) scan (*i.e.* nodular or cavitary opacities, multifocal bronchiectasis and multiple nodules), smear positive for acid-alcohol-fast bacilli (AFB) or positive culture (≥2 positive sputum cultures or positive culture from ≥1 bronchoalveolar lavage (BAL)) and other infections, such as TB should be excluded (Table 5) (21). When performing NTM culture, the samples used should preferably be three early-morning samples obtained on different days.

Table 5. NTM-LD diagnosis (21).

NTM-LD diagnosis	
Presence of symptoms	Smear positive for AFB
Abnormal chest X-ray or CT scan	Positive culture
Exclusion of other infections	

4.2.1 Staining techniques

The Ziehl-Neelsen (ZN) staining technique is used to stain mycobacteria for its low cost, rapidity and specificity (Table 6 and Fig. 6). The ZN consists of the staining with carbol fuchsin and the resistance to decolorization with acid-alcohol solutions by AFB due to their characteristic cell wall. Non-AFB are decolorized by the acid-alcohol solution and appear blue as a result of the counter stain. However, AFB retain the carbol fuchsin stain and appear red against a blue background. After the staining procedure, the smear is observed with light microscopy. Another technique with the same principle is the Kinyoun staining, a cold-acid fast staining that does not require heating. Instead, more chemical mordant is necessary.

On the other side, the auramine-rhodamine staining uses fluorescent dyes (*i.e.* auramine and rhodamine) as primary stains and potassium permanganate is the counter stain that inactivates the unbound fluorochrome dyes. Mycobacteria appear yellow-green against a black background. This staining is more sensitive but it requires fluorescent microscopy, which is not accessible for every microbiology laboratory.

Table 6. Ziehl-Neelsen staining procedure.

Ziehl-Neelsen staining procedure			
Reagent	Time	AFB	Non-AFB
Carbol fuchsin (primary dye)	10 min	Red	Red
Acid-alcohol (decolorizer)	3 min	Red	Colorless
Methylene blue (counter stain)	1 min	Red	Blue

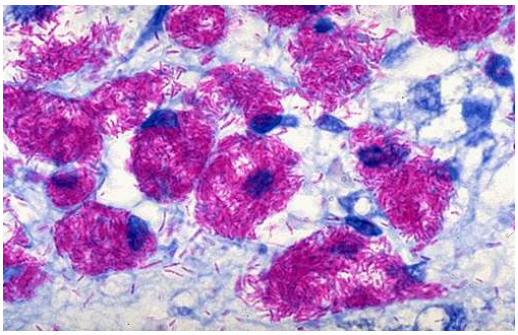

Figure 6. Ziehl-Neelsen stain (https://webpath.med.utah.edu/INFEHTML/INFEC037.html).

4.2.2 Culture techniques

NTM generally are not distinguishable based on the morphologic features observed under the microscope. Therefore, culture is required and remains the gold-standard technique. For growth on agar-based medium, Middlebrook solid medium (Fig. 7 a) is selective for NTM isolation. It contains nutrients required for the growth of mycobacteria (*i.e.* salts, vitamins, OADC, glycerol and glucose) and malachite green to inhibit Gram-positive bacteria. Culture and isolation of NTM can also be done on egg-based Lowenstein-Jensen (LJ) solid medium (Fig. 7 b) LJ is a selective medium that contains glycerol, potato flour, salts and egg and malachite green. Solid media can also be used for drug susceptibility testing (DST) and allow observing colonies and detect any possible contamination. However, NTM growth on solid media usually takes place within 2 to 3 weeks and up to 8 weeks in some cases.

Figure 7. Solid media for culture of NTM. a) Middlebrook 7H10 (https://catalog.hardydiagnostics.com), b) Lowenstein-Jensen (https://labmedicineblog.com/tag/mycobacterium).

Regarding liquid media, Middlebrook 7H9 broth is the most appropriate for SGM and Mueller-Hinton broth for RGM. There are also automated systems on the market. The most broadly used is the mycobacteria growth indicator tube (MGIT), which consists of a modified Middlebrook 7H9 broth that includes an oxygen sensor which detects growth and determines DST. The main advantage of liquid media is that growth occurs sooner compared to solid media. Nevertheless, the detection of contamination is less evident.

4.2.3 Molecular techniques

Nowadays, with the development and improvement of molecular techniques they have gained importance in identification of NTM. The main benefits are that results are obtained in <24 hours and their high sensibility. Most of them are based on nucleic acid amplification (NAA), the most reliable technique for mycobacterial identification at the strain level. Among these molecular techniques, DNA probes, DNA hybridization, polymerase chain reaction

(PCR), multiplex PCR, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), WGS and high performance liquid chromatography (HPLC) are the most frequently employed (Fig. 8).

The DNA probe assay is a rapid phenotypic method to identify NTM; however, is expensive and may lack accuracy. This method is based on the release of 16S rRNA. The partial gene sequencing of the 16S rRNA, the *hsp65* and the *rpoB* genes is often used to identify NTM

species as well. The multilocus sequencing typing (MLST) sequences multiple genes in order to provide greater information when identifying NTM.

On the other side, MALDI-TOF detects characteristic proteins of bacteria, resulting in a fingerprint that can be used for identification. On the market, there are different available commercial databases (*e.g.* Bruker, bioMérieux) where to compare data (28). WGS allows species identification with even greater detail and also can detect genes and mutations involved in antibiotic resistance. Moreover, HPLC analyses NTM mycolic acids and is the preferred method for species identification. Unfortunately, it remains inaccessible to many microbiology laboratories. Nevertheless, and despite being time-consuming, culture still remains the gold standard technique for NTM diagnosis and identification (29, 30, 31).

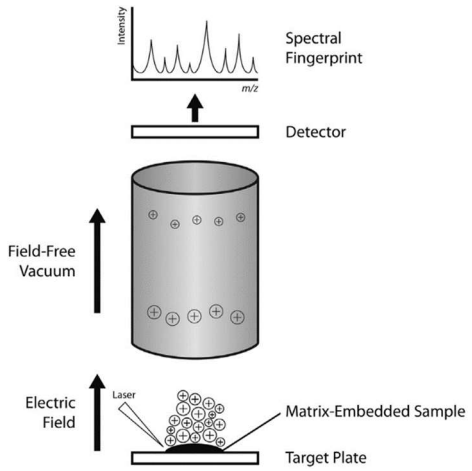

Figure 8. Principles of MALDI-TOF (https://www.labmed.theclinics.com/article/S0272-2712(13)00010-3/fulltext).

4.2.4 Drug susceptibility testing

DST is useful for identification at the species level and mainly informs of the antibiotic susceptibility profile, which indicates the most adequate antibiotic regimen. Nevertheless, no clear correlation between the *in vitro* DST and the *in vivo* treatment outcome is observed in NTM (32). Two of the main DST methods are:

- Broth microdilution: establishes the MIC by inoculating small volumes of liquid media (≈ 100 μl) with 5 x 10^5 colony forming unit (CFU)/ml in 96-well plates (Fig.

9). The MIC is determined by visual reading in comparison with the growth control well, which is antibiotic-free. This method is available as a commercial test (*e.g.* Sensititre™) but in-house broth microdiluton can be performed as well. Broth microdilution for RGM is performed in cation adjusted Mueller-Hinton (CAMH) media. For broth microdilution in SGM, Middlebrook 7H9 broth is used. The MICs obtained in broth media are lower than those on agar-based media (32).

- Broth macrodilution: larger volumes of broth media (≈ 1 ml) are inoculated with the antibiotic tested at the desired concentration. Then, the inoculum (5 x 10^5 CFU/ml) is added in order to establish MIC. Being the MIC the lowest antibiotic concentration that inhibits bacterial growth, and thus contains less growth than the antibiotic-free tube. In other words, the lowest antibiotic concentration that kills >99% of the inoculum (32). On the other side, the minimum bactericidal concentration (MBC) is the lowest antibiotic concentration that kills bacteria. The MBC is generally 2 times higher compared to the MIC.

Figure 9. 96-well plate (https://www.diagenode.com/en/p/96-well-microplates-for-ip-star-10-pc).

4.2.4.1 Phenotypic techniques

There are commercial systems available for phenotypic DST, such as the plates AST SLOMYCO Sensititre™ for SGM which test thirteen different antibiotics (clarithromycin, rifabutin, ethambutol, isoniazid, moxifloxacin, rifampin, trimethoprim/sulfamethoxazole, amikacin, linezolid, ciprofloxacin, streptomycin, doxycycline and ethionamide) in a concentration range and the plates AST RAPMYCO Sensititre™ for RGM which test fifteen different antibiotics (amikacin, amoxicillin/clavulanic acid, cefepime, cefoxitin, ceftriaxone,

ciprofloxacin, clarithromycin, doxycycline, imipenem, linezolid, minocycline, moxifloxacin, tigecycline, tobramycin and trimethoprim/sulfamethoxazole) in a range of concentrations (Table 7 and 8).

Table 7. Antibiotics and concentrations tested in the SLOMYCO Sensititre™ plates.

Antibiotic	Range of concentrations (µg/mL)
Clarithromycin	0.06-8
Rifabutin	0.25-8
Ethambutol	0.3-20
Isoniazid	0.25-8
Moxifloxacin	0.12-8
Rifampin	0.12-8
Trimethoprim/sulfamethoxazole	0.12-8
Amikacin	1-64
Linezolid	1-64
Ciprofloxacin	0.12-16
Streptomycin	0.5-64
Doxycycline	0.12-16
Ethionamide	0.3-20

Table 8. Antibiotics and concentrations tested in the RAPMYCO Sensititre™ plates.

Antibiotic	Range of concentrations (µg/mL)
Amikacin	1-64
Amoxicillin/clavulanic acid	2-64
Cefepime	1-32
Cefoxitin	4-128
Ceftriaxone	4-64
Ciprofloxacin	0.12-4
Clarithromycin	0.06-16
Doxycycline	0.12-16
Imipenem	2-64
Linezolid	1-32
Minocycline	1-8
Moxifloxacin	0.25-8
Tigecycline	0.015-4
Tobramycin	1-16
Trimethoprim/sulfamethoxazole	0.25-8

4.2.4.1.1 Degradation of antibiotics

An important factor that needs to be carefully addressed when doing DST is the stability of the antibiotics tested. It is well-known that some commonly used antibiotics are rapidly

degraded when diluted in the media used for DST. For instance, doxycycline degrades in 14 days in Mueller-Hinton medium, imipenem degrades rapidly in liquid medium and daily addition of fresh antibiotic is required, tigecycline is very unstable in aqueous environments and photosensitive as well, losing in 24 hours 80% of its activity, and thus daily addition and protection from light are necessary. Furthermore, in Middlebrook 7H10 there is a <50% activity reduction in 1 week for minocycline and trimethroprim and in 2 weeks for kanamycin, cotrimoxazole, amikacin and rifampin.

Another critical factor is the pH of the medium used for DST since it can alter the MIC obtained for a specific antibiotic, especially macrolides. Such is the case of clarithromycin, which activity is higher at a pH of 7.3-7.4 (*i.e.* CAMH pH), obtaining two-fold lower MICs than at an acidic pH of 6.8. Equivalent to what happens with ciprofloxacin, clofazimine and ethambutol (32).

4.2.4.2 Genotypic techniques

Nowadays, there is a commercially available test (GenoType NTM-DR VER 1.0) for the detection of genes that confer resistance to macrolides and aminoglycosides in several clinically relevant NTM. Furthermore, in-house systems can sequence target genes for later comparison with wild-type isolates. The most used are the 23S rRNA and the *erm*(41) mutation which predicts macrolide resistance in MAC and RGM. Additionally, 16S rRNA sequencing is used to assess aminoglycoside susceptibility in MAC and MABSC.

4.3 Treatment of NTM-LD

4.3.1 Antibiotic treatment

Antibiotic treatment for NTM-LD is neither always recommended nor necessary. Currently, there is no standardized treatment but most guidelines recommend the administration of antibiotic combination regimens in order to prevent the appearance and spread of antibiotic

resistance. However, available treatment regimens are of long duration, costly and have many side effects. In some cases, surgery alone or in combination with antibiotic therapy, particularly in well localized infections, is also advised. Pulmonary resection of the cavitary lesions is useful in removing a significant mycobacterial burden (33). NTM antibiotic treatment radically differs by species, particularly between SGM and RGM.

4.3.2 Antibiotic treatment for MAC-LD

At the moment and according to the British Thoracic Society (BTS) guidelines for the management of NTM-LD, the following antibiotic regime against MAC-LD is recommended: administration of rifampicin, ethambutol, azithromycin and clarithromycin for a minimum of twelve months after negative cultures (Table 9). However, in those MAC isolates which are resistant to clarithromycin, isoniazid or moxifloxacin should be used instead of clarithromycin. Furthermore, in patients suffering from a severe MAC-LD or caused by MAC isolates resistant to clarithromycin, iv amikacin for a maximum of three months or nebulised amikacin should be considered (34).

Table 9. Antibiotic treatment for MAC-LD (34).

Antibiotic treatment for adults with MAC-LD	
Non-severe MAC-LD	rifampicin 600 mg 3x per week and ethambutol 25 mg/kg 3x per week and azithromycin 500 mg 3x per week or clarithromycin 1g in two divided doses 3x per week antibiotic treatment should continue for a minimum of 12 months after culture conversion
Severe MAC-LD	rifampicin 600 mg daily and ethambutol 15 mg/kg daily and azithromycin 250 mg daily or clarithroycin 500 mg twice daily and consider iv amikacin for up to 3 months or nebulised amikacin antibiotic treatment should continue for a minimum of 12 months after culture conversion
Clarithromycin-resistant MAC-LD	rifampicin 600 mg daily and ethambutol 15 mg/kg daily and isoniazid 300 mg (pyridoxine 10 mg) daily or moxifloxacin 400 mg daily and consider iv amikacin for up to 3 months or nebulised amikacin antibiotic treatment should continue for a minimum of 12 months after culture conversion

4.3.3 Antibiotic treatment for MABSC-LD

On the other side, the BTS suggests the following antibiotic regimen for the treatment of MABSC-LD (Table 10). In the case of sensitive or inducible macrolide resistant isolates, an initial phase of at least one month of iv amikacin and iv tigecycline, when tolerated iv imipenem and when tolerated oral clarithromycin or oral azithromycin. This should be followed by a continuation phase consisting of nebulised amikacin and oral clarithromycin or azithromycin and one to three of the following antibiotics based on the DST results and patient tolerance: oral clofazimine, oral linezolid or oral minocycline, oral moxifloxacin and oral co-trimoxazole.

Nevertheless, in constitutive macrolide resistant MABSC this another antibiotic regimen is advised: an initial phase of iv amikacin and iv tigecycline and when tolerated iv imipenem for at least one month. This should be followed by a continuation phase of nebulised amikacin and two to four of the following antibiotics according to the DST results and patient tolerance: oral clofazimine, oral linezolid or oral minocycline, oral moxifloxacin and oral co-trimoxazole (34).

Table 10. Antibiotic treatment for MABSC-LD (34).

Antibiotic treatment for adults with MABSC-LD	
Clarithromycin sensitive isolates or inducible macrolide-resistant isolates	**Initial phase:** iv amikacin 15 mg/kg daily or 3x per week and iv tigecycline 50 mg twice daily and where tolerated iv imipenem 1 g twice daily and where tolerated oral clarithromycin 500 mg twice daily or oral azithromycin 250-500 mg daily **Continuation phase:** nebulized amikacin and oral clarithromycin 500 mg twice daily or azithromycin 250-500 mg daily and 1-3 of the following antibiotics guided by DST and patient tolerance: oral clofazimine 50-100 mg daily oral linezolid 600 mg daily or twice daily oral minocycline 100 mg twice daily oral moxifloxacin 400 mg daily oral co-trimoxazole 960 mg twice daily
Constitutive macrolide-resistant isolates	**Initial phase:** iv amikacin 15 mg/kg daily or 3x per week and iv tigecycline 50 mg twice daily and where tolerated iv imipenem 1 g twice daily **Continuation phase:** nebulized amikacin and 2-4 of the following antibiotics guided by the DST and patient tolerance: oral clofazimine 50-100 mg daily oral linezolid 600 mg daily or twice daily oral minocycline 100 mg twice daily oral moxifloxacin 400 mg daily oral co-trimoxazole 960 mg twice daily

4.3.4 Antibiotics classification

Antibiotics are classified as either bacteriostatic or bactericidal. These two terms are defined based on the MBC/MIC ratio. Bacteriostatic antibiotics inhibit bacterial growth and the MBC/MIC ratio is >4. Whereas bactericidal antibiotics kill bacteria and the MBC/MIC ratio is ≤4. They kill at higher rates in comparison with bacteriostatic antibiotics (Fig. 10) (35, 36).

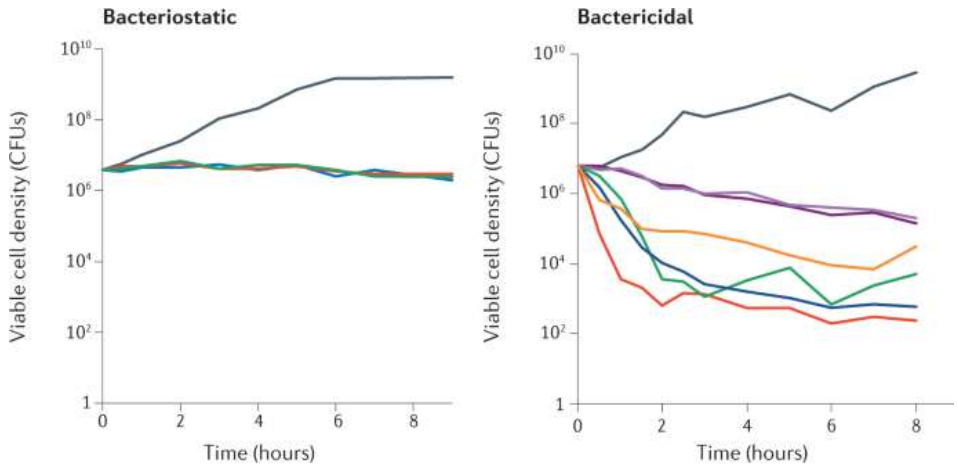

Figure 10. Pharmacodynamics of bacteriostatic and bactericidal antibiotics (36).

4.3.5 Antibiotics mode of action

For most classes of antibiotics, the major killing effect against a microorganism is produced by either the time or the concentration of the antibiotic at the binding site (Fig. 11). Therefore, two modes of action can be clearly established. The antibiotics that are effective because of the prolonged time that they bind to the bacteria are referred to as time-dependent antibiotics. In this case, the inhibitory effect can be effective because the antibiotic concentration exceeds the MIC of the bacteria. For time-dependent drugs, the pharmacodynamic parameter can be simplified to the time that serum concentrations remain above the MIC during the dosing interval (t>MIC) (37).

Other antibiotics reach high concentrations at their binding sites, resulting in the eradication of the bacteria. These antibiotics have a different mode of action and are referred to as

concentration-dependent antibiotics. For these antibiotics, the pharmacodynamic parameter can be simplified as a peak/MIC ratio. Moreover, the clinical response can be predicted as well as the peak/MIC ratio by measuring the area under the curve (AUC) over the dosing interval and dividing that value by the antibiotic MIC against the target bacteria (Fig. 11) (37).

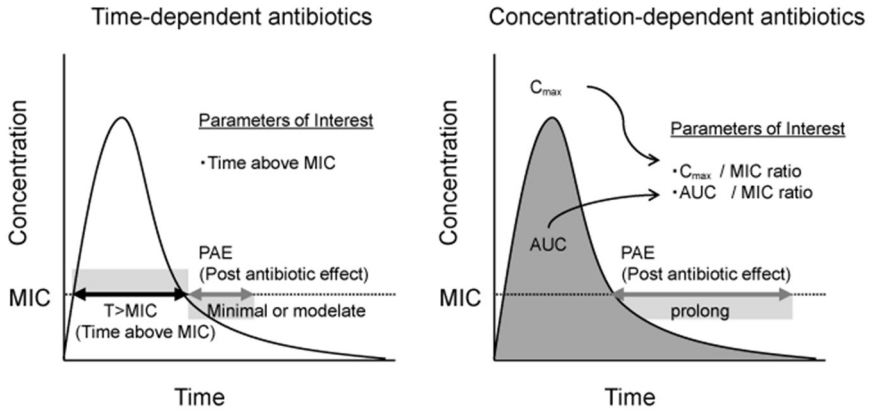

Figure 11. Time-dependent versus concentration-dependent antibiotics (37).

4.3.6 Anti-NTM antibiotics

4.3.6.1 Aminoglycosides

Aminoglycosides have common amino sugars and a shared cyclohexane ring. They inhibit the protein synthesis by binding to the 30S ribosomal subunit and thus producing the misreading of the mRNA. Aminoglycosides show a bactericidal activity. However, they can exhibit side effects, such as ototoxicity, hepatotoxicity, loss of balance, nauseas and allergic reactions (38).

4.3.6.1.1 Amikacin $C_{22}H_{43}N_5O_{13}$

Amikacin is a semisynthetic aminoglycoside antibiotic that is derived from kanamycin A. Amikacin is refractory to enzymatic modification by many aminoglycoside-modifying enzymes. Consequently, it is the most widely used semisynthetic aminoglycoside, particularly against resistant

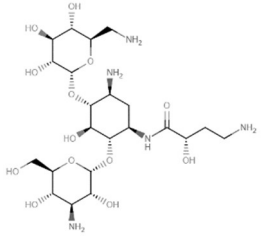

Figure 12. Amikacin structure (https://go.drugbank.com/drugs/DB00479).

Gram-negative bacilli such as *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. Amikacin also shows excellent activity against most aerobic Gram-positive bacilli from the Enterobacteriaceae family, including Nocardia and some Mycobacterium (*i.e.* MAC, *M. chelonae*, and *M. fortuitum*). Nonetheless, resistance to amikacin in mycobacterial species is mainly caused by substitutions in the 16S ribosomal RNA. Currently, amikacin is administered to patients intravenously, intramuscularly or via nebulization. Moreover, a liposomal inhalation suspension was approved for the treatment of MAC-LD in a reduced group of patients who did not respond to traditional treatment (Fig. 12) (39).

4.3.6.2 Macrolides

Macrolides have a lactone ring with 12 to 22 carbon atoms to which one or more sugars bind. They inhibit protein synthesis by binding to the 50S ribosomal subunit and thus preventing the mRNA translation. Macrolides are classified as bacteriostatic; however, they can be bactericidal when administered at high doses. They can cause gastrointestinal side effects, such as nausea and diarrhoea. Furthermore, macrolides show dose-dependent activation of intestinal motilin receptors, which stimulate gastric motility (40).

4.3.6.2.1 Clarithromycin $C_{38}H_{69}NO_{13}$

Clarithromycin is a semisynthetic macrolide antibiotic derived from erythromycin. It inhibits bacterial protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit. Clarithromycin may be bacteriostatic or bactericidal depending on the organism and drug concentration. It can be orally administered (Fig. 13) (39).

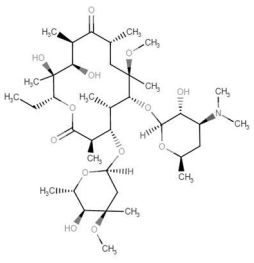

Figure 13. Clarithromycin structure (https://go.drugbank.com/drugs/DB01211).

4.3.6.2.2 Azithromycin $C_{38}H_{72}N_2O_{12}$

Azithromycin is a broad-spectrum macrolide with a long half-life and high tissue and intracellular accumulation. It is structurally related to erythromycin and, the presence of 15-membered ring with a methyl-substituted nitrogen instead of a carbonyl group at the 9a position on the aglycone ring, differentiates azithromycin from other macrolides. Azithromycin is effective against bacteria such as *Chlamydia trachomatis*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, and mycobacteria (*e.g. M. avium*). Additionally, given its activity against *Streptococcus pneumoniae*, *Hemophilus influenzae* and *Moraxella catarrhalis*, azithromycin is indicated for the treatment of community-acquired pneumonia (CAP). It is available in both oral and intravenous formulations (Fig. 14) (39, 41).

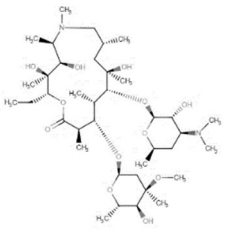

Figure 14. Azithromycin structure (https://go.drugbank.com/drugs/DB00207

4.3.6.3 Carbapenems

Carbapenems are beta lactam antibiotics, hence they bind to penicillin-binding proteins (PBPs) and inhibit the bacterial cell wall synthesis. However, they exhibit a broader activity, spectrum and resistance to beta-lactamases compared to most cephalosporins and penicillins. Consequently, they are generally used as the "antibiotic of last resort" in infections caused by resistant bacteria. Nonetheless, nephrotoxicity, neurotoxicity, and immunomodulation have been described.

4.3.6.3.1 Imipenem $C_{12}H_{17}N_3O_4S$

Imipenem is a semisynthetic carbapenem with a wide spectrum of antibacterial activity. It is effective against Gram-negative and Gram-positive bacteria such as *Pseudomonas aeruginosa* and the Enterococcus, including multiresistant strains. Imipenem is stable to many beta-lactamases. It is commonly used in combination with

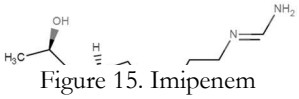

Figure 15. Imipenem structure (https://go.drugbank.com/drugs/DB01598).

cilastatin and is available in a three-drug compound with cilastatin and relebactam. Carbapenems have low oral bioavailability and do not cross gastrointestinal barriers readily. However, imipenem-cilastatin and ertapenem can also be administered intramuscularly (Fig. 15) (39).

4.3.6.4 Rifamycins

The rifamycins are a group of antibiotics that act as transcriptional inhibitors by binding specifically to the β subunit of the RNA polymerase and supress RNA synthesis. In addition, they have little or no activity against the human RNA polymerase (43).

4.3.6.4.1 Rifampin $C_{43}H_{58}N_4O_{12}$

Rifampin is a semisynthetic antibiotic derivative of rifamycin. It has a broad antibacterial spectrum against many Gram-positive cocci, mycobacteria, *Clostridium difficle*, and limited Gram-negative bacteria, *Neisseria meningitides*, *N. gonorrhoeae*, and *Hemophilus influenzae*. Rifampin is bactericidal, and acts on both intracellular and extracellular organisms. It is available as oral and intravenous formulations. Dose-dependent side effects comprise orange discoloration of body fluids, hepatotoxicity and gastrointestinal symptoms such as nausea, anorexia, and diarrhoea. Dose-independent adverse effects consist of hypersensitive reactions (*e.g.* urticaria, flu-like symptoms, thrombocytopenia, hemolysis, and renal failure) (Fig. 16) (39, 44).

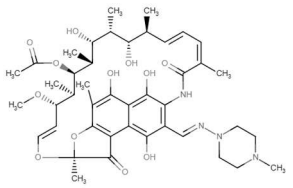

Figure 16. Rifampin structure (https://go.drugbank.com/drugs/DB01045).

4.3.6.5 Tetracyclines

The common structure of tetracyclines consists of a four-ring skeleton to which multiple side chains bind. Tetracyclines bind to the 30S ribosomal subunit and inhibit the protein synthesis by halting the union of the aminoacyl-tRNA to the A-site of the ribosome. They

are bacteriostatic and the administration of high doses can cause diarrhoea, nauseas, yellow teeth in children and hepatic and renal lesions (45).

4.3.6.5.1 Tigecycline $C_{29}H_{39}N_5O_8$

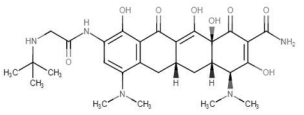

Figure 17. Tigecylcine structure (https://go.drugbank.com/drugs/DB00560).

Tigecycline is a glycylcycline antibiotic used in the treatment of complicated skin and soft tissue infections and complicated intra-abdominal infections. It is active against RGM. However, it is poorly tolerated and has no oral formulation. In combination with amikacin, imipenem and linezolid, tigecycline shows good outcomes in the treatment of *M. abscessus*, although side effects (*i.e.* ototoxicity, gastrointestinal distress or myelosupression) have been reported (Fig. 17) (39).

4.3.6.6 Fluoroquinolones

Fluoroquinolones are synthetic antibiotics that contain a 4-quinolone ring. They inhibit the synthesis of nucleic acids by halting the DNA gyrase and the topoisomerase IV. The inhibition of the DNA gyrase interrupts the replication and the reparation of DNA and, the separation of the bacterial chromosome during division. Fluoroquinolones also inhibit topoisomerase IV, another enzyme that uncoils DNA during replication. With all, fluoroquinolones are bactericidal. They are orally administered and can cause several side effects, particularly, gastrointestinal symptoms.

4.3.6.6.1 Moxifloxacin $C_{21}H_{24}FN_3O_4$

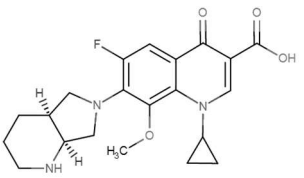

Figure 18. Moxifloxacin structure (https://go.drugbank.com/drugs/DB00218).

Moxifloxacin is a synthetic fourth generation fluoroquinolone for oral administration. It is bactericidal with activity against Gram-positive and Gram-negative bacteria including *M. tuberculosis*. Resistance to moxifloxacin

and other fluoroquinolones occurs as a consequence of point mutations within DNA gyrase A (*gyrA*) and gyrase B (*gyrB*) genes (Fig. 18) (39, 46).

4.3.7 Other anti-NTM antibiotics

4.3.7.1 Ethambutol $C_{10}H_{24}N_2O_2$

Ethambutol is an antitubercular agent that inhibits arabinosyl transferase, which transfers mycolic acids during cell wall biosynthesis. Its activity is typically bactericidal and has a good level of penetration of the membranes. Ethambutol is active against actively growing species of the genus *Mycobacterium*, including *M. tuberculosis*. Practically all strains of *M. tuberculosis* and *M. kansasii* as well as several strains of MAC are sensitive to ethambutol. However, resistance to ethambutol is caused by mutations in the *emb* genes. The most common side effects are optic neuropathy and hepatotoxicity. Further side effects that have also been reported are pruritus, joint pain, gastrointestinal symptoms, abdominal pain, malaise, and headache. (Fig. 19) (39, 47).

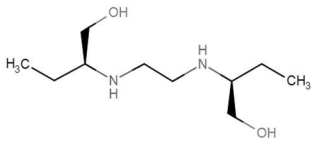

Figure 19. Ethambutol structure (https://go.drugbank.com/drugs/DB00330).

4.3.7.2 Isoniazid $C_6H_7N_3O$

Isoniazid is a prodrug and hence not effective unless activated by the enzyme KatG, a catalase-peroxidase. This enzyme produces reactive species that result in the inhibition of the production of mycolic acids. Therefore, isoniazid displays bactericidal activity. It remains the first-line treatment against *M. tuberculosis*. Resistance to isoniazid is mediated by mutations in the *katG* gene. Isoniazid is orally, intravenously and intramuscularly available (Fig. 20) (39, 48).

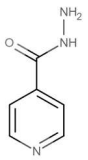

Figure 20. Isoniazid structure (https://go.drugbank.com/drugs/DB00951).

4.3.7.3 Co-trimoxazole: Trimethoprim-Sulfamethoxazole

4.3.7.3.1 Trimethoprim $C_{14}H_{18}N_4O_3$

Trimethoprim is a synthetic antibiotic that interferes the synthesis of folic acid. It binds to the dihydrofolate reductase (DHFR), a critical enzyme that catalyzes the formation of tetrahydrofolic acid (THF), thus preventing the synthesis of DNA. Trimethoprim is frequently used in combination with sulfamethoxazole due to their synergistic activity but can be administered individually in the treatment of urinary tract infections (Fig. 21) (36).

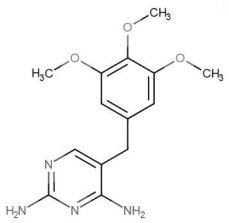

Figure 21. Trimethoprim structure (https://go.drugbank.com/drugs/DB00440).

4.3.7.3.2 Sulfamethoxazole $C_{10}H_{11}N_3O_3S$

Sulfamethoxazole is a bacteriostatic sulfonamide antibiotic that interferes folic acid synthesis. It is normally given combined to trimethoprim. They show synergism blocking two steps in the synthesis of nucleic acids and proteins which are essential for growth and division. Moreover, using them in combination prevents the appearance of antibiotic resistance. Sulfamethoxazole is used for the treatment of a range of infections, including urinary, respiratory, and gastrointestinal tract infections (Fig. 22) (39).

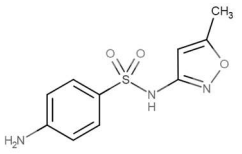

Figure 22. Sulfamethoxazole structure (https://go.drugbank.com/drugs/DB01015).

4.3.7.4 Linezolid $C_{16}H_{20}FN_3O_4$

Linezolid is a synthetic antibiotic belonging to the oxazolidinone class. It is used for the treatment of hospital-acquired pneumonia, complicated skin infections, community-acquired pneumonia caused by *S. pneumoniae* and pneumococcal meningitis. Linezolid inhibits the protein synthesis by binding to both rRNA subunits, 30S and 50S, being the last one surrounded by 23S rRNA nucleotides. Consequently, a common mechanism of resistance to linezolid is the presence of mutations in the 23S rRNA. Linezolid is available as in

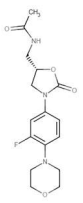

Figure 23. Linezolid structure (https://go.drugbank.com/drugs/DB00601).

oral and intravenous formulations. Among linezolid side effects, neuropathy, anaemia, thrombocytopenia, hyperlactatemia, diarrhoea, nausea, headache and hypoglycaemia have been reported (Fig. 23) (39, 49).

4.3.7.5 Clofazimine $C_{27}H_{22}Cl_2N_4$

Clofazimine is a riminophenazine dye that has been traditionally used for the treatment of leprosy. It exhibits a very good inhibitory activity against MAC. For RGM, *in vitro* studies have demonstrated a low MIC and *in vitro* synergy when combined with clarithromycin, amikacin and tigecycline. Moreover, clofazimine prevents the regrowth of *M. abscessus* previously exposed to amikacin and clarithromycin. Nonetheless, its clinical use results in long-lasting discoloration of the skin and body fluids (Fig. 24) (39).

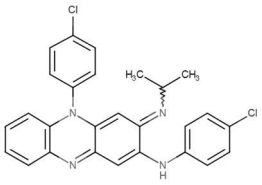

Figure 24. Clofazimine structure (https://go.drugbank.com/drugs/DB00845).

4.3.7.6 Inhaled antibiotics

New antibiotic delivery systems such as aerosolization can improve treatment outcome by enabling the administration of higher concentrations at the site of infection and thus reduce systemic side effects. For instance, inhaled antibiotics are engulfed by macrophages, which facilitates bacilli eradication given that greater antibiotic concentrations are achieved where NTM reside and proliferate. Nonetheless, not all of the antibiotics can be aerosolized due to their physicochemical properties.

One antibiotic that is currently administered via aerosolized formulation and with good results is amikacin. Furthermore, inhaled tigecycline has shown promising results *in vitro* and *in vivo* against MABSC infection by displaying great efficacy in human macrophages as well as in mice where it showed a dose-dependent activity. Nonetheless, the presence of CF, sputum and anatomical changes such as abscesses hampered its activity (50). Moreover, patients receiving aerosolized antibiotics need a nebuliser to convert large antibiotic particles

into smaller ones that can be readily inhaled (Fig. 25). However, these devices can be expensive and require a power supply. In addition, undesired effects of aerosolized antibiotics can occur, such as respiratory tract irritation, cough and allergies (25).

Figure 25. Nebuliser (https://www.stressnomore.co.uk/beper-nebuliser-12439.htm).

4.3.8 Treatment outcome

Cure is defined as culture conversion for more than 12 months after treatment cessation. Nonetheless, treatment outcomes remain very poor with cure rates of only 50-70% in MAC patients and of 30-50% in those patients suffering from *M. abscessus* disease (21). The cure rates for the *M. abscessus* disease caused by *M. abscessus* subsp. *massiliense* are better than those of other subspecies given its susceptibility to macrolides (17). These low cure rates prove that continued clinical improvement and culture conversion is not achievable for all patients. Moreover, many patients suffer from reinfection or relapse after treatment (*i.e.* MAC patients have 8.3-48% of recurrence) (21).

The poor treatment outcomes could be explained, in part, by the discrepancies between the DST *in vitro* and the antibiotic activity *in vivo* due to technical difficulties in performing DST, the lack of standardized methods and the lack of clinical validation. Moreover, DST is performed with mycobacteria at the exponential phase of growth and under optimal conditions of oxygen and nutrients. Differing from the environment where bacilli are located in the human host. Lung lesions in NTM-LD are complex and dynamic, with many

microenvironments under diverse conditions that lead NTM to different morphological and physiological states linked to antibiotic resistance.

Under such conditions (*i.e.* starvation and oxygen limitation) NTM enter a viable but non-culturable (VBNC) state characterized by antibiotic resistance. The resistance mechanism involved appears to be the reduced antibiotic uptake (51). In addition, the poor penetration of antibiotics to the site of infection can also explain the lack of correlation between the DST and the treatment outcome. Finally, the conditions encountered by NTM within macrophages (*e.g.* reactive oxygen species -ROS-, low oxygen and pH levels, the carbon source composition and nutrient starvation) also result in a VBNC state, being of great concern since many antibiotics are only effective against replicating bacteria (26).

4.3.9 Side effects

During the course of NTM-LD antibiotic treatment, it is necessary to monitor for potential side effects due to their high frequency. Among them, nephrotoxicity, ototoxicity, neurotoxicity, allergies and dermatological and gastrointestinal symptoms (*e.g.* nausea and diarrhoea) are the most common.

4.3.10 Antibiotic resistance

Another characteristic feature of NTM is their high level of natural and acquired resistance to commonly used antibiotics, hence making NTM treatment even more complicated than that of TB.

4.3.10.1 Natural resistance

There are many described factors that make NTM naturally resistant to most classes of antibiotics (Table 11). For instance, factors such as, the presence of drug export systems and porins, the biotransformation of antibiotics, the decrease in the affinity with the antibiotic target, the production of enzymes, the modification of permeability, hydrophobicity and the

content are all involved in the natural resistance. Of note, the waxy mycobacterial cell wall acts as an important physical and chemical barrier which directly interferes with the antibiotic uptake (52). Additionally, Whib7 is a conserved transcription factor which regulates different genes involved in antibiotic resistance (*e.g.* resistance to clarithromycin, amikacin and tigecycline) in NTM. The increased expression of Whib7 results in the upregulation of efflux pumps and drug-modifying genes (26).

On the other side, aminoglycoside resistance in NTM is caused by single-point mutations in the 16S rRNA gene. Moreover, MAC resistance to linezolid is mediated by mutations in the 23S rRNA *rrl*, *rplC* and *rplD* genes. NTM also have high levels of resistance to beta-lactams due to their impermeable membrane and the production of beta-lactamases that display broad-spectrum activity against cephalosporins. In MABSC, Bla_{Mab} is present and when beta-lactamase inhibitors, such as avibactam, are added to imipenem and amikacin there is a higher reduction in MABSC burden. Vaborbactam and relebactam are a new generation of beta-lactamase inhibitors (14).

The natural and intrinsic resistance in NTM could be explained by the fact that they are broadly found in the environment, where they cohabitate with other bacteria that produce antibiotics as secondary metabolites. Hence and as a survival response, NTM have developed resistance mechanisms to evade the antibiotics present in the environment and in response to competition for habitat and nutrients as well (26, 53).

Table 11. Mechanisms of antibiotic resistance in *M. avium* and MABSC (53).

Antibiotic	**Target**	***M. avium* genes**	**MABSC genes**
Isoniazid	InhA	Presumably efflux pumps	Presumably efflux pumps
Rifampicin	RNA	NA	Inactivation of drug (arr_{Mab})
Ethambutol	Arabinosyl transferase	NA	Polymorphisms in target gene *embB*
Pyrazinamide	PanD	Presumably due to active efflux of POA	Presumably owing to active efflux of POA
Aminoglycosides	16S rRNA	NA	Inactivation of drug [*aac(2'), eis2-whiB7*]

Fluoroquinolones	DNA gyrase	Polymorphisms in target gene *gyrA*	Polymorphisms in target gene *gyrA*
beta-lactams	Penicillin-binding protein	beta-lactamase with mild activity. Other unknown reasons	Inactivation of drug (*bla*$_{Mab}$)
Thiacetazone	FAS-II dehydratase	The target is not essential: redundant dehydratase present	The target is not essential: redundant dehydratase present
BTZ043	DprE1	Polymorphisms in target gene *dprE1*	Polymorphisms in target gene *dprE1*
BRD4592	Tryptophan synthase	NA	Polymorphisms in target gene *trpA*
Macrolides	23S rRNA	Efflux pumps (MAV_1695, MAV_1406)	Modification of drug target [erm(41)-*whiB7*]

4.3.10.2 Acquired resistance

The most likely phenomenon involved in acquired resistance is spontaneous mutations in genes which act as antibiotic targets. These mutations can be transmitted to the offspring, resulting in the spread of antibiotic resistance among NTM. Furthermore, acquired resistance has been favoured by the lengthy antibiotic treatments and particularly monotherapy, leading to the emergence of resistant isolates (26). For instance, a point mutation in the *rrl* gene confers constitutive resistance to macrolides in MABSC, commonly from macrolide monotherapy. With all, MABSC has been dubbed the "antibiotic nightmare" due to its resistance to most classes of antibiotics (26, 50). However, many other factors may be involved with the acquired antibiotic resistance in NTM (52).

At the moment, there is limited evidence that acquired resistance due to genotypic changes in NTM is caused by mobile elements such as transposons and plasmids (52). Nonetheless, WGS determined that *M. abscessus* shares several genes with other bacteria involved in CF, such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Therefore, horizontal gene transfer could be a route antibiotic resistance acquisition in NTM (26).

Worryingly, many patients suffering from COPD and non-CF bronchiectasis are being administered macrolide monotherapy in order to decrease the risk of exacerbations but

leading to macrolide resistance. Therefore, these patients should be screened for NTM prior beginning of macrolide monotherapy so that resistance is not favoured (20).

4.4 Methods to test antibiotics in NTM

Nowadays, there are different available methods to study the activity of antibiotics against NTM. Among these methods, we distinguish the *in vitro* methods which are carried out in a dish or test tube, the *ex vivo* methods which are performed with cells or tissues obtained from organisms and the *in vivo* methods which are carried out in animals.

4.4.1 *In vitro* models

4.4.1.1 Time-kill assay

The time-kill assay allows establishing the rate at which NTM die at a certain antibiotic and antibiotic concentration along a defined period of time. That is, NTM are exposed to an established antibiotic concentration over time and at the defined time points sampling is performed for CFU determination. Moreover, and after plotting the results, graphics displaying the time-kill curves are obtained. This method assesses the pharmacodynamics of antibiotics and has been widely used in the microbiology laboratory (54).

In addition, it allows studying antibiotics alone or in combination, being of great interest given that current treatments are based on the administration of different antibiotics at the same time. The results obtained with the time-kill assay are interpreted as follows: indifference, synergy and antagonism (Fig. 28). These concepts are established based on the activity of the most effective individual antibiotic in comparison with the antibiotic combination. A difference of less than 2 $\log_{10}$ lower or higher is considered indifference. Synergy is determined when the activity of the antibiotic combination is 2 $\log_{10}$ higher than the most active individual antibiotic. Antagonism when the activity of the combination is 2 $\log_{10}$ lower compared to the most active individual antibiotic (55).

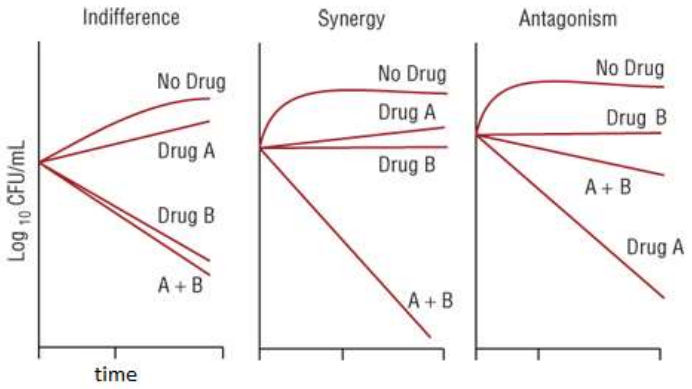

Figure 28. Antibiotic interactions in the time-kill assay (55).

4.4.1.2 Checkerboard assay

The checkerboard assay is used to study possible synergies between different antibiotics. In this assay, different antibiotics are tested in a wide range of concentrations, including the MIC and concentrations above and below the MIC. Afterwards, the fractional inhibitory concentration (FIC) can be calculated using the following formula:

$$\Sigma FIC = FIC\ A + FIC\ B$$

Where FIC A is the MIC of antibiotic A in the combination/MIC of antibiotic A alone, and FIC B is the MIC of antibiotic B in the combination/MIC of antibiotic B alone (Fig. 29). The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <4 and antagonistic when the ΣFIC is ≥4 (56). When performed in triple antibiotic combinations ΣFIC = FIC A + FIC B + FIC C, where FIC A is the MIC of antibiotic A in the combination/MIC of antibiotic A alone, FIC B is the MIC of antibiotic B in the combination/MIC of antibiotic B alone and FIC C is the MIC of antibiotic C in the combination/MIC of antibiotic C alone (57). In this case, synergism is considered when FIC is ≤0.75.

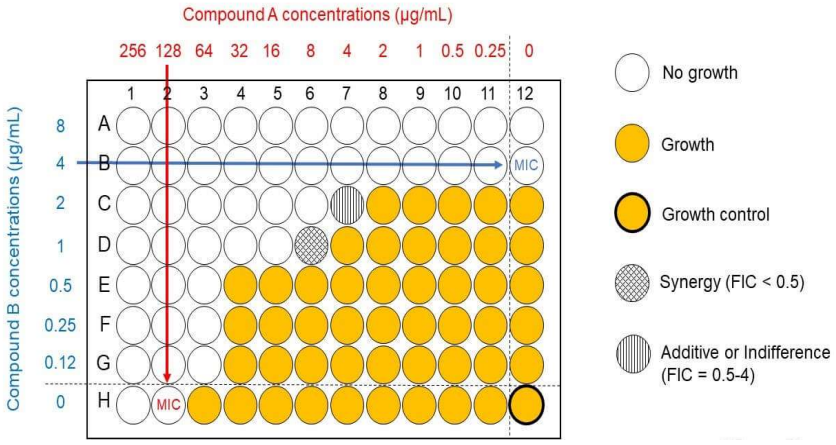

Figure 29. Interpretation of the checkerboard assay (56).

4.4.2 *Ex vivo* models

4.4.2.1 Cell infection assay

This *ex vivo* technique consists of culturing cells (*e.g.* THP-1 macrophages) and then infecting them with the NTM species to be studied (Fig. 30). These cells can be readily obtained from commercial companies and have different origins. For instance, THP-1 monocytes are obtained from peripheral blood from acute monocytic leukaemia patients. When grown under the right conditions of temperature, $C0_2$ levels and time, they are able to turn into an immortalized cell line with the capacity to proliferate indefinitely and that can be cultured for long periods of time. Then, the cell line can be treated with different antibiotics and antibiotic combinations to determine the intracellular activity. This method is closer to the actual environment found on NTM-LD since macrophages play a great role on mycobacterial infections by phagocyting mycobacteria and hampering the entry of antibiotics at the site of infection (58).

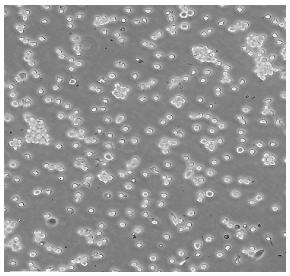

Figure 30. THP-1 macrophages (https://www.atcc.org/Products/All/tib-202.aspx).

4.4.3 *In vivo* models

Animal models

These models use animals (*e.g.* mice, guinea pigs, zebrafish and macaques) to study human diseases and their evolution as well as their treatment before it is administered to humans (Fig. 31). These models need prior approval from the ethics committee since they are performed in animals and ethical issues may be involved. Moreover, they are more expensive and more complex to maintain. *In silico* methods could be a good alternative to animal models. However, further development of *in silico* models is needed so that they can better reproduce the conditions and evolution of the infection and its treatment.

Figure 31. Laboratory mice (ttps://www.shutterstock.com/es/video/clip-1019518030-white-lab-mouse-albino-close-up-on-background).

4.4.4 Clinical trials

Clinical trials are studies performed in humans to test new treatments and assess their possible side effects. Therefore, they need prior approval by the corresponding authorities. Previous start of clinical trials, the new drugs and treatments have to be tested *in vitro* in the

laboratory and in animal models as well. With all, clinical trials have four phases which test the drug or treatment, determine the right dose and look for any side effects:

- In phase I the treatment is tested in a small group of healthy individuals to come across with possible side effects and tests out the safety and the adequate dose.
- Phase II studies a larger group of individuals and is aimed to assess the effectiveness of the treatment. This phase also analyses whether the treatment is successful in individuals who have a disease or medical condition.
- Phase III analyses the drug in different populations and doses, being administered in combination with other drugs. If the results are positive the drug will be approved.
- Phase IV monitors the effectiveness and the safety after approval given that side effects may not be evident until the drug is used by a large and diverse population for a long period of time (59).

4.5 Biofilm formation in NTM

Most NTM species have the ability to produce biofilm as a result of their hydrophobicity. Biofilms are a community of microorganisms attached to a biotic (*e.g.* respiratory system) and/or abiotic surfaces (*e.g.* pipelines and showerheads) protected by a matrix of extracellular polymeric substances (EPS) which confers protection from the environment, antibiotics, the immune response, ultraviolet radiation, disinfectants and others. Two classes of biofilm can be distinguished according to spatial location, the biofilms found on the bottom surfaces and biofilms produced in the air-liquid interphases as pellicles (60, 61).

NTM species that can potentially produce biofilm are more difficult to eradicate given that biofilms, among others, complicate antibiotic entry at the site of infection. Thus, making NTM treatment even more difficult and requiring much higher antibiotic concentrations for a complete eradication than those needed to inhibit planktonic forms. As a result, biofilms are one of the main NTM virulence factors. Moreover, NTM thriving within a biofilm

community show enhanced antibiotic resistance due to horizontal gene transfer and quorum sensing. That is, within the biofilm community NTM communicate with each other and modify gene expression, according to population density, by releasing chemical substances into the environment (62).

Moreover, a subpopulation of persister cells which show no metabolic activity and are resistant to antibiotics can be distinguished within the biofilm (63). Additionally, the presence of extracellular DNA (eDNA) inside the biofilm is also linked to antibiotic resistance (26). This increased resistance in biofilm communities can be explained by the protection provided by the EPS, the reduced transport through the EPS and the changes in gene regulation. Additionally, the absence of O_2 boosts antibiotic tolerance, and the bactericidal activity of many antibiotics is increased by aerobic respiration (13). NTM differ from other bacterial biofilms by having an extracellular matrix rich in lipids rather than polysaccharides (63). With all, *in vitro* DST may not be useful for those infections where biofilm production is involved (64). Nonetheless, this temporary and phenotypic antibiotic resistance is lost when NTM leave the biofilm community (65).

The poor penetration and the limited accumulation of antibiotics at the site of infection can derive in subtherapeutic concentrations where NTM reside (*e.g.* macrophages), resulting in decreased treatment efficacy. Furthermore, the exposure to sub-inhibitory antibiotic concentrations causes enhanced biofilm production since the genes expressed during the stress response are associated to biofilm formation. On the other side, the coinfection with other bacteria in, for example, CF patients makes the presence of mixed-species biofilms very likely (26).

The process of biofilm formation is a dynamic progress that includes four different stages: reversible attachment, irreversible attachment, maturation and dispersion (Fig. 26). The attachment phase depends on the electrostatic interaction between bacteria and the surface.

NTM will only attach to a surface if they encounter adequate levels of nutrients, temperature and oxygen. Once the biofilm is completely mature, social interaction occurs before biofilm aggregates are dispersed. Within the biofilm aggregates, planktonic NTM are also released and the entire process may start over again given that they have the ability to form a new biofilm (65).

The glycopeptidolipid (GPL) is located in the outer part of the mycobacterial cell wall, playing an important role in the development of biofilm. In *M. avium*, the first action for GPL biosynthesis consists on the addition of four amino acids to the lipopeptide by a peptide synthetase which is encoded by an operon with two genes, *pstA* and *pstB*. These two genes influence the adherence on polyvinyl chloride (PVC) materials given that they are involved in the GLP synthesis. Guanosine diphosphate (GDP)-fucose is used as a main substrate in the synthesis of GPL. Therefore, GDP-mannose possibly is the substrate for *M. avium* biofilm by using some of the GPL. *M. avium* isolates with deficient or no GPL production show less sliding capacity and rougher colony phenotype and have no ability to form biofilm. The same is observed in MABSC.

The mycolic acids are components of the cell wall which are essential for pathogenesis, immune evasion and biofilm production. GroEL1 is a chaperone involved in biofilm production and has the potential to interact with the type II fatty acid synthase (FAS-II) responsible for the biosynthesis of C56-C68 short-chain fatty acids. Mutations in genes of FAS-II result in a defective biofilm.

Proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) are families of proteins. PPE38 is a protein that is associated to the surface and can promote proinflammatory cytokines. The presence of a mutation results in poor sliding and biofilm formation abilities (66).

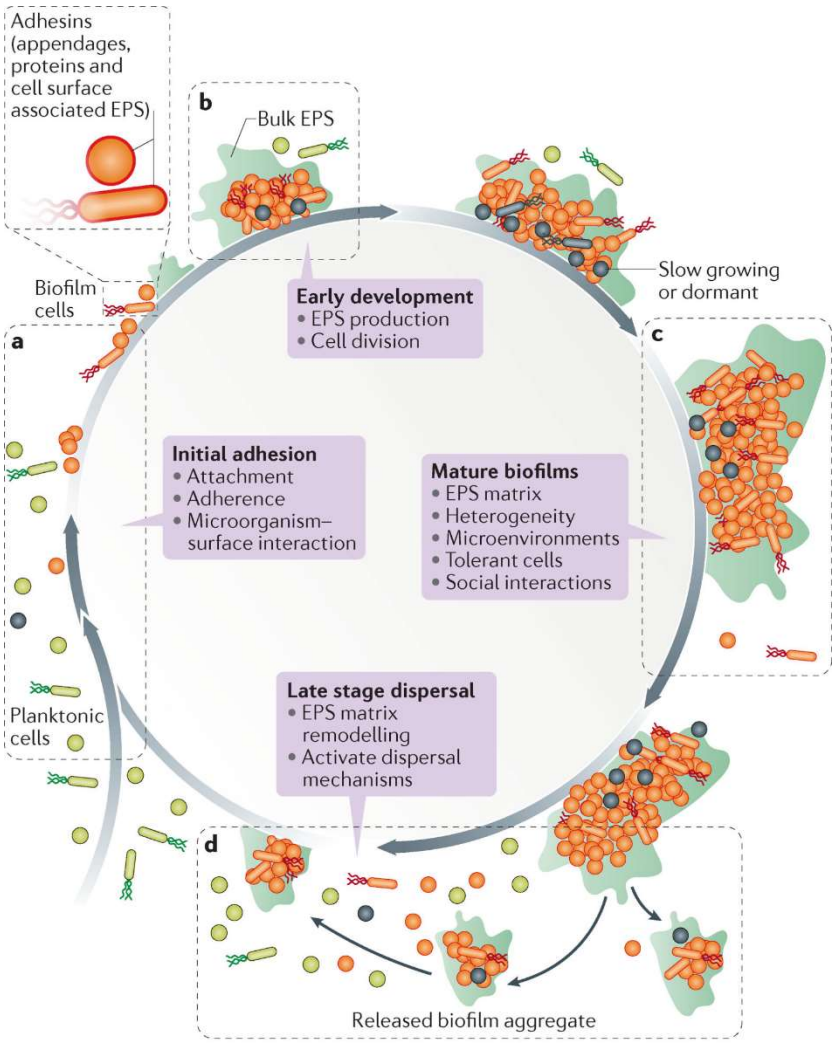

Figure 26. Process of biofilm formation. a, reversible attachment; b, irreversible attachment; c, maturation and d, dispersion (66).

4.6 Methods to assess biofilm formation in NTM

Although there is no current standardised method, there are some *in vitro* methods that can be used to assess biofilm production in NTM. These methods can also be used to test the activity of different antibiotics and antibiofilm agents against NTM biofilms. Thus, obtaining data closer to the actual scenario found in NTM-LD given that a great proportion of NTM involved in lung disease can produce biofilm.

The tube method described by Christensen *et al.* 1982 (67) enables the determination of biofilm production by inoculating tubes that contain liquid media. Then, the tubes are

incubated, stained with crystal violet, dried and finally the visual reading is performed by observing the formation of a line on the walls of the tubes, indicative of biofilm production.

In other methods, the NTM culture is seeded in 96-well plates (*e.g.* polystyrene non-treated plates and PVC plates) containing broth media. The plates are then incubated for different periods of time and at the corresponding temperature depending on the NTM species. Afterwards, the non-adherent components are washed off and the plates are completely dried and stained with crystal violet or similar dyes. Finally, the optical density (OD) is determined by using a microplate spectrophotometer. When studying biofilm production in some NTM species and particularly in RGM, the Calgary Biofilm Device (CBD), consisting of a 96-well plate with a pegged-lid, is necessary given that the biofilm is formed on the pegs (Fig. 27). Furthermore, microscopy techniques are also used when studying biofilms since NTM aggregation can be readily observed (68, 69).

Figure 27. Calgary 96-well plate (https://cced.cdeworld.com/courses/760-supragingival-and-subgingival-plaque-paradigm-biofilms).

4.7 Future of NTM-LD treatment

4.7.1 New anti-NTM antibiotics

At present, NTM-LD treatment is based on the administration of multiple antibiotics during long periods of time. Due to the poor treatment outcomes and the high levels of antibiotic resistance in NTM it is necessary to develop other treatment options that result in better patient management, improved quality of life and better treatment outcomes. The repurposing of existing antibiotics, particularly those approved for the treatment of TB (*e.g.*

bedaquiline, clofazimine, rifabutin and tedizolid), or the discovery of new antibiotics with higher efficacy against NTM would improve the NTM-LD treatment (53).

New antibiotics should preferably be bactericidal and have broad-spectrum activity, oral formulation, good penetration, intracellular activity, low price and reduced side effects (26). Moreover, NTM thrive and proliferate inside macrophages, thus antibiotic uptake and penetration into the macrophages is a key factor for an efficient antimicrobial activity (25). Other factors that need to be considered are the fact that the efficiency of antibiotics depends on the metabolic activity thus; higher metabolism leads to increased antibiotic uptake and increased expression of antibiotic targets. Moreover, aerobic metabolism leads to the formation of ROS which contribute to the bactericidal activity of antibiotics. Nevertheless, the lack of new agents in the antibiotic development pipeline is of great concern (Table 12).

Table 12. Clinical trials of MAC and MABSC treatment (26).

Discovery		Phase I/II	Phase III	Phase IV
LCB01-0371: Target 50S ribosome For *M. abs*	**Clofazimine**: Target NDH-2 For *M. abs*	**Clofazimine**: Target NDH-2 For *M. avium* LD	**LAI**: Target 30S ribosome For refractory MAC LD	**Linezolid**: Target 50S ribosome For NTM disease
PIPD1: Target MmpL3 For *M. abs*	**Tedizolid**: Target 50S ribosome For NTM	**Liposomal amikacin for inhalation (LAI)**: Target 30S ribosome For *M. abs* LD	**Clarithromycin vs azithromycin**: Target 50S ribosome For MAC LD	
Indole-2-carboxamides: Target MmpL3 For *M. abs*	**Bedaquiline**: Target ATP synthase For NTM	**Nitric Oxide (NO)**: Enhance host defence Produce reactive nitrogen intermediates For CF patients with NTM (especially *M. abs*)	**Clarithromycin vs moxifloxacin**: Target DNA gyrase For *M. xenopi* LD	

Thiacetazone derivatives: Target FAS-II dehydratase For *M. avium* and *M. abs*	**beta-lactams with avibactam**: Target PBP For *M. avium* and *M. abs*	**Gaseous NO**: Enhance host defence Produce reactive nitrogen intermediates For NTM
	Rifabutin: Target RNA polymerase For *M. abs*	

Mechanisms of action:
Inhibition of cell wall synthesis
Inhibition of protein synthesis
Inhibition of nucleic acid synthesis
Other mechanisms

Some of the repurposed or lately discovered antibiotics are:

4.7.1.1 Bedaquiline $C_{32}H_{31}BrN_2O_2$

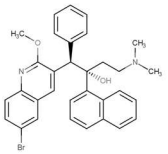

Figure 32. Bedaquiline structure (https://go.drugbank.com/drugs/DB08903).

Bedaquiline is a member of the diaryquinolines. Its mode of action is through the inhibition of the ATP synthase. Bedaquiline is bacteriostatic against MAC and *M. abscessus*. It showed MICs ranging from 0.016 μg/mL to 1 in clinical isolates of *M. abscessus*. Nonetheless, a considerable proportion of the isolates had MICs of 16 μg/mL or higher (Fig. 32) (70).

4.7.1.2 Delamanid $C_{25}H_{25}F_3N_4O_6$

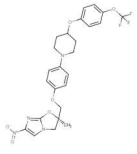

Figure 33. Delamanid structure (https://go.drugbank.com/drugs/DB11637).

Delamanid is a nitroimidazole antibiotic that inhibits the synthesis of methoxycholic and ketomicolic acids, components of the mycobacterial cell wall. It displays modest activity against some SGM species. Resistance mechanisms to delamanid have been associated with genes in the F420-dependent deazoflavin nitroreductase pathway, found in different mycobacterial species (Fig. 33) (64, 70).

4.7.1.3 Tedizolid $C_{17}H_{15}FN_6O_3$

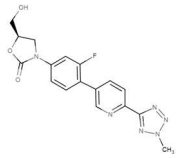

Figure 34. Tedizolid structure (https://go.drugbank.com/drugs/DB14569).

Tedizolid is an antibiotic from the family of the oxazolidinones. A number of studies have established *in vitro* activity of tedizolid against *M. abscessus*, alone and in combination with for instance, clarithromycin and amikacin. Tedizolid also displayed intracellular activity in a macrophage infection model, and more pronounced when combined with imipenem in the presence or absence of avibactam. It has an improved side effects profile compared to that of linezolid (Fig. 34) (70).

4.7.1.4 Omadacycline $C_{29}H_{40}N_4O_7$

Omadacycline is an aminomethylcyline and a member of the tetracycline class of antibiotics. It has broad-spectrum activity and it is promising in the management of NTM infections and particularly of lower respiratory tract infections caused by resistant *M. abscessus*. Omadacycline has similar *in vitro* activity to that of tigecycline against *M. abscessus* but it has better PK profiles, showing higher and more constant concentrations in plasma, epithelial lining fluid and alveolar cells. Furthermore, it is also formulated for oral administration (Fig. 35) (70).

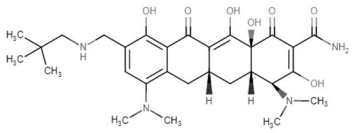

Figure 35. Omadacycline structure (https://go.drugbank.com/drugs/DB12455).

4.7.1.5 Eravacycline $C_{27}H_{31}FN_4O_8$

Eravacycline is a fluorocycline and thus a new tetracycline. It shows similar activity to that of tigecycline, but it is better tolerated. Eravacycline is parenterally administered for the treatment of problematic intraabdominal infections (Fig. 36) (70).

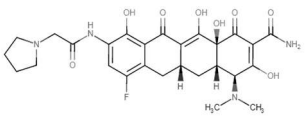

Figure 36. Eravacycline structure (https://go.drugbank.com/drugs/DB12329).

4.7.2 Other agents and treatment options against NTM-LD

Other less common agents have shown antimycobacterial activity as well. For instance, essential oils, nitric oxide (NO) (*i.e.* in inhaled therapies displays broad-spectrum activity, resulting in reduced inflammation and increased respiratory function), animal venoms (*i.e.* source of natural antimicrobial substances including proteins, amines, peptides, toxins and enzymes), medicinal plants (*i.e.* source of multiple bioactive compounds) and spices (*i.e.* some spices apart from having antimicrobial activity display synergistic activity when combined with antibiotics) (14, 53, 64).

4.7.2.1 Beta-lactamase inhibitors

Beta-lactam resistance in *M. abscessus* is mediated, among others, by chromosomally-encoded Ambler-Class A beta-lactamases (BlaMab). Despite being several *M. abscessus* beta-lactamases inhibited by classical (*e.g.* clavulanic acid) or new generation (*e.g.* avibactam) inhibitors, BlaMab is not inhibited by clavulanic acid, tazobactam or sulbactam. However, avibactam seems to inhibit BlaMab. The combination of avibactam with amoxicillin or piperacillin proved to be effective against *M. abscessus* clinical isolates. Avibactam also enhanced the *in vitro* and *in vivo* effect of imipenem, supposedly unaffected by BlaMab, indicating that avibactam could have some level of intrinsic activity (70).

4.7.2.2 Dual beta-lactams

The use of two beta-lactams is based on the selective inhibition of non-redundant target enzymes in mycobacteria. Each beta-lactam has different inhibitory activity on different transpeptidases, hence the combination of two beta-lactams may display synergy. For instance, imipenem with cefoxitin or cefdinir, as well as with avibactam shad a synergistic activity. Additionally, the combination of ceftazidime with ceftaroline or ceftazidime with imipenem, have good *in vitro* MIC values and time-kill curve activities. They also are

effective against infected THP-1 human macrophages. Consequently, the dual β-lactam treatment against *M. abscessus* may be of benefit (70).

4.7.2.3 Disulfiram $C_{10}H_{20}N_2S_4$

Disulfiram is a drug that has been longly used in the treatment of alcohol abuse. In addition, it has a potent antimicrobial and anti-parasitic activity. Disulfiram is an electrophile and acts forming disulphides with substances containing thiol. It exhibits a concentration and time-dependent bactericidal activity against *M. abscessus* and synergistic activity with other antibiotics used during treatment. Disulfiram also showed to reduce the bacterial load within macrophages (Fig. 37) (39).

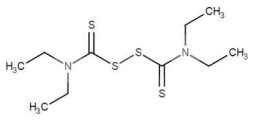

Figure 37. Disulfiram structure (https://go.drugbank.com/drugs/DB00822).

4.7.2.4 D-cycloserine $C_3H_6N_2O_2$

D-cycloserine inhibits two enzymes involved in the peptidoglycan biosynthesis, alanine racemase (Alr) and D-Ala:D-Ala ligase (Ddl). It belongs to the second-line treatment against multidrug and extensively drug-resistant-TB (MDR/XDR-TB). Moreover, D-cycloserine is very active against *M. abscessus*; hence either alone or in combination with other antibiotics may be promising for the treatment of *M. abscessus* (Fig. 38) (39).

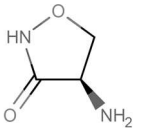

Figure 38. D-cycloserine structure (https://go.drugbank.com/drugs/DB00260).

4.7.2.5 Auranofin $C_{20}H_{34}AuO_9PS$

Auranofin is a gold-based anti-rheumatic agent. It inhibits the thioredoxin reductase enzyme (TrxR). The inhibition of this enzyme results in the disruption of different essential processes such as the maintenance of the intracellular environment, the regulation of redox enzymes and proteins by oxidoreduction and the detoxification of ROS. Auranofin has been tested alone and in

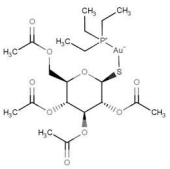

Figure 39. Auranofin structure (https://go.drugbank.com/drugs/DB00995).

combination with antibiotics in NTM, showing synergism with amikacin against *M. abscessus* (Fig. 39) (39).

4.7.2.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) are powerful natural antibiotics produced by all life forms from microorganisms to humans. AMPs tend to be relatively short (20-60 amino acid residues), amphipathic and positively charged. Four main structural AMPs classes have been defined: α-helix, β-hairpin, β-sheet, and linear non α-helical. Several AMPs databases, such as the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/main.html), Dragon Antimicrobial Peptide Database (DAMPD) (http://apps.sanbi.ac.za/dampd) and the database Linking Antimicrobial Peptide (LAMP) (http://biotechlab.fudan.edu.cn/database/lamp/) are available, providing comprehensive information about their antimicrobial activity and mechanisms of action (71).

AMPs play an important role in the innate immune response and have the ability to modulate host defences. They promote pathogen clearance with their broad-spectrum antimicrobial activity against bacteria, viruses, parasites and fungi. Most AMPs target the cell wall by different modes of action, such as pore formation, thinning, altered curvature, localized perturbations and modified electrostatics. Hence, the interaction between AMPs and the cell wall may result in its disruption. Different studies have demonstrated that AMPs increase the permeability of the mycobacterial wall, which may facilitate the translocation of AMPs and other drugs across the membrane and into the cytoplasm. Additionally, acquisition of resistance to AMPs is very rare since they are structurally stable and only few bacteria show resistance. Moreover, they do not present haemolytic properties, they do not show toxicity to host cells and can be easily synthesized and modified (53). As a result, AMPs have promising clinical applications and the potential to combat mycobacterial infections (Fig. 40).

Figure 40. Mastoparan structure (https://en.wikipedia.org/wiki/Mastoparan).

4.7.2.7 Nanoparticles

Nanoparticles are small coated particles with a range of size from 1 to 100 nm. They have characteristic physical and chemical properties which can be used with medical purposes. Moreover, nanoparticles can be synthesized *de novo* with the desired properties. Accordingly, the nanoparticles coat can be loaded with different molecules which will confer different properties based on the sought purpose (Fig. 41).

4.7.2.8 Liposomes

Liposomes are nanoparticles whose properties make them suitable as drug delivery systems. They are sphere-shaped vesicles mainly composed of phospholipids and usually have a size ranging from 50 to 450 nm. Given the amphipathic properties of phospholipids, these molecules tend to form membranes when in contact with aqueous environments. Hence, their stable structure consists of an aqueous core and a phospholipid bilayer. Liposomes can respectively carry lipophilic and hydrophilic compounds in their lipidic bilayer and in their aqueous phase. Moreover, they can be readily modified to become optimal delivery systems of the desired compound (Fig. 41) (72).

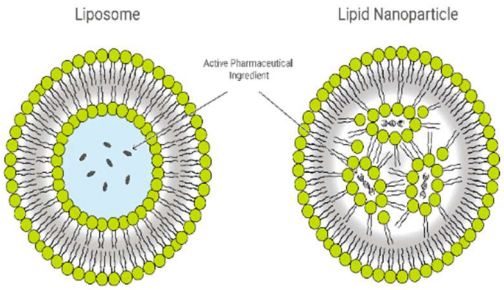

Figure 41. Liposome and nanoparticle structures (https://www.exeleadbiopharma.com/news/liposomes-and-lipid-nanoparticles-as-delivery-vehicles-for-personalized-medicine).

4.7.2.9 Bacteriophages

Bacteriophages are viruses that specifically infect bacteria and ultimately some produce their lysis. They are highly specific, making them very safe for the human host. They are able to replicate inside the host cell and therefore the infective dose needed is very low. In addition, their mode of action is quicker than that of antibiotics, making of them promising therapy options with few side effects.

However, bacteriophages are not capable of penetrating eukaryotic cells and delivery systems, such as liposomes are required in order to gain access to the site of infection. One of the most studied mycobacteriophages is TM4, which has shown to effectively reduce *in vitro* and *in vivo M. avium* bacilli. At the moment, 4200 mycobacteriophages are known to specifically infect mycobacteria. Their potential use in the treatment of NTM-LD should be further investigated (Fig. 42) (73).

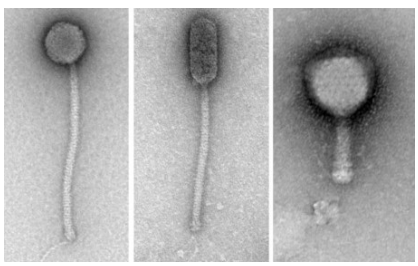

Figure 42. Different morphologies of mycobacteriophages (http://europepmc.org/article/PMC/4199240).

4.7.2.10 Host-directed therapies

Host-directed therapies (HDT) target the human host instead of bacteria, in contrast with antibiotics and other drugs which target bacteria. For instance, by boosting the immune system and weakening the inflammatory response the HDT could clear the NTM infection and reduce the damage of the inflammatory response. Moreover, HDT enable to reduce the emergence of antibiotic resistance and allow using lower doses of antibiotic. In NTM infections, the interferon-γ immune response has an important role and the enhancement of this immune pathway has proved to be effective in *in vitro* and *in vivo* studies by reducing the burden of bacilli in *M. avium* infections (73).

5. WORK JUSTIFICATION AND HYPOTHESES

The high-level of antibiotic resistance, the ability to form biofilm as well as the emergence of nontuberculous mycobacteria-lung disease (NTM-LD) by *Mycobacterium avium-intracellulare* complex (MAC) and *Mycobacterium abscessus* has led to the need for effective treatments against NTM-LD. Currently available treatments are lengthy, consist on the administration of at least two to three antibiotics, have numerous side effects and show poor treatment outcomes. Further knowledge of MAC and *M. abscessus* response to different antibiotics is key in finding novel treatment options which result in improved patient management, less side effects and better treatment outcomes.

IV. Natural and acquired antibiotic resistance in MAC and *M. abscessus* is of great concern. Thus, a good characterization of the antibiotic susceptibility profile is necessary. Moreover, alternative treatments could help in the fight against NTM-LD.

   a. The antimicrobial peptides (AMPs) could be effective against NTM

V. NTM-LD treatment requires the administration of multiple antibiotics, however, few antibiotics show good activity. On the other side, even though drug susceptibility testing (DST) is performed individually, during treatment antibiotics are administered in combination. Furthermore, few studies have tested antibiotic combinations, and particularly triple-antibiotic combinations against NTM.

   b. The study of triple-antibiotic combinations could assess synergistic activity
   c. The adaptation of the time-kill assay to NTM could assess synergistic activity and determine the pharmacodynamics of antibiotics
   d. The adaptation of the macrophage infection model to NTM could assess synergistic activity and determine the intracellular activity of antibiotics

VI. Biofilm formation plays an important role in NTM-LD by offering extra protection from antibiotics and other agents. Nonetheless, there is no standardized method for *in vitro* biofilm formation in NTM. Additionally, little is known about the activity of antibiotics and antibiofilm agents against NTM biofilm.

e. The standardization of an *in vitro* model for biofilm formation could assess biofilm formation as well as determine the activity of antibiotics and antibiofilm agents against NTM biofilm

6. OBJECTIVES

1. To determine the MICs of 12 antimicrobial peptides (AMPs) against *M. avium* clinical isolates.

2. To assess the *in vitro* and *ex vivo* activity of a triple-antibiotic combination (amikacin/ethambutol/clarithromycin) against MAC clinical isolates using the time-kill assay and the macrophage infection model.

3. To assess the *in vitro* activity of two triple-antibiotic combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) against *M. abscessus* clinical isolates using the time-kill assay.

4. To standardize a method for the assessment of the *in vitro* biofilm formation in MAC clinical isolates.

5. To assess the *in vitro* activity of 9 different combinations, including 4 antibiotics and 5 antibiofilm agents, against biofilm forming MAC clinical isolates.

7. METHODS AND RESULTS

Paper 1: *In vitro* activity of 12 antimicrobial peptides against *Mycobacterium tuberculosis* and *Mycobacterium avium* clinical isolates

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Impact Factor: 2.156-JCR Microbiology Q3; Scimago Medicine Q1. (Objective 1).

Introduction: Tuberculosis (TB) remains a major health problem and is one of the leading causes of death by a single infectious agent, *Mycobacterium tuberculosis*. Infections produced by non-tuberculous mycobacteria (NTM) have dramatically increased, mainly in immunocompromised patients and individuals with pre-existing pulmonary diseases. Among these NTM, *Mycobacterium avium* is gaining increasingly more relevant clinical significance. The high levels of natural drug resistance of NTM leads to poor treatment outcomes, requiring novel drug regimens and compounds. Antimicrobial peptides (AMPs) are powerful natural antibiotics produced by all life forms from micro-organisms to humans. They promote pathogen clearance with their broadspectrum antimicrobial activity against bacteria, viruses, parasites and fungi. Additionally, acquisition of resistance to AMPs is very rare, and only a few bacteria show resistance.

Objectives: To investigate the activity of 12 different AMPs (bactenecin, buforin I, mastoparan, indolicidin, histatin 5, histatin 8, magainin I, magainin II, cecropin PI, cecropin A, cecropin B and melittin) against clinical isolates of *M. tuberculosis* and *M. avium*.

Materials and methods: Six clinical isolates of *M. tuberculosis* and four clinical isolates of *M. avium* were studied. AMPs were tested at concentrations ranging from 128 μg ml^{-1} to 0.125

μg ml^{-1}. The minimum inhibitory concentration (MIC) of each peptide was determined using the resazurin assay in 96-well microtitre plates.

Results: When tested against *M. tuberculosis*, mastoparan and melittin showed the best activity, with MIC values ranging from 32 to 64 μg ml^{-1}. Indolicidin showed MIC values of 32 μg ml^{-1} against two of the isolates and of 64 μg ml^{-1} against one isolate. The other AMPs showed higher MICs (>128 μg ml^{-1}). Regarding *M. avium*, indolicidin had the greatest activity with MIC values of 128 μg ml^{-1}. All the remaining AMPs showed MICs >128 μg ml^{-1}.

Conclusions: AMPs are promising alternatives in the treatment of mycobacterial infections. Nonetheless, since the AMPs studied may exhibit even greater activity, further evaluation of these AMPs in combination and particularly associated with conventional antibiotics would be of great interest. Moreover, new methods of drug administration may improve the therapeutical potential of AMPs.

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SHORT COMMUNICATION

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MICROBIOLOGY SOCIETY

In vitro activity of 12 antimicrobial peptides against *Mycobacterium tuberculosis* and *Mycobacterium avium* clinical isolates

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Abstract

Tuberculosis (TB) remains a major threat to human health worldwide. The increasing incidence of non-tuberculous mycobacterial infections and particularly those produced by *Mycobacterium avium* has emphasized the need to develop new drugs. Additionally, high levels of natural drug resistance in non-tuberculous mycobacteria (NTM) and the emergence of multidrug-resistant (MDR) TB is of great concern. Antimicrobial peptides (AMPs) are antibiotics with broad-spectrum antimicrobial activity. The objective was to assess the activity of AMPs against *Mycobacterium tuberculosis* and *M. avium* clinical isolates. MICs were determined using microtitre plates and the resazurin assay. Mastoparan and melittin showed the greatest activity against *M. tuberculosis*, while indolicidin had the lowest MIC against *M. avium*. In conclusion, AMPs could be alternatives for the treatment of mycobacterial infections. Further investigation of AMPs' activity in combination and associated with conventional antibiotics and their loading into drug-delivery systems could lead to their use in clinical practice.

Tuberculosis (TB) remains a major health problem worldwide and is one of the leading causes of death by a single infectious agent, that is, *Mycobacterium tuberculosis*. According to the World Health Organization in 2016 there were an estimated 10.4 million new TB cases and 1.6 million deaths due to TB [1]. Furthermore, the emergence of multidrug-resistant (MDR) TB and extensively drug-resistant (XDR) TB has led to the need for the development of new treatment options [2].

Infections produced by non-tuberculous mycobacteria (NTM) have dramatically increased in recent years, mainly in immunocompromised patients and individuals with pre-existing pulmonary diseases. Among these NTM, *Mycobacterium avium* is of note and is gaining increasingly more relevant clinical significance. The high levels of natural drug resistance of NTM leads to poor treatment outcomes, requiring novel drug regimens and compounds [3].

Antimicrobial peptides (AMPs) are powerful natural antibiotics produced by all life forms from micro-organisms to humans [4–6]. AMPs tend to be relatively short (20–60 amino acid residues), amphipathic and positively charged. Four main structural AMP classes have been defined: α-helix, β-hairpin, β-sheet and linear non-α-helical [7]. In addition, several AMP databases, such as the Antimicrobial Peptide Database (APD), Dragon Antimicrobial Peptide Database (DAMPD) and the database Linking Antimicrobial Peptide (LAMP) are available, providing comprehensive information about their antimicrobial activity and mechanisms of action [7, 8].

AMPs play an important role in the innate immune response and have the ability to modulate host defences. They promote pathogen clearance with their broad-spectrum antimicrobial activity against bacteria, viruses, parasites and fungi. Most AMPs target the cell wall by different modes of action, such as pore formation, thinning, altered curvature, localized perturbations and modified

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Keywords: antimicrobial peptides; resazurin assay; minimum inhibitory concentration; mycobacterial infections; Mycobacterium tuberculosis; Mycobacterium avium.

Abbreviations: AMP, antimicrobial peptide; APD, antimicrobial peptide database; DAMPD, dragon antimicrobial peptide database; HNP, human neutrophil peptides; HNP, human neutrophil peptides; LAMP, linking antimicrobial peptide; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; NTM, non-tuberculous mycobacteria; TB, tuberculosis; XDR, extensively drug-resistant.

Table 1. Characteristics of the antimicrobial peptides used in the present study

Antimicrobial peptide	Sequence	Length	Source	Activity
Bactenecin	RLCRIVVIRVCR	12	*Bos taurus*	Antibacterial
Buforin I	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVH RLLRKGNY	39	*Bufo bufo gargarizans*	Antibacterial, Antifungal
Mastoparan	INLKALAALAKKIL	14	*Vespula lewisii*	Antibacterial
Indolicidin	ILPWKWPWWPWRR	13	*Bos taurus*	Antibacterial, Antifungal, Antiviral
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	24	*Homo sapiens*	Antibacterial, Antifungal, Antiviral
Histatin 8	KFHEKHHSHRGY	12	*Homo sapiens*	Antibacterial, Antifungal
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	23	*Xenopus laevis*	Antibacterial, Antiviral
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	23	*Xenopus laevis*	Antibacterial, Antifungal, Antiviral, Antiparasitic
Cecropin PI	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	31	*Ascaris suum*	Antibacterial
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQA TQIAK	37	*Hyalophora cecropia*	Antibacterial, Antiviral, Antiparasitic
Cecropin B	KWKIFKKIEKVGRNIRNGIIKAGPAVAVLGEAKAL	35	*Antheraea pernyi*	Antibacterial, Antifungal
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	*Apis mellifera*	Antibacterial, Antifungal, Antiviral, Antiparasitic

The AMP features were obtained from the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP/main.php).

electrostatics [9]. Hence, the interaction between AMPs and the membrane may result in disruption of the bacterial membrane. Different studies have demonstrated that AMPs increase the permeability of the mycobacterial wall, which may facilitate the translocation of AMPs and other drugs across the membrane and into the cytoplasm. Additionally, acquisition of resistance to AMPs is very rare, and only a few bacteria show resistance. As a result, AMPs have promising clinical applications and the potential to combat mycobacterial infections [10–12].

The objective of the present study was to investigate the activity of 12 different AMPs (bactenecin, buforin I, mastoparan, indolicidin, histatin 5, histatin 8, magainin I, magainin II, cecropin PI, cecropin A, cecropin B and melittin) against clinical isolates of *M. tuberculosis* and *M. avium* using microtitre plates and the resazurin assay.

Six clinical isolates of *M. tuberculosis* susceptible to first-line anti-TB drugs and four clinical isolates of *M. avium* were studied. Two of the *M. avium* isolates were susceptible to clarithromycin, rifampicin and amikacin but resistant to ethambutol. The other two *M. avium* isolates were susceptible to clarithromycin, rifampicin amikacin and ethambutol. All of the isolates were obtained from the Laboratory of Microbiology of the Hospital Clínic of Barcelona (Barcelona, Spain).

Of the 12 AMPs studied, histatin 5, magainin I, magainin II, cecropin PI, cecropin A and cecropin B were provided by Sigma-Aldrich (St. Louis, MO, USA). On the other hand, bactenecin, buforin I, mastoparan, indolicidin, histatin 8 and melittin were purchased from BionovaCientifica (S.L., Madrid, Spain) (Table 1) [12]. The lyophilized AMPs were dissolved in sterile distilled water and sterilized by filtration. Then, they were stored at −20 °C in aliquots until use. All the AMPs were tested at concentrations ranging from 128 µg ml^{-1} to 0.125 µg ml^{-1}. The AMPs were selected based on the activity shown against mycobacteria and other bacterial species described in previous studies as well as their availability and affordability [8].

M. tuberculosis isolates were grown in Lowenstein–Jensen medium slants (Becton Dickinson, Sparks, MD) and *M. avium* isolates were grown in BD Columbia Agar with 5 % sheep blood plates (Becton Dickinson). Afterwards, all of the isolates were subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) (Comercial Bellés, Tarragona, Spain) and 0.25 % Tween 80 (Merck, Darmstadt, Germany) to avoid bacilli clump formation. After 7 days of incubation, *M. tuberculosis* cultures were subjected to disaggregation techniques by being vigorously vortexed in a tub containing glass beads (Sigma-Aldrich) and the use of insulin syringes (Becton Dickinson). *M. avium* cultures were homogenized by agitation. Finally, the inoculum was adjusted to 1.5×10^8 cells ml^{-1} using a nephelometer (CrystalSpec; Becton Dickinson).

The minimum inhibitory concentration (MIC) of each peptide was determined using the resazurin assay in 96-well microtitre plates (Smartech Biosciences, Barcelona, Spain) as described by Palomino *et al.* [13]. Schena *et al.* [14] demonstrated that the resazurin assay is useful for the rapid and accurate MIC determination of delamanid in *M. tuberculosis* isolates, showing great concordance with the agar reference method. Briefly, 100 µl of Middlebrook 7H9 liquid media were added to each well. Then, serial dilutions of the AMPs ranging from 128 µg ml^{-1} to 0.125 µg ml^{-1} were made. Finally, 100 µl of inoculum at a final concentration of

5×10^5 cells ml^{-1} were added. Positive control wells consisted of 100 µl of Middlebrook 7H9 and 100 µl of inoculum (5×10^5 cells ml^{-1}). Negative control wells were prepared by adding 200 µl of Middlebrook 7H9. Plates were incubated at 37 °C in a 5 % CO_2 atmosphere for 7 days, after which 20 µl (10 % of the final volume) of fresh resazurin were added (alamarBlue Invitrogen, Life Technologies, Belgium). Plates were covered with aluminium foil for protection from light. After overnight incubation, visual reading was performed. Resazurin is a colorimetric reagent used to assess cell viability. This active agent, is blue and is reduced to resorufin (pink) by viable cells. A colourimetric change from blue to pink was interpreted as mycobacterial growth. The MIC was interpreted as the first concentration at which there was no colourimetric change. All the experiments were performed in duplicate for each isolate.

Twelve different AMPs were tested against clinical isolates of *M. tuberculosis* and *M. avium*. The AMPs tested showed moderate activity against *M. tuberculosis* and *M. avium* clinical isolates.

The MIC values obtained are shown in Table 2. When tested against *M. tuberculosis*, mastoparan and melittin showed the best activity, with MIC values ranging from 32 to 64 µg ml^{-1}. Indolicidin showed MIC values of 32 µg ml^{-1} against two of the isolates and of 64 µg ml^{-1} against one isolate. The other AMPs showed higher MICs (>128 µg ml^{-1}).

Regarding *M. avium*, indolicidin had the greatest activity with MIC values of 128 µg ml^{-1}. All the remaining AMPs showed MICs >128 µg ml^{-1}.

To our knowledge, there are few studies on the *in vitro* effect of the AMPs investigated in the present study against *M. tuberculosis*, and especially against *M. avium*. Human neutrophil peptides (HNP) are the most frequently studied against mycobacteria [15]. Sharma *et al.* [16] reported that HNP-1 has effective bactericidal activity against *M. tuberculosis* H37Rv *in vitro* as well as mycobacteria replicating within macrophages. Furthermore, Ogata *et al.* [17] demonstrated that HNP-1 and HNP-2 killed all of the *M. avium-M. intracellulare* isolates. Therefore, the present study aimed to assess the antimicrobial activity of 12 AMPs with antibacterial activity against other bacteria in *M. tuberculosis* and *M. avium* clinical isolates.

The activity of AMPs against mycobacteria is probably lower than in Gram-negative and other Gram-positive bacteria [15]. In the present study, we tested a set of 12 AMPs previously tested against other bacteria but rarely studied or with unknown activity against mycobacteria. As shown in Table 2, mastoparan and melittin showed better results against *M. tuberculosis* than the other AMPs. In a previous study these two AMPs also showed a good activity against *Acinetobacter baumanii* [12]. However, these AMPs were less effective against the *M. avium* isolates. Differences in membrane composition may affect the activity of the AMPs. Therefore, we hypothesized that they may present a different efficacy in other mycobacterial species, suggesting that each AMP should be specifically tested in each species. Moreover, the mycobacterial cell membrane has a distinct architecture with a lipid-rich envelope, and taking into account the cationic and amphipathic nature of these peptides AMPs targeting mycobacterial membrane lipids could be further explored.

Several AMPs have been shown to cause membrane permeabilization, which would facilitate the entry of drugs into the mycobacterial cell and allow interaction with intracellular targets [18]. From this point of view, AMPs could have greater activity or even synergism when combined with antibiotics. Sharma *et al.* [19] observed that the bacterial peptide deformylase (PDF) showed synergism with isoniazid and rifampicin against *M. tuberculosis* H37Rv. Therefore, further studies are needed to establish the potential synergism of any AMP in combination with antimycobacterial antibiotics [20]. Combination therapy including AMPs and common antibiotics could be an excellent therapeutic option to facilitate antibiotic uptake and decrease the MICs. Moreover, this novel treatment could reduce the therapeutic dose, prevent the appearance of resistance and reduce side effects. In a recent study, Li *et al.* [21] demonstrated that the combination of PA-824 (pretomanid) and *Cordyceps sinensis* had greater bacteriostatic activity against *M. tuberculosis* than pretomanid alone.

Table 2. MICs of the 12 antimicrobial peptides tested against *M. tuberculosis* clinical isolates

Isolate	Antimicrobial peptide MICs (µg ml^{-1})											
	Bac	Buf I	Mas	Ind	His 5	His 8	Mag I	Mag II	Cec PI	Cec A	Cec B	Mel
M. tuberculosis 1	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 2	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 3	>128	>128	64	>128	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 4	>128	>128	32	32	>128	>128	>128	>128	>128	>128	>128	32
M. tuberculosis 5	>128	>128	64	64	>128	>128	>128	>128	>128	>128	>128	64
M. tuberculosis 6	>128	>128	32	32	>128	>128	>128	>128	>128	>128	>128	64

Bac, bactenecin; Buf I, buforin I; Mas, mastoparan; Ind, indolicidin; His 5, histatin 5; His 8, histatin 8; Mag I, magainin I; Mag II, magainin II; Cec PI, cecropin PI; Cec A, cecropin A; Cec B, cecropin B; Mel, melittin.

It is mandatory to find novel drugs with minimum or no toxicity to human cells. In general, many AMPs are considered to have low toxicity to eukaryotic cells. In addition, AMPs are cell selective, having the ability to kill bacterial cells without being toxic to human host cells. Furthermore, AMP selectivity could be increased by modifying the molecule, thus altering the physicochemical properties of the AMPs, which would thereby enhance the safety profile [7, 9]. Nonetheless, some studies have described the *in vitro* cytotoxicity of some AMPs. For instance, Fu *et al.* [22] reported that HNP-1 exhibits important cytotoxicity to different types of cells when found at high concentrations. On the other hand, in order to improve their activity and to target AMPs to specific sites of infection, they could be loaded in drug-delivery systems such as liposomes and nanoparticles. These methods of drug administration reduce side effects by allowing the delivery of high drug concentrations directly to the site of infection. However, this system of administration should be further optimized [7].

In conclusion, AMPs are promising alternatives in the treatment of mycobacterial infections. Nonetheless, since the AMPs studied may exhibit even greater activity, further evaluation of these AMPs in combination and particularly associated with conventional antibiotics would be of great interest. Moreover, new methods of drug administration may improve the therapeutical potential of AMPs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval was received from the Ethical Committee of the Hospital Clínic de Barcelona (Barcelona, Spain) [ref.no. 2013/8147].

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Paper 2: Comparison of two drug combinations, amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin against *Mycobacteroides abscessus* subsp. *abscessus* using the *in vitro* time-kill assay.

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Introduction: *Mycobacteroides abscessus* causes lung infections which are difficult to treat due to its resistance to most classes of antibiotics. The treatment regimens available for *M. abscessus* lung infections consist of the administration of different antibiotics in combination. Therefore, it is necessary to determine the efficacy of these combinations as well as of that of the antibiotics alone.

Objectives: To compare the *in vitro* activity of four different antibiotics, amikacin, tigecycline, imipenem and clarithromycin alone and in two different three-drug combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) against *M. abscessus* subsp *abscessus* clinical isolates.

Materials and methods: Seven clinical isolates of *M. abscessus* subsp. *abscessus* were studied using the *in vitro* time-kill assay. Amikacin and imipenem were tested at 2 x MIC and tigecycline and clarithromycin at 4 x MIC. Time points were established at days 0, 1, 3 and 6.

Results: The most active individual antibiotic was amikacin followed by imipenem and clarithromycin. Both combinations were indifferent (neither synergistic nor antagonistic) showing more activity than the antibiotics tested individually. Moreover, no significant

differences ($P \geq 0.05$) were observed between the activities of the two three-drug combinations.

Conclusions: Both three-drug combinations, amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin, showed similar *in vitro* activity against *M. abscessus* subsp. *abscessus* clinical isolates as well as no antagonistic activity. The combination including imipenem is not a reliable alternative against *M. abscessus* subsp. *abscessus* lung infections caused by isolates susceptible to clarithromycin. However, this combination should be further studied against clarithromycin resistant isolates.

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JARA Japan Antibiotics Research Association
The Society for Actinomycetes Japan

BRIEF COMMUNICATION

Comparison of two-drug combinations, amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin against *Mycobacteroides abscessus* subsp. *abscessus* using the in vitro time-kill assay

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Abstract

Nontuberculous mycobacteria include 198 mycobacterial species. Among these, *Mycobacteroides abscessus* is a rapidly growing mycobacteria that causes lung and skin infection. *M. abscessus* lung infections are difficult to treat due to the high levels of resistance to several classes of antibiotics. The current treatment is based on combining at least two or three antibiotics. However, treatment outcomes remain very poor. The objective was to compare the in vitro activity of amikacin, tigecycline, imipenem, and clarithromycin, alone and in two different three-drug combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) against seven *M. abscessus* subsp. *abscessus* clinical isolates using the time-kill assay. The two combinations showed greater activity than the antibiotics tested individually. Even though both combinations showed similar activity as well as no antagonistic activity, the combination including imipenem could not be an alternative treatment against *M. abscessus* subsp. *abscessus* lung infections caused by clarithromycin susceptible isolates. However, this combination could be considered against clarithromycin resistant isolates. Future studies are necessary to confirm this hypothesis.

Introduction

The nontuberculous mycobacteria (NTM) family includes 198 species, the majority of which do not cause disease in humans [1]. NTM are naturally found in the environment, from soil to water systems [2]. Recently, mycobacterial species have been divided into five new genera: *Mycobacterium, Mycolicibacter, Mycolicibacterium, Mycolicibacillus, and Mycobacteroides* [3]. Over the last years, infections caused by NTM have significantly increased [4]. Among these NTM, *Mycobacteroides abscessus* is a rapidly growing mycobacteria (RGM) that causes lung and skin infections which are difficult to treat due to its resistance to most classes of antibiotics [2, 4]. This RGM is classified into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* [2, 3, 5]. The identification to subspecies level is important since their resistance profiles to macrolides are different. Macrolides, and specifically clarithromycin are among the most active antibiotics against *M. abscessus*. The resistance to macrolides can be either constitutive (*rrl* gene) or inducible (*erm*41 gene). The most frequent is the inducible resistance, due to the presence of a functional *erm*41 gene that codes for a methylase. Accordingly, *M. abscessus* subsp. *massiliense*

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is usually susceptible to macrolides since the *erm*41 gene is truncated and not functional. *M. abscessus* subsp. *bolletii* is innately resistant to macrolides. Most of *M. abscessus* subsp. *abscessus* also exhibit inducible macrolide resistance, but some isolates remain susceptible due to a non-functional *erm*41 gene. Although there are geographical differences, the most commonly isolated is *M. abscessus* subsp. *abscessus* [4, 6].

M. abscessus lung infections are particularly common in patients with underlying respiratory conditions, such as cystic fibrosis and bronchiectasis [5]. These infections require treatment, but management is difficult due to high levels of natural and acquired resistance to frequently used antibiotics. Moreover, the presence of mucus and other secretions at the site of infection makes the entry of antibiotics difficult. Depending on the macrolide resistance profile, the current guidelines of the British Thoracic Society recommend the administration of different intravenous (IV) and oral antibiotics (e.g., IV amikacin, IV tigecycline, IV imipenem, and oral clarithromycin) for 1 month during the initial phase followed by oral and inhaled antibiotics (e.g., oral clofazimine, oral moxifloxacin and nebulized amikacin) for a minimum of 12 months during the continuation phase. The use of macrolides is recommended even in the presence of inducible resistance, but not when constitutive resistance associated to the *rrl* gene is found [6, 7]. On the other side and despite its IV administration, tigecycline has better activity than former drugs used in combination with amikacin, such as doxycycline, tetracycline and minocycline [8]. Nevertheless, side effects are common and treatment outcomes are very often poor [2]. Hence, it is of great importance to develop new antibiotics and antibiotic combinations.

The treatment regimens available for *M. abscessus* lung infections consist of the administration of different antibiotics in combination. Therefore, it is necessary to determine the efficacy of these combinations as well as of that of the antibiotics alone. The time-kill assay establishes the in vitro pharmacodynamics of antibiotics by detecting the rate at which an antibiotic concentration kills bacteria along time [9].The main goal was to compare the in vitro activity of four different antibiotics, amikacin, tigecycline, imipenem, and clarithromycin alone and in two different three-drug combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) against *M. abscessus* subsp *abscessus* clinical isolates using the in vitro time-kill assay.

Material and methods

Mycobacteroides abscessus subsp. abscessus clinical isolates

Seven clinical isolates of *M. abscessus* subsp. *abscessus* were selected from a strain collection of the Microbiology Department of the Hospital Clinic of Barcelona (Spain). They were previously identified at subspecies level using GenoType® NTM-DR (Hain Lifescience GmbH, Nehren, Germany). In addition, Sensititre™ AST RAPMYCO plates (Thermo Fisher Scientific, MA, USA) were used in accordance to manufacturers recommendations for antibiotic susceptibility testing, incubating the plates for 3 days, except for clarithromycin whose incubation was prolonged for up to 7 and 14 days to detect the presence of inducible resistance. The seven isolates selected had the following range of minimum inhibitory concentrations (MICs) to the antibiotics tested: 4–16 μg ml^{-1} for amikacin, 0.125–0.5 μg ml^{-1} for tigecycline, 8–64 μg ml^{-1} for imipenem, and 0.5–4 μg ml^{-1} for clarithromycin (Table 1). Stocks of each isolate were preserved at −80 °C in skim milk and were thawed for each assay.

Antibiotics

The four antibiotics tested were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amikacin and imipenem were dissolved in sterile distilled water. Clarithromycin was dissolved in dimethyl sulfoxide (DMSO) (0.002% final concentration) and sterile distilled water. All the antibiotics were sterilized by filtration and stored at −20 °C.

Tigecycline preparation

Tigecycline was prepared as described by Jitkova et al. [10] due to its poor stability. It was dissolved in DMSO (1 mg ml^{-1})

Table 1 Minimum inhibitory concentrations (MICs) of the *M. abscessus* clinical isolates studied

Isolate	MICs (μg ml^{-1})			
	AMK	TGC	IPM	CLR
M. abscessus 1	8	0.125	64	0.5
M. abscessus 2	8	0.5	16	4
M. abscessus 3	16	0.25	8	1
M. abscessus 4	16	0.25	64	0.5
M. abscessus 5	8	0.25	8	2
M. abscessus 6	8	0.25	32	0.5
M. abscessus 7	4	0.5	8	0.5
M. abscessus MIC_{90}	16	0.5	64	4

AMK amikacin, *TGC* tigecycline, *IPM* imipenem, *CLR* clarithromycin

and saline solution containing ascorbic acid (3 mg ml^{-1}) (Sigma-Aldrich) and pyruvate (60 mg ml^{-1}) (Sigma-Aldrich) and adjusted to pH 7.0. With this formulation, tigecycline remained stable for up to 7 days when protected from light [10].

Inoculum preparation

All of the isolates were grown in BD™ Columbia Agar with 5% Sheep Blood plates (Becton Dickinson, Sparks, MD). Then, they were subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) supplemented with 10% oleic acid-albumin-dextrose-catalase (Comercial Bellés, Tarragona, Spain) and 0.25% Tween 80 (Merck, Darmstadt, Germany) to avoid bacilli clump formation. Finally, the *M. abscessus* subsp *abscessus* cultures were homogenized by agitation and adjusted to the desired concentration using a nephelometer (CrystalSpec™; Becton Dickinson).

Minimum inhibitory concentrations

The MICs of each antibiotic were determined in 96-well plates (Smartech Biosciences, Barcelona, Spain). Briefly, 100 µl of Mueller Hinton broth were added to each well. Then, serial dilutions of the antibiotics ranging from 512 to 0.25 µg ml^{-1} were made. Finally, 100 µl of inoculum at a concentration of 0.5 McFarland (5×10^6 CFU ml^{-1}) were added. Positive control wells consisted of 100 µl of Mueller Hinton and 100 µl of inoculum (5×10^6 CFU ml^{-1}). Negative control wells were prepared by adding 200 µl of Mueller Hinton. Plates were incubated at 30 °C for 3 days and for up to 14 days in the case of clarithromycin. After incubation, visual reading was performed. The MIC was interpreted as the lowest antibiotic concentration preventing growth. All the experiments were performed in duplicate.

Time-kill assays

A previously described protocol was adapted for the present study [11]. Briefly, the time-kill assays were performed by dispensing 600 µl of the corresponding antibiotic concentration (amikacin, tigecycline, imipenem, or clarithromycin) and 500 µl of inoculum (final concentration of 5×10^5 CFU ml^{-1}) into tubes with 7.8 ml of Mueller Hinton broth. For the three-drug combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin), 200 µl of each of the three antibiotics were dispensed into the tubes. Amikacin and imipenem were tested at 2× MIC and tigecycline and clarithromycin at 4× MIC. Control growth tubes containing 500 µl of sterile distilled water instead of antibiotic were also included. All of the tubes were incubated at 37 °C in a 5% CO_2 atmosphere for 6 days. Time points were established at days 0, 1, 3, and 6. At the defined time points, a volume of 500 µl was removed from each liquid culture. The number of viable mycobacteria in each culture was determined by plating 10-fold serial dilutions on Middlebrook 7H11 medium (Becton Dickinson). The 7H11 agar plates were incubated at 37 °C in a 5% CO_2 atmosphere for 3 days, after which colony-forming units were counted. All the experiments were performed in duplicate.

Data analysis of the time-kill assays

The means of the $\log_{10}$ CFU ml^{-1} values were plotted against time for each isolate. The results were interpreted by the effect of the combinations compared with the most active antibiotic tested individually. Synergy was considered when the activity of the combinations was 2 $\log_{10}$ higher than the most active antibiotic alone. Antagonism was determined when the activity of the combinations was 2 $\log_{10}$ lower compared to the most active antibiotic alone. Finally, a difference of less than 2 $\log_{10}$ lower or higher was considered indifferent.

Statistical analysis

Significant differences between the two combinations (amikacin/tigecycline/imipenem or amikacin/tigecycline/clarithromycin) were analyzed using the Wilcoxon Mann–Whitney test. Results showing $P \leq 0.05$ were considered as statistically significant. Calculations were performed using STATA 13.0 software (Stata Corporation, College Station, TX, USA).

Results

Four different antibiotics, amikacin, tigecycline, imipenem, and clarithromycin, alone and in combination (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) were tested against clinical isolates of *M. abscessus* subsp. *abscessus* using the time-kill assay. The results observed during the 6-day period are summarized in Table 2.

In the present study at day 6, amikacin and tigecycline showed a mean 2.01 and 0.46 $\log_{10}$ CFU ml^{-1} decrease, respectively, compared with the initial inoculum (5.70 $\log_{10}$ CFU ml^{-1}). Imipenem and clarithromycin displayed a mean 1.48 and 1.27 $\log_{10}$ CFU ml^{-1} decrease, respectively. The combination of amikacin/tigecycline/imipenem showed a mean decrease of 2.58 $\log_{10}$ CFU ml^{-1}. Finally, the combination of amikacin/tigecycline/clarithromycin displayed a

Table 2 Mean colony-forming values (log_{10} CFU ml^{-1}) and reduction in growth compared with the initial inoculum (Δ) at the defined time points of the time-kill assay

Antibiotic or antibiotic combination	Day 0 (log_{10} CFU ml^{-1})	Day 1 (log_{10} CFU ml^{-1})	ΔDay 1	Day 3 (log_{10} CFU ml^{-1})	ΔDay 3	Day 6 (log_{10} CFU ml^{-1})	ΔDay 6
GC	6.85 ± 0.09	7.35 ± 0.42	0.5	8.18 ± 0.39	1.33	8.80 ± 0.65	1.95
AMK	6.85 ± 0.10	6.37 ± 0.38	−0.48	5.62 ± 0.49	−1.23	4.84 ± 0.92	−2.01
TGC	6.85 ± 0.11	6.73 ± 0.43	−0.12	6.50 ± 0.33	−0.35	6.39 ± 0.31	−0.46
IPM	6.85 ± 0.12	6.12 ± 0.64	−0.73	5.47 ± 0.94	−1.38	5.37 ± 0.35	−1.48
CLR	6.85 ± 0.13	6.58 ± 0.42	−0.27	6.02 ± 0.40	−0.83	5.58 ± 0.60	−1.27
AMK/TGC/IPM	6.85 ± 0.14	6.10 ± 0.75	−0.75	5.42 ± 0.60	−1.43	4.27 ± 0.67	−2.58
AMK/TGC/CLR	6.85 ± 0.15	6.20 ± 0.49	−0.65	5.46 ± 0.70	−1.39	3.83 ± 0.90	−3.02

CFU colony-forming unit, *GC* growth control, *AMK* amikacin, *TGC* tigecycline, *IPM* imipenem, *CLR* clarithromycin

mean decrease of 3.02 log_{10} CFU ml^{-1}. Although being less pronounced, these decreases were also observed at day 3.

No significant differences were observed among the two replicates of each isolate. The most active individual antibiotic was amikacin followed by imipenem and clarithromycin. Both combinations were indifferent (neither synergistic nor antagonistic) showing more activity than the antibiotics tested individually. Moreover, no significant differences ($P \geq 0.05$) were observed between the activities of the two three-drug combinations.

Discussion

In this study, the effects of amikacin, tigecycline, imipenem, and clarithromycin individually and in two three-drug combinations were investigated as these antibiotics are usually administered during the treatment of lung infections caused by *M. abscessus*. To our knowledge, there are few studies on the effect of these antibiotics against *M. abscessus* clinical isolates using the time-kill assay [12, 13]. This method is adequate for studying the activity of antibiotics and determining their pharmacodynamics [14]. Furthermore, the time-kill assay has been largely used in clinical microbiology laboratories and has demonstrated to be reliable and consistent in the study of different microorganisms, including NTM [11, 15–18]. The antibiotic susceptibility testing for *M. abscessus* is read at day 3 in microbiological practice. However, in this study the time points were established at days 3 and 6 in order to study the accumulated activity of the antibiotics alone and of the three-drug combinations. Regarding the antibiotics, it is well-known that tigecycline has poor stability. In this study, it was prepared as described by Jitkova et al. and remained stable for up to 7 days [10]. This novel formulation allows the testing of tigecycline, a drug frequently used in the treatment of *M. abscessus* lung infections given that it is more effective than doxycycline and other tetracyclines [8]. Concerning imipenem, it shows poor stability in formulations parenterally administered to patients. This poor stability has been attributed to changes in pH and in the concentrations of sodium bisulfite and L-cysteine [19]. Nonetheless, our in vitro results showed activity of all the antibiotics and antibiotic combinations at both days 3 and 6.

Currently, the majority of patients with lung infections by *M. abscessus* receive more than two antibiotics in combination during the course of treatment. However, there is no standard combination and most of the treatments are based on empirical experience. In addition, most studies only investigate the activity of antibiotics in two-drug combinations. Some of them include few of the antibiotics analyzed in the present study, but none includes three-drug combinations [12, 15, 16].

In this study we used isolates of *M. abscessus* subsp. *abscessus*, the most isolated subspecies in our area. From a strain collection we selected seven clinical isolates susceptible to macrolides, with the aim to compare the two three-drug combinations. The results show that both combinations had good activity and did not show statistically significant differences, although the reduction in log_{10} CFU ml^{-1} with the combination including imipenem was lower than of that including clarithromycin. For this reason and due to the IV administration of imipenem, the combination of amikacin/tigecycline/imipenem could not be recommended as an alternative in the treatment of *M. abscessus* subsp. *abscessus* lung infections caused by clarithromycin susceptible isolates. However, in the light of the results obtained, this combination could be considered against clarithromycin resistant isolates. Future studies are required to confirm this hypothesis. This is especially important, since at least 14 months of treatment are necessary and outcomes remain very poor, with success rates of only 30–50% [20].

Furthermore, systems of antibiotic administration that have already proven to be effective against NTM lung infections, such as inhaled liposomal antibiotics and other nanoparticle-based antibiotic delivery systems, should be further developed. These forms of administration are currently used for amikacin [21]. In addition, imipenem

physicochemical properties also allow its administration using these new delivery systems [22].

In conclusion, both three-drug combinations, amikacin/tigecycline/imipenem, and amikacin/tigecycline/clarithromycin, showed similar in vitro activity against *M. abscessus* subsp. *abscessus* clinical isolates as well as no antagonistic activity. The combination including imipenem is not a reliable alternative against *M. abscessus* subsp. *abscessus* lung infections caused by isolates susceptible to clarithromycin. However, this combination should be further studied against clarithromycin resistant isolates.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethical approval was received from the Ethical Committee of the *Hospital Clínic de Barcelona* (Barcelona, Spain) [HCB/2016/0344].

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Paper 3: *In vitro* biofilm formation in *Mycobacterium avium-intracellulare* complex

Authors: **Portell-Buj E**, López-Gavín A, González-Martín J, Tudó G.

Journal, volume(issue): pages, date of publication: Arch Bronconeumol, S0300-2896(20)30263-5, September 2020.

Impact Factor: 4.95-Q1. (Objective 4).

Introduction: The *Mycobacterium avium-intracellulare* complex (MAC) is composed of different species, being *M. avium* and *M. intracellulare* the most representative members. Respiratory infections produced by MAC have significantly increased. Moreover, MAC isolates have the potential to produce biofilm. These communities display different characteristics compared to planktonic forms, such as persistence and increased levels of antibiotic resistance. Little is known about the characteristics of MAC biofilms, and there is no current standardized method for the determination of biofilm formation.

Objectives: To standarize a metohd for the assessment of *in vitro* biofilm formation in MAC clinical isolates by using a protocol based on the adherence to 96-well plates and the crystal violet method.

Materials and methods: Seventeen *M. avium* and 21 *M. intracellulare* clinical isolates were studied by seeding in 96-well non-treated polystyrene plates. The optimal conditions for biofilm formation in *M. avium* were incubation at 42 ˚C for 4 weeks and at 37 ˚C for 4 weeks in *M. intracellulare*. After incubation, the OD was calculated by determining the A580 with a microplate spectrophotometer. The A580 readings were classified into three categories of biofilm formation:<0.3 nm: none/weak; 0.3-0.6 nm: moderate; >0.6 nm: strong.

Results: Slight non-significant differences between *M. avium* and *M. intracellulare* were observed, producing *M. avium* stronger biofilm than *M. intracellulare*. Thus, 82% of *M. avium*

and 62% of *M. intracellulare* formed moderate to strong biofilm. On the contrary, 18% of *M. avium* and 38% of *M. intracellulare* produced none/weak biofilm, respectively.

Conclusions: We determined that a significant number of MAC clinical isolates can produce biofilm. Moreover, the method described showed excellent reproducibility and could be easily applied to screen for MAC biofilm production. This is of great interest given the important role that biofilms play in MAC respiratory infections. Further understanding of MAC biofilm would result in improved patient management.

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Scientific Letter

In Vitro Biofilm Formation in *Mycobacterium avium-intracellulare* Complex

Formación in vitro *de biopelículas en el complejo* Mycobacterium avium-intracellulare

Dear Editor:

Non-tuberculous mycobacteria (NTM) are widely found in the environment and can cause respiratory infections.[1] The *Mycobacterium avium-intracellulare* complex (MAC) is composed of different species, being *M. avium* and *M. intracellulare* the most representative members, although in the last years *Mycobacterium chimaera* has become also relevant since several outbreaks of *M. chimaera* infections have been reported in patients who underwent cardiac surgery. The heater-cooler devices used in the surgical procedure contained *M. chimaera* biofilms in their water systems, leading to airborne infections in these patients.[2]

Over the past decades, respiratory infections produced by MAC have significantly increased, particularly among immunocompromised individuals and patients with lung disease.[1] Moreover, MAC isolates have the potential to produce biofilm.[3] That is, a microbial community attached to a surface and surrounded by a matrix, which provides protection from the environment, the immune system and antibiotics.[4] These communities display different characteristics compared to planktonic forms, such as persistence and increased levels of antibiotic resistance.[4–6] Little is known about the characteristics of MAC biofilms,[7] and there is no current standardized method for the determination of biofilm formation. Besides, biofilm formation makes MAC respiratory infections even more difficult to treat given that biofilms offer extra protection from antibiotics and other agents.[4,5] The antibiotic concentrations required to eradicate mycobacterial biofilms can be a thousand times higher than those needed to inhibit planktonic forms.[8,12] Due to the increased antibiotic resistance, these complicated MAC respiratory infections need higher doses of antibiotics in order to improve patient outcome.[9,10,13,14] Hence, common antibiotic susceptibility testing may not be useful for those chronic MAC infections where biofilms are involved.[8,12] However, there is no unified protocol to assess *in vitro* biofilm formation in MAC. Therefore, a standardized protocol is needed in order to study the activity of antibiotics and other agents against MAC biofilm. Ideally, standard methods should be easy to perform, affordable, quick to execute and should not require highly specialized equipment.[11,15]

We report *in vitro* biofilm formation in MAC clinical isolates by using a protocol based on the adherence to 96-well plates and the crystal violet method. First, we performed the tube method described by Christensen et al.[8,12] using 6 *M. avium* and 6 *M. intracellulare* clinical isolates obtained from the Microbiology Department of the Hospital Clínic of Barcelona. Briefly, MAC cultures were inoculated in tubes with Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD, USA) at a concentration of 5×10^6 CFU/mL. The tubes were incubated at 37 °C for 1 week. After incubation, they were decanted, rinsed with 1×PBS (Gibco, Life Technologies, Belgium) and dried at room temperature. Finally, the tubes were stained with 1% of crystal violet (Comercial Bellés) for 10 min. Biofilm formation was considered positive when a visible line appeared. All the 12 MAC isolates showed biofilm formation.

After that, we studied the optimal conditions for the *in vitro* biofilm formation, based on the adherence to 96-well Plates[9,13] and incorporating some modifications previously described by Kumar et al.[10] The isolates were grown in tubes containing Middlebrook 7H9 and incubated at 37 °C for 1 week. Afterwards, the cultures were centrifuged and the supernatant was discarded. Then, fresh Middlebrook 7H9 was added to each culture. Finally, MAC cultures were homogenized and ajusted to 1×10^7 CFU/mL (CrystalSpec™, Becton Dickinson).

Biofilm formation was determined by seeding 200 μL (1×10^7 CFU/mL) of the corresponding MAC culture in 96-well non-treated polystyrene plates (Thermo Fisher Scientific, MA, USA). Negative controls containing Middlebrook 7H9 were also included. The following temperatures and times of incubation were tested, 37 °C and 42 °C for 3 and 4 weeks each. To minimize evaporation, the plates were covered with a lid. All the experiments were performed in duplicate.

For the biofilm determination, the supernatant of the plates was discarded and each well was rinsed with 1xPBS. The plates were dried at 60 °C for 1 h and the wells were stained with 1% crystal violet and incubated at room temperature for 10 min. Each well was rinsed with 1xPBS and dried at 60 °C for 2 h. Finally, 33% acetic acid (Vidra Foc, Barcelona, Spain) was added to solubilize the biofilm. The OD was calculated by determining the A_{580} with a microplate spectrophotometer (Bio Tek, VT, USA). The wells with only Middlebrook 7H9 were used as blanks and their mean A_{580} values were subtracted from wells containing biofilm. The A_{580} readings were classified into three categories of biofilm formation: <0.3 nm: none/weak; 0.3–0.6 nm: moderate; >0.6 nm: strong. The optimal conditions for biofilm formation in *M. avium* were incubation at 42 °C for 4 weeks and at 37 °C for 4 weeks in *M. intracellulare*. Finally, this protocol, with the optimal incubation conditions, was performed in a total of 17 *M. avium* isolates and 21 *M. intracellulare* clinical isolates.

Slight non-significant differences between *M. avium* and *M. intracellulare* were observed, producing *M. avium* stronger biofilm than *M. intracellulare*. Thus, 82% of *M. avium* and 62% of *M. intracellulare* formed moderate to strong biofilm. On the contrary, 18% of *M. avium* and 38% of *M. intracellulare* produced none/weak biofilm, respectively (Table 1).

The results of this study corroborate that an important proportion of MAC clinical isolates can produce biofilm.[10,11,14,15] Hence,

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Table 1
Distribution of MAC clinical isolates according to the degree of *in vitro* biofilm formation.

MAC species	Degree of biofilm formation	A_{580} (nm)	Number of isolates
M. avium	None/weak	<0.3	3
	Moderate	0.3–0.6	8
	Strong	>0.6	6
M. intracellulare	None/weak	<0.3	8
	Moderate	0.3–0.6	9
	Strong	>0.6	4

the assessment of biofilm production in MAC isolates would contribute to a better management of patients with MAC respiratory infections. It is worthy of note that *M. avium* appears to produce stronger biofilm in comparison with *M. intracellulare*. Although further data is needed to confirm this fact, it highlights the importance of identifying MAC isolates to the species level. Moreover, the protocol described probed excellent reproducibility, robustness and was easy to perform. In addition, it can be useful to study the effect of antibiotics, alone and in combination, against MAC biofilm.

Summarizing, we determined that a significant number of MAC clinical isolates can produce biofilm. Moreover, the method described showed excellent reproducibility and could be easily applied to screen for MAC biofilm production. This is of great interest given the important role that biofilms play in MAC respiratory infections. Further understanding of MAC biofilm would result in improved patient management.

Ethical statement

Ethical approval was received from the Ethical Committee of the Hospital Clínic de Barcelona (Barcelona, Spain) [HCB/2016/0344].

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Conflict of interest

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Manuscript 1: **Portell-Buj E**, Bonet-Rossinyol Q, López-Gavín A, Tudó G, González-Martín J. Activity of amikacin, ethambutol and clarithromycin against *Mycobacterium avium* and *Mycobacterium intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model. (Objective 2).

Activity of amikacin, ethambutol and clarithromycin against *Mycobacterium avium* and *Mycobacterium intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model.

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Running title: Time-kill assays and THP-1 macrophage infection model in MAC clinical isolates

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Abstract

Background: Non-tuberculous mycobacteria (NTM) are widely distributed in the environment. Among these NTM, the *Mycobacterium avium-intracellulare* complex (MAC) is one of the most representative members, being the MAC lung disease of increasing clinical relevance. Even though *M. avium* and *M. intracellulare* are usually included in the same complex, differences in the clinical manifestation as well as in the antibiotic susceptibility pattern have been observed. The high levels of antibiotic resistance and the poor treatment outcomes have led to the need of new therapy options. The objective was to study the activity of amikacin, ethambutol and clarithromycin, alone and in combination, against 6 *M. avium* and 8 *M. intracellulare* clinical isolates using the time-kill assay as well as against 4 *M. avium* and 4 *M. intracellulare* isolates with the THP-1 macrophage infection model.

Results: In both methods, amikacin, ethambutol, clarithromycin and particularly the combination of amikacin/ethambutol/clarithromycin showed good activity against MAC clinical isolates as well as no antagonistic activity. Moreover, amikacin, ethambutol and the three-drug combination showed significantly higher activity against *M. intracellulare* than against *M. avium* with the time-kill assay.

Conclusions: The antibiotics tested, alone and in combination, showed good activity, and the finding that with the time-kill assay, amikacin, ethambutol and the three-drug combination showed significantly higher activity against *M. intracellulare* highlights the importance of performing specific identification for each MAC species. Finally, the time-kill assay and the THP-1 macrophage infection model are good methods to test new treatment regimens and establish their pharmacodynamics. Further studies on the antibiotic interactions are necessary to improve the MAC lung disease treatment.

Keywords: Non-tuberculous mycobacteria, *Mycobacterium avium-intracellulare* complex, Time-kill assay, THP-1 macrophages, Antibiotics, Combinations

Background

Non-tuberculous mycobacteria (NTM) are a large group of mycobacteria found in the environment, including water and soil, which have the potential to colonize the respiratory tract when inhaled. Moreover, the *Mycobacterium avium-intracellulare* complex (MAC) consists of different slowly-growing NTM species, being *Mycobacterium avium* and *Mycobacterium intracellulare* the most representative members of the complex. In the last years, the number of cases of MAC lung disease has significantly increased, mainly in immunosuppressed patients and individuals with pre-existing pulmonary diseases, such as cystic fibrosis and bronchiectasis [1, 2].

Although in most guidelines *M. avium* and *M. intracellulare* are commonly included in the same complex, significant differences in the clinical presentation as well as in the antibiotic susceptibility pattern have been observed among them. In general, *M. avium* shows more severe clinical manifestations and higher levels of antibiotic resistance in comparison with *M. intracellulare*. However, despite the existing differences in the antibiotic susceptibility patterns, they have barely been determined when antibiotic combinations are administrated [3, 4]. Consequently, not considering the differences between these two mycobacterial species may affect the patient's outcome. On the other side, the patient's location should also be regarded since geographical variations in the antibiotic susceptibility patterns of the MAC isolates have been demonstrated [3, 5].

Carrying MAC is not always indicative of needing treatment and differentiating between colonization and infection can be very difficult. Additionally, the current treatments are of long duration, have many side effects and the outcomes are not optimal with many patients having reinfection or relapse. In patients requiring treatment, combination regimens including a macrolide (i.e. clarithromycin) are the most advised in guidelines due to the high levels of natural resistance among MAC isolates [6]. The other antibiotics included in therapy regimens are clofazimine, rifamycins (e.g. rifampicin and rifabutin), ethambutol, fluoroquinolones (e.g. moxifloxacin and levofloxacin), linezolid and aminoglycosides (e.g. amikacin) [1, 2]. Thus, there is a real need to develop and investigate novel treatment regimens against MAC lung disease which include new and old antibiotics and show better outcomes, resulting in improved patient management [7].

Considering that the antibiotics used to treat MAC lung disease are administered in combination, it is necessary to determine the efficacy of these combinations as well as of these antibiotics alone. Additionally, there is little knowledge of their pharmacodynamics and interactions. The time-kill assay allows establishing the pharmacodynamics of

different antibiotics by observing the rate at which a certain antibiotic concentration kills bacteria over time [8]. Furthermore, macrophage infection models are used to determine the activity of antibiotics against intracellular mycobacteria [9]. The main objective was to investigate the activity of amikacin, ethambutol and clarithromycin, alone and in combination against *M. avium* and *M. intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model and, to compare the antibiotic activities between both MAC species.

Results

Time-kill assay results

Three different antibiotics, amikacin, ethambutol and clarithromycin, alone and in combination, were tested against clinical isolates of *M. avium* and *M. intracellulare* using the time-kill assay. The results observed during the 14 days period are summarized in Table 2. In addition, Fig. 1 shows an example of the time-kill assays obtained in both *M. avium* and *M. intracellulare* isolates.

When tested against *M. avium*, amikacin and ethambutol showed a mean 3.85 and 3.41 $\log_{10}$ CFU/mL decrease, respectively, compared with the initial inoculum (5.70 $\log_{10}$ CFU/mL). Clarithromycin showed a mean decrease of 2.92 $\log_{10}$ CFU/mL. The combination of amikacin/ethambutol/clarithromycin displayed a mean decrease of 4.67 $\log_{10}$ CFU/mL.

Regarding *M. intracellulare*, amikacin and ethambutol caused a mean 4.34 and 4.59 $\log_{10}$ CFU/mL decrease, respectively, compared with the initial inoculum (5.70 $\log_{10}$ CFU/mL). Clarithromycin showed a mean decrease of 2.94 $\log_{10}$ CFU/mL. The combination of amikacin/ethambutol/clarithromycin displayed a mean decrease of 4.72 $\log_{10}$ CFU/mL.

No significant differences were observed among the replicates or between the isolates of the same species. However, amikacin, ethambutol and the three-drug combination showed significantly better activity ($P \leq 0.05$) on the 14th day, against *M. intracellulare* than against *M. avium*. In addition, the combination of amikacin/ethambutol/clarithromycin was more effective than the antibiotics tested individually although displaying an indifferent activity compared to the most active antibiotic tested alone.

THP-1 macrophage infection results

The antibiotics and the three-drug combination tested where also studied in a THP-1 macrophage infection model. The results observed over the 4 days period are shown in Table 3.

When tested against *M. avium*, clarithromycin and amikacin showed a mean 1.76 and 1.46 $\log_{10}$ CFU/mL decrease, respectively, compared with the initial inoculum (5.30 $\log_{10}$ CFU/mL). Ethambutol showed a mean decrease of 1.06 $\log_{10}$ CFU/mL. The combination of amikacin/ethambutol/clarithromycin displayed a mean decrease of 2.08 $\log_{10}$ CFU/mL.

Regarding *M. intracellulare*, clarithromycin and amikacin caused a mean 1.78 and 1.63 $\log_{10}$ CFU/mL decrease, respectively, compared with the initial inoculum (5.30 $\log_{10}$ CFU/mL). Ethambutol showed a mean decrease of 1.40 $\log_{10}$ CFU/mL. The combination of amikacin/ethambutol/clarithromycin displayed a mean decrease of 2.00 $\log_{10}$ CFU/mL.

No significant differences were observed among the replicates or between the isolates of the same species. The combination of amikacin/ethambutol/clarithromycin was more effective than the antibiotics tested individually although displaying an indifferent activity compared to the most active antibiotic tested alone.

Discussion

In this study, the effects of amikacin, ethambutol and clarithromycin, individually and in a three-drug combination were investigated as these antibiotics are usually included in the regimens for the treatment of MAC lung disease. To the best of our knowledge, there are few studies on the effect of these antibiotics against *M. avium* and *M. intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model. The time-kill assay has been broadly used in the clinical microbiology field and has proved to be robust [8, 10]. This method is an excellent approach to study the activity of antibiotics and establish their pharmacodynamics [11]. Furthermore, the THP-1 macrophage infection model allows establishing the effect of antibiotics against mycobacteria within macrophages [12]. In addition, our group had prior experience using the time-kill assay and the THP-1 macrophage infection model in *Mycobacterium tuberculosis* clinical isolates; hence in this study we slightly adapted the protocol to *M. avium* and *M. intracellulare* [12, 13].

As shown in Table 2 and even though *M. avium* and *M. intracellulare* are mostly included in the same complex, amikacin, ethambutol and the combination of amikacin/ethambutol/clarithromycin, showed significantly better activity against *M.*

intracellulare than against *M. avium* in the time-kill assay. Therefore, we hypothesized that the current antibiotics used to treat MAC lung disease could display different activity depending on the MAC species, hence being important to specifically identify the clinical isolates and test them for each antibiotic. This could improve patients outcome due to these significant differences in the response to antibiotics, which have been particularly observed between these two mycobacterial species and being generally *M. avium* more resistant to antibiotics [3-5].

Using the THP-1 macrophage infection model we also assessed the intracellular activity of the antibiotics over a 4 days period. No significant differences between *M. avium* and *M. intracellulare* clinical isolates were detected, in contrast with the time-kill assay. However, our *ex vivo* model had duration of 4 days while the time-kill assay is incubated until day 14. In our opinion, a time extension of the *ex vivo* model would show differences between these two MAC species, as observed in the time-kill assay results. Nonetheless, the viability of the infected macrophages could be affected for incubations longer than 4 days, being not recommended.

We aimed to study the *in vitro* activity of antibiotics in a three-drug combination given that many MAC lung disease patients receive a three-drug combination regimen. However, most studies have tested these antibiotics in a two-drug combination. Ferro et al. employed the time-kill assay to describe the synergy of the combination of clofazimine with amikacin or clarithromycin in *M. avium* isolates [14]. In a recent *in vitro* study, Ruth et al. reported that a bedaquiline/clofazimine combination increased the activity to the therapy against MAC [15]. On the other side, it has been described that ethambutol improves cell wall permeability in *M. avium* isolates, thus allowing better entry of rifampicin and perhaps of other antibiotics as well [16]. Consistent with this, van Ingen et al. reported that the combination of rifampicin/ethambutol presented synergistic activity against *M. avium* and *M. intracellulare* [17]. The above-mentioned studies include different antibiotic combinations that show a promising utility against *M. avium* and *M. intracellulare*. Furthermore, the combination tested in the present study includes antibiotics usually administrated to patients and the results obtained with both methods prove their applicability in the treatment of MAC lung disease.

Additionally, the lengthy current MAC treatment should be improved, since at least 18-24 months of treatment are required, and the rate of long-term culture conversion is 70 % [15]. Moreover, the methods for evaluating treatment regimens should include the study of antibiotic combinations, using *in vitro* methods such as the time-kill assay and

ex vivo models of cell infection since they allow establishing synergism and antagonism between antibiotics [9, 12]. In addition, it is important to explore new systems of antibiotic delivery, such as liposomes and nanoparticles. For instance, Rose et al. reported that in *in vitro* models, liposomal amikacin for inhalation was more successful than free amikacin in eradicating *M. avium* [18]. These methods of antibiotic administration reduce side effects by allowing the sustained delivery of high concentrations directly to the site of infection.

Conclusions

In summary, amikacin, ethambutol, clarithromycin and particularly the combination of amikacin/ethambutol/clarithromycin showed good activity against *M. avium* and *M. intracellulare* as well as no antagonistic activity using the time-kill assay and the THP-1 macrophage infection model. Moreover, amikacin, ethambutol and the three-drug combination showed significantly higher activity against *M. intracellulare* than against *M. avium* with the time-kill assay, highlighting the importance of performing specific identification for each MAC species. Finally, the time-kill assay and the THP-1 macrophage infection model are good methods to test new treatment regimens against MAC lung disease.

Methods

Mycobacterium avium and *Mycobacterium intracellulare* clinical isolates

Six clinical isolates of *M. avium* and 8 clinical isolates of *M. intracellulare* were studied. They were selected based on their susceptibility to the antibiotics tested, with minimum inhibitory concentrations (MICs) ranging from 8 μg/mL to 16 μg/mL for amikacin, from 2 μg/mL to 8 μg/mL for ethambutol and from 1 μg/mL to 8 μg/mL for clarithromycin (Table 1). All of the isolates were obtained from the Laboratory of Microbiology of the Hospital Clínic of Barcelona (Barcelona, Spain). Stocks of each isolate were preserved at -80 ºC in skim milk and were thawed for each assay.

Antibiotics

The antibiotics tested, amikacin, ethambutol and clarithromycin, were provided by Sigma-Aldrich (St. Louis, MO, USA). Amikacin and ethambutol were dissolved in sterile distilled water. Clarithromycin was dissolved in dimethyl sulfoxide (DMSO) (0.002%

final concentration). Afterwards, the antibiotics were sterilized by filtration and stored at -20 °C in aliquots until use.

Inoculum preparation

All of the isolates were subcultured in Middlebrook 7H9 liquid medium (Becton Dickinson) supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) (Comercial Bellés, Tarragona, Spain) and 0.25 % Tween 80 (Merck, Darmstadt, Germany). The mycobacterial cultures were homogenised by agitation and ajusted to 0.5 McFarland (1.5 x 10^8 CFU/mL) using a nephelometer (CrystalSpec™; Becton Dickinson) [13].

Minimum inhibitory concentrations

The MICs of each antibiotic were determined by microdilution in 96-well microplates (Smartech Biosciences, Barcelona, Spain) according to CLSI guidelines [19]. Briefly, 100 µL of Middlebrook 7H9 liquid media were added to each well. Then, serial dilutions of the antibiotics ranging from 512 µg/mL to 0.25 µg/mL were made. Finally, 100 µL of inoculum at a concentration of 1 x 10^6 CFU/mL were added. Positive control wells consisted of 100 µL of Middlebrook 7H9 and 100 µL of inoculum (final concentration of 5 x 10^5 CFU/mL). Negative control wells were prepared by adding 200 µL of Middlebrook 7H9. The microplates were incubated at 37 °C for 7 days. After incubation, visual reading was performed. The MIC was interpreted as the lowest antibiotic concentration preventing mycobacterial growth. All the experiments were performed in duplicate for each isolate.

Time-kill assays

In the present study, the protocol previously described by López-Gavín et al. was adapted [13]. Briefly, the time-kill assays were performed by dispensing in 7.8 mL tubes of Middlebrook 7H9 600 µL of the corresponding antibiotic (amikacin, ethambutol or clarithromycin) and 500 µL of inoculum (final concentration of 5 x 10^5 CFU/mL). For the three-drug combination (amikacin/ethambutol/clarithromycin), tubes contained 200 µL of each antibiotic. All of the antibiotics were tested at 2 x MIC. Control growth tubes containing 500 µL of sterile distilled water instead of antibiotic were also included. All of the tubes were incubated at 37 °C in a 5 % CO_2 atmosphere for 14 days. Time points were established at days 0, 3, 7, 10 and 14. At the defined time points, a volume of 500

µL was removed from each liquid culture. Then, the number of viable mycobacteria in each culture was determined by plating 10-fold serial dilutions on Middlebrook 7H11 solid medium (Becton Dickinson). The 7H11 agar plates were incubated at 37 °C in a 5 % CO_2 atmosphere for 14 days, after which CFU were determined. All the experiments were performed in duplicate for each isolate.

Time-kill assays data analysis

The means of the $\log_{10}$ CFU/mL values were plotted against time for each isolate. Due to the dilutions used in the Middlebrook 7H11 solid medium subcultures, the limit of detection was 100 CFU/mL. Therefore, when the CFU/mL counts were 0 we conveyed to express them as 1 $\log_{10}$ CFU/mL. The results were interpreted by the effect of the combination compared to the most active antibiotic tested individually. Synergism was determined when the activity of the three-drug combination was 2 $\log_{10}$ higher than the most active individual antibiotic alone. Antagonism when the activity of the combination was 2 $\log_{10}$ lower compared to the most active individual antibiotic alone. A difference of less than 2 $\log_{10}$ lower or higher was considered indifferent activity. Graph Pad Prism (La Jolla, CA, USA) was used for graphical outputs.

THP-1 cells culture

A protocol previously described was adapted [12]. Human THP-1 monocytic cells were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10 % fetal bovine serum (FBS) (Lonza) and 1 % penicillin/streptomycin (Sigma Aldrich). Incubation was in cell culture flasks at 37 °C in 5 % CO_2. When exponential growth was achieved, THP-1 monocytic cells (2 x 10^5 cells/mL) were seeded in 24-well plates. Then, 100 nM of phorbol-12-myristate-13-acetate (PMA) was added to differentiate THP-1 monocytic cells into THP-1 macrophages. Incubation was at 37 °C in 5 % CO_2 for 48 h. Changes in morphology were checked by viewing the 24-well plates under the microscope.

THP-1 macrophage infection

After THP-1 macrophage differentiation, they were washed once with antibiotic-free RPMI-1640. After 2 h of incubation, the number of viable THP-1 macrophages was determined using the trypan blue exclusion test. The inoculum of 4 *M. avium* and 4 *M. intracellulare* clinical isolates (2 x 10^5 CFU/mL) was added into the THP-1 macrophage

culture at a multiplicity of infection (MOI) of 1:1. After 3 h of incubation, THP-1 macrophages were then washed three times with antibiotic-free RPMI-1640. Finally, they were incubated at 37 °C in 5 % CO_2 for 72 h to allow THP-1 macrophages phagocyte mycobacteria.

THP-1 macrophage antibiotic treatment

After THP-1 macrophage infection, they were washed once with antibiotic-free RPMI-1640. Then, the corresponding antibiotics (amikacin, ethambutol, clarithromycin and the combination of amikacin/ethambutol/clarithromycin) were added at 4 x MIC, according to the maximum concentrations detected in human serum. This process was repeated daily over a 4-days period. As negative control wells, antibiotic-free uninfected THP-1 macrophages were also included. Positive control wells consisted of antibiotic-free infected THP-1 macrophages. At day 0, lysis of the positive control wells was performed by adding 0.5 % NP40 detergent solution (Roche, Basel, Switzerland). After washing twice with phosphate-buffered saline (PBS) (Lonza), the number of viable mycobacteria was determined by plating 10-fold serial dilutions of the lysates on Middlebrook 7H11 solid medium (Becton Dickinson). The 7H11 agar plates were incubated at 37 °C in 5 % CO_2 for 14 days, after which colony forming units (CFU) were determined. At day 4, all of the THP-1 macrophages were lysed and the respective lysates were cultured as previously described. All the experiments were performed in duplicate for each isolate.

THP-1 macrophage infection model data analysis

The activity of the antibiotics alone and of the three-drug combination was defined as the reduction in growth ($\log_{10}$ CFU/mL) compared with the initial inoculum (Δ) after the 4-days period. The results were interpreted by the effect of the three-drug combination compared with the most active antibiotic tested alone. Then, interpreted as synergy, antagonism or indifference.

Statistical analysis

Significant differences between *M. avium* and *M. intracellulare* clinical isolates were determined using the Wilcoxon Mann-Whitney test, and results showing $P \leq 0.05$ were considered as statistically significant. Calculations were performed using Graph Pad Prism.

Abbreviations

NTM: Non-tuberculous mycobacteria; MAC: *Mycobacterium avium-intracellulare* complex; *M. avium*: *Mycobacterium avium*; *M. intracellulare*: *Mycobacterium intracellulare;* MIC: Minimum inhibitory concentrations; DMSO: Dimethyl sulfoxide; OADC: Oleic acid-albumin-dextrose-catalase; FBS: Fetal bovine serum; PMA: Phorbol-12-myristate-13-acetate; MOI: Multiplicity of infection; PBS: Phosphate-buffered saline; CFU: Colony forming units; AMK: Amikacin; EMB: Ethambutol; CLR: Clarithromycin GC: Growth control.

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Authors' contributions

Conception and design of the study: EPB, ALG and JGM. Performed the experiments: EPB and QBR. Analysis and interpretation of data: EPB, QBR, GT, JGM. Manuscript drafting: EPB. Revised the manuscript: GT and JGM. Study supervision: GT and JGM. All authors read, revised and approved the final draft.

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Availability of data and materials

The dataset(s) supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

The study received ethical approval from the Ethical Committee of the *Hospital Clínic de Barcelona* (Barcelona, Spain) [HCB/2016/0344].

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no completing interests.

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Table 1 Minimum inhibitory concentrations (MICs) of the studied *M. avium* and *M. intracellulare* clinical isolates

Isolate	MICs (μg/mL)		
	AMK	EMB	CLR
M. avium 1	8	8	8
M. avium 2	16	8	8
M. avium 3	16	8	8
M. avium 4	8	4	8
M. avium 5	16	8	8
M. avium 6	16	8	1
M. avium MIC_{90}	16	8	8
M. intracellulare 1	8	8	2
M. intracellulare 2	8	4	2
M. intracellulare 3	16	2	4
M. intracellulare 4	8	8	2
M. intracellulare 5	8	8	4
M. intracellulare 6	8	4	2
M. intracellulare 7	8	4	2
M. intracellulare 8	16	2	4
M. intracellulare MIC_{90}	16	8	4

AMK: Amikacin; EMB: Ethambutol; CLR: Clarithromycin

Table 2 Mean colony forming values (log_{10} CFU/mL) and reduction in growth compared with the initial inoculum (Δ) at the defined time points of the time-kill assay

Antibiotic or antibiotic combination	Day 0 (log_{10} CFU/mL)	Day 3 (log_{10} CFU/mL)	ΔDay 3	Day 7 (log_{10} CFU/mL)	ΔDay 7	Day 10 (log_{10} CFU/mL)	ΔDay 10	Day 14 (log_{10} CFU/ mL)	ΔDay 14
M. avium									
GC	6.31 ± 0.09	7.38 ± 0.37	1.07	7.52 ± 0.44	1.21	8.07 ± 0.19	1.76	8.24 ± 0.34	1.93
AMK	6.31 ± 0.10	4.83 ± 1.01	-1.48	3.90 ± 0.50	-2.41	3.63 ± 0.31	-2.68	2.46 ± 0.37	-3.85
EMB	6.31 ± 0.11	4.65 ± 0.64	-1.66	3.77 ± 0.90	-2.54	2.98 ± 0.94	-3.33	2.90 ± 0.82	-3.41
CLR	6.31 ± 0.12	5.99 ± 0.45	-0.32	5.08 ± 0.96	-1.23	4.48 ± 0.97	-1.83	3.39 ± 1.04	-2.92
AMK/EMB/CLR	6.31 ± 0.13	3.79 ± 0.36	-2.52	2.41 ± 0.92	-3.9	1.85 ± 1.09	-4.46	1.64 ± 1.10	-4.67
M. intracellulare									
GC	5.72 ± 0.09	6.48 ± 0.37	0.76	7.07 ± 0.46	1.35	7.39 ± 0.54	1.67	7.86 ± 0.27	2.14
AMK	5.72 ± 0.10	2.52 ± 1.01	-3.2*	1.79 ± 1.04	-3.93*	1.5 ± 1	-4.22*	1.38 ± 1.04	-4.34*
EMB	5.72 ± 0.11	2.74 ± 0.52	-2.98*	2.14 ± 0.68	-3.58*	1.62 ± 1.06	-4.1	1.13 ± 0.74	-4.59*
CLR	5.72 ± 0.12	4.66 ± 0.18	-1.06*	3.94 ± 0.60	-1.78*	3.61 ± 0.66	-2.11	2.78 ± 0.54	-2.94
AMK/EMB/CLR	5.72 ± 0.13	2.85 ± 0.48	-2.87	1.77 ± 1.15	-3.95	1.06 ± 0.59	-4.66*	1 ± 0	-4.72*

GC: Growth control; AMK: Amikacin; EMB: Ethambutol; CLR: Clarithromycin. *Significant differences between *M. avium* and *M. intracellulare* clinical isolates ($P \leq 0.05$)

Table 3. Mean colony forming values (log_{10} CFU/mL) and reduction in growth compared with the initial inoculum (Δ) at day 4 of the THP1-macrophage model.

Antibiotic or antibiotic combination	Day 0 (log_{10} CFU/mL)	Day 4 (log_{10} CFU/mL)	ΔDay 4
M. avium			
GC	5.26 ± 0.3	5.85 ± 0.6	0.59
AMK	5.26 ± 0.4	4.39 ± 0.7	-1.46
EMB	5.26 ± 0.5	4.79 ± 0.3	-1.06
CLR	5.26 ± 0.6	4.09 ± 0.4	-1.76
AMK/EMB/CLR	5.26 ± 0.7	3.77 ± 0.2	-2.08
M. intracellulare			
GC	4.70 ± 0.3	5.35 ± 0.5	0.65
AMK	4.70 ± 0.4	3.72 ± 0.2	-1.63
EMB	4.70 ± 0.5	3.95 ± 0.4	-1.40
CLR	4.70 ± 0.6	3.57 ± 0.2	-1.78
AMK/EMB/CLR	4.70 ± 0.7	3.35 ± 0.3	-2.00

GC: Growth control; AMK: Amikacin; EMB: Ethambutol; CLR: Clarithromycin

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Activity of antibiotics and potential antibiofilm agents against *Mycobacterium avium-intracellulare* complex biofilm.

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Running title: Antibiotics and potential antibiofilm agents against MAC biofilm

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ABSTRACT

Nontuberculous mycobacteria (NTM) generally do not cause disease in humans. Nevertheless, NTM can cause lung infections in immunosuppressed patients and those with underlying pulmonary diseases. Furthermore, the *Mycobacterium avium-intracellulare* complex (MAC) is frequently involved in NTM-pulmonary disease (NTM-PD). The current MAC-PD treatment is based on the administration of multiple antibiotics for long periods of time. Nonetheless, treatment outcomes remain very poor and reinfection and relapse are common. Moreover, MAC has the ability to form biofilm. The antibiotic concentrations needed to eradicate biofilm infections are much higher than those required in non-biofilm forming infections. Given the poor MAC-PD treatment outcomes it is essential to develop novel treatments that not only target the planktonic forms but also MAC biofilm. The main goal was to study the *in vitro* activity of different antibiotics and potential antibiofilm agents, alone and in combination, against MAC biofilm. A total of 4 antibiotics and 6 potential antibiofilm agents were tested in five *M. avium* and 6 *M. intracellulare* clinical isolates. When tested against biofilm forming forms, the combinations of CLR/AMK and CLR/EMB were synergistic. In addition, when a potential antibiofilm was included in the antibiotic combinations the MBECs obtained were lower. Finally, the administration of potential antibiofilm agents combined to antibiotics, could be advantageous given their activity against the biofilm matrix and hence increase antibiotic activity.

Keywords: non-tuberculous mycobacteria, biofilm, antibiotics, potential antibiofilm agents, minimum biofilm inhibitory concentration.

Introduction

Nontuberculous mycobacteria (NTM) include over 180 mycobacterial species other than *Mycobacterium tuberculosis* and *Mycobacterium leprae*. They are broadly found in the environment (*e.g.* soil and water systems) and are generally non-pathogenic to humans.[1] However, NTM can originate a diverse range of infections, from lung to skin infections, in susceptible individuals such as immunosuppressed patients and those with pre-existing pulmonary diseases (*e.g.* bronchiectasis, cystic fibrosis and chronic obstructive pulmonary disease -COPD-).The *Mycobacterium avium-intracellulare* complex (MAC) is one of the most clinically significant NTM, being frequently involved in NTM-pulmonary disease (NTM-PD).[2] There is currently no standardized treatment against MAC-pulmonary disease (MAC-PD). However, the British Thoracic Society (BTS) guidelines recommend the administration of multiple antibiotics (*i.e.* rifampicin, ethambutol, azithromycin and clarithromycin) for a minimum of twelve months.[3] Nonetheless, treatment outcomes remain very poor and reinfection and relapse after treatment are remarkably common. Currently, patients suffering from MAC-PD show 8.3-48% of recurrence.[4]

MAC as well as other NTM and *Mycobacterium tuberculosis* have the ability to form biofilms.[5] At the moment, there is little knowledge about the proportion of isolates that can form biofilm and about its association to virulence. Nevertheless, it has been suggested that non biofilm forming isolates have less ability to colonize the human mucosa and tissues.[6] Biofilms are defined as a community of microorganisms embedded in a surface and surrounded by a self-made matrix.[7] Biofilms offer protection from the environment, the host immune response and antibiotics, among others. Thus, microorganisms thriving within biofilms display differential traits compared to their planktonic counterparts, such as increased antibiotic tolerance and persistence.[8,9] The antibiotic concentrations needed to eradicate biofilm infections can be several thousand times higher than those required in non-biofilm forming infections.[10] As a result, biofilms are a key virulence factor in chronic infections, making MAC-PD treatment even more difficult.[6,11] In microbiology diagnostic laboratories, however, antibiotic susceptibility testing (AST) is routinely performed with planktonic forms. Consequently, the MICs determined with common methods cannot predict the antibiotic concentrations needed to eradicate biofilm infections.[10]

Furthermore, given the poor MAC-PD treatment outcome it is essential to develop novel treatments that not only target the planktonic forms but also MAC biofilm. Worryingly, at the moment there are limited clinically available options. However, few studies have used novel approaches against MAC biofilm by targeting the biofilm matrix.[11] For instance, agents such as Tween 80 and N-acetyl-L-Cysteine (NAC) have proved to be effective in disrupting the biofilm matrix, and thus improving antibiotic penetration at the site of infection.[7] On the other side, anti-inflammatory drugs have shown to reduce inflammation in animal models of tuberculosis, contributing to a better response to the treatment.[12] Further understanding of MAC biofilm could improve MAC-PD patient management and result in better treatment outcomes. Substances such as acetyl-salicylic acid, paracetamol and ibuprofen could act as potential antibiofilm agents. In addition, diallyl disulphide, an organosulfur compound derived from garlic was also tested in the present study. The objective of the present work was to study the *in vitro* activity of different antibiotics and potential antibiofilm agents, alone and in combination, against MAC biofilm.

Methods

M. avium-intracellulare complex isolates

Five *M. avium* and 6 *M. intracellulare* clinical isolates obtained from the Microbiology Department of the Hospital Clínic of Barcelona were used in the present study. All of the isolates had previously shown the ability to form biofilm *in vitro*. The reference strain *M. avium* ACTT25291 was also included.

Antibiotics and potential antibiofilm agents

Amikacin (AMK), clarithromycin (CLR), ethambutol (EMB), moxifloxacin (MXF) and the potential antibiofilm agents, NAC, Tween 80, acetyl-salicylic acid, ibuprofen, paracetamol and diallyl disulphide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Amikacin, ethambutol, moxifloxacin, NAC, Tween 80, acetyl-salicylic acid and paracetamol were dissolved in sterile distilled water. Clarithromycin was dissolved in acetone and sterile distilled water. Ibuprofen was dissolved in dimethyl sulfoxide (DMSO) (final concentration of 0.002%) (Panreac Applichem, Barcelona, Spain) and sterile distilled water. Diallyl disulphide was dissolved in ethanol and sterile

distilled water. All the antibiotics and potential antibiofilm agents were sterilised by filtration and stored at -20 ºC until use.

Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations (MICs) for AMK, CLR, EMB, MXF, NAC, Tween 80, acetyl-salicylic acid, ibuprofen, paracetamol and diallyl disulphide, alone and in combination, were determined in 96-well plates (Smartech Biosciences, Barcelona). In brief, 100 µL of Middlebrook 7H9 liquid media (Becton Dickinson, Sparks, MD) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Comercial Bellés, Tarragona, Spain) were added to each well. Then, two-fold serial dilutions of the antibiotics and antibiofilm agents ranging from 0.5 µg/mL to 64 µg/mL were made. Finally, 100 µL of inoculum at a concentration of 0.5 McFarland (5×10^5 CFU/mL) were added. Positive control wells contained 100 µL of Middlebrook 7H9 and 100 µL of inoculum. Negative control wells were also included by adding 200 µL of Middlebrook 7H9. The microplates were incubated at 37 °C for 7 days. After incubation, the plates were read using Sensititre Vizion Digital MIC Viewing System (ThermoFisher Scientific, Barcelona, Spain). The MIC value was interpreted as the lowest antibiotic concentration inhibiting mycobacterial growth. All the experiments were performed in duplicate for each isolate.

Biofilm formation

In vitro biofilm was formed as previously described by Portell-Buj et al.[13] Briefly, the isolates were grown in Middlebrook 7H9. Then, the mycobacterial cultures were homogenised by agitation and adjusted to a concentration of 1×10^7 CFU/mL using a nephelometer (PhoenixSpec, Becton Dickinson). Afterwards, 200 µL of inoculum (1×10^7 CFU/mL) were seeded in non-treated polystyrene plates (ThermoFisher Scientific). The plates were incubated for 4 weeks at 42 ºC in the case of *M. avium* and at 37 ºC regarding *M. intracellulare*. Negative controls containing 200 µL of Middlebrook 7H9 were also included. In order to minimize evaporation, sterile distilled water was added to the surrounding well and the plates were covered with a lid.

Minimum biofilm eradication concentrations (MBEC)

After biofilm formation, the plates were treated with different antibiotics and potential antibiofilm agents at concentrations ranging from 32 µg/mL to 4096 µg/mL by adding in each well 100 µL of an aqueous solution containing the desired drug concentration. The plates were incubated again for 1 week at 37 ºC. Then, the supernatant of the plates was discarded and each well was rinsed once with 200 µL of 1 x phosphate-buffered saline (PBS) (ThermoFisher Scientific, Barcelona, Spain). The plates were dried at 60 °C for 1 h and the wells were died with 200 µL of 1% crystal violet. The plates were incubated at room temperature for 10 min and blotted on paper towels. Each well was rinsed once with 200 µL of 1 x PBS and dried at 60 °C for 1 h. Then, 200 µL of 33% acetic acid were added in order to solubilize the biofilm. Finally, the A_{580} was determined using a microplate spectrophotometer (BioTek Instruments, Inc. Winooski, VT, USA). The wells containing only Middlebrook 7H9 medium as well as only antibiotics or potential antibiofilms agents were used as blanks and their mean A_{580} values were subtracted from wells containing biofilm. In the present study, he minimum biofilm inhibitory concentrations (MBICs) was defined as the lowest concentration of antibiotic and/or potential antibiofilm agent decreasing 40% or more the A_{580} value of the control without antibiotic.[14,15]

Before the A_{580} readings, subcultures of the plates were made in a second plate by seeding 20 µL of each well in a new well containing 180 µL of Middlebrook 7H9. These plates were then incubated for 1 week at 37 ºC. After incubation, the plates were checked for growth using Sensititre Vizion Digital MIC Viewing System.

The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration of antibiotic and/or potential antibiofilm agent inhibiting biofilm formation.

Data analysis

The A_{580} values from wells without antibiotic were considered 100%. The wells containing antibiotics were interpreted as percentage of these values.

The $MBEC_{50}$ and $MBEC_{90}$ values were defined as the lowest concentration that produced 50% and 90%, respectively, inhibition of the biofilm formation.

Results

Table 1 shows the MIC_{50} and MIC_{90} of 4 clinical isolates of *M. avium* and 6 clinical isolates of *M. intracellulare*, against 4 antibiotics (CLR, AMK, MXF and EMB) and 6 potential antibiofilm agents (NAC, Tween 80, acetyl-salicylic acid, ibuprofen, paracetamol and diallyl disulphide). The planktonic forms were susceptible to CLR, AMK and EMB, intermediate to MXF and resistant to all the potential antibiofilm agents. Table 1 also shows the $MBEC_{50}$ and $MBEC_{90}$ against all the drugs tested. All of them showed $MBEC_{90}$ concentrations of 4096 µg/mL.

Table 2 shows the MICs for three two-antibiotic combinations (CLR/AMK, AMK/MXF and CLR/EMB). In planktonic forms, the FICs of the combinations were indifferent, not synergistic. However, in biofilm forming forms, the combinations CLR/AMK and CLR/EMB were synergistic and the combination of AMK/MXF was indifferent.

Table 3 shows six three-drug combinations including two antibiotics (CLR and AMK) and a potential antibiofilm agent. The MBECs obtained were 1-2 dilutions lower than those observed with the two-antibiotic combinations.

In addition, all of the tables show the MBICs values for the biofilm forming forms, against individual drugs and against combinations of two and three drugs. An overall correlation between $MBEC_{90}$ and MBIC was observed for individual drugs and for combinations of three drugs. Nevertheless, when testing combinations of two drugs the MBEC was lower than the MBIC.

Discussion

In the present work we studied the *in vitro* activity of 4 antibiotics, alone and in combination, against planktonic and biofilm forming forms of 5 *M. avium* and 6 *M. intracellulare* clinical isolates. The antibiotic combinations were studied with and without potential antibiofilm agents.

The main finding is the synergic activity of the three combinations of CLR-AMK, AMK-MXF and CLR-EMB against biofilm forming forms, as can be observed with the MBECs. In addition, we observed a reduction in the MBICs when potential antibiofilm agents were added to the antibiotic combinations, which could be associated to the disruption of the biofilm matrix.

There is no standardized method to study biofilm formation in mycobacteria, complicating the comparison of the results between the different studies. However, a few methods to assess biofilm formation have been described such as, the 96-well plate and crystal violet, method employed in the present work, and the use of bioreactors or of the Calgary device.[16] In general, biofilm assessment is based on macroscopically dye the biofilm structure or on determining the ultrastructure of the biofilm with methods such as, confocal laser scanning microscopy (CLSM) in combination with fluorescent dyes which allow observing the matrix and the viability of the bacteria within the biofilm.

In the biofilm forming infections it is of great interest to study the activity of antibiotics. Respiratory infections associated to biofilm formation, mainly occur in patients suffering from bronchiectasis and cystic fibrosis, and being most of the literature about *Pseudomonas aeruginosa* and very scarce regarding mycobacteria. Moreover, the existing literature is focused on rapidly growing mycobacteria (RGM). Differences in the antibiofilm activity of antibiotics have been observed in RGM. For instance, Muñoz-Egea et al.[17] observed a higher activity of ciprofloxacin when combined to NAC or Tween 80, than to clarithromycin or amikacin, in *Mycobacterium smegmatis* and *Mycobacterium fortuitum*, while in the planktonic forms the opposite occurred. This fact could be related to the chemical properties of each drug. Moreover, in the presence of biofilm the concentrations required to eradicate mycobacteria can be up to 100 to 100.000 times higher than the MICs of the planktonic forms. The addition of detergents and mucolytics such as Tween 80 or NAC facilitated the activity of antibiotics and reduced the MBECs in two dilutions.[17] In our study, MBECs remained high (2048 µg/mL) for each drug and without significant differences, albeit in this study ciprofloxacin was not included. Nguyen et al.[18] studied the antibiofilm activity of different imidazole-amines being two of them 12j and 12g, (4-(4-(Pentyloxy)phenyl)-5-(trifluoromethyl)-1H-imidazol-2-amine and 4-(4-Hexylphenyl)-5-(trifluoromethyl)-1H-imidazol-2-amine), that in combination with izoniazid and rifampin showed synergistic activity against *M. smegmatis* biofilm.

Here three combinations were studied, showing indifferent activity against the planktonic forms with FICs ranging from 1 to 3. Nonetheless, against biofilm forming forms the three combinations showed synergistic activity since they reduced the MBEC in more than 2 dilutions and with FICs lower than 0.5. This is the most relevant finding of the study given that it shows that these combinations are synergic against MAC

biofilm. Although these concentrations are not low enough to be achieved in the bloodstream, they could potentially be used for inhaled administration.

On the other side, in this study 6 potential antibiofilm agents were also tested. Two of them, NAC and Tween 80, had been previously studied, showing antibiofilm activity.[17] Three of them were analgesic/anti-inflammatory drugs, acetyl-salicylic acid, ibuprofen and paracetamol. They reduce the local inflammation, modulate tissue destruction and the host response against tuberculosis.[19] Despite *in vitro* models are far from *in vivo* activity, biofilm is formed by bacteria and also occurs *in vivo* in chronic lung infections. Diallyl disulphide is an agent derived from garlic and was included due to its antibacterial activity.[20]

When the potential antibiofilm agents were added to the antibiotic combination of CLR-AMK, an increase in the synergistic activity in the MBEC was not observed, but a reduction in the MBIC (*i.e.* A_{580} values) was measured. This suggests that these 6 agents could have activity against the biofilm matrix. Accordingly, they would have a role in enabling the activity of antibiotics. Further studies are needed to confirm this hypothesis.

From a methodological perspective, it has been proved that biofilm production in 96-well plates is a good method to assess biofilm formation in MAC. Nevertheless, the activity of antibiotics it is not directly correlated to the absorbance. Here, the MBEC was established by subculturing each of the wells. Other studies have used the same methodology and/or confocal microscopy.[7,17] A limitation of the present work is that the conclusions are directly associated to the studied combinations and with the methodology used.

The main conclusions are that the combinations of CLR-AMK, CLR-EMB and AMK-MXF showed synergy when compared to the individual drugs against the biofilm producing forms. Moreover, the crystal violet method as well as the technique used to test drugs against MAC biofilm have proved useful. These results lead the path for further studies with other combinations including two or three antibiotics. These data, can also open new therapeutic options based on the clinical use of these drugs, particularly in inhaled formulations that allow the administration of higher doses. Furthermore, the administration of potential antibiofilm agents combined to antibiotics, could be advantageous given their activity against the biofilm matrix and hence increase antibiotic activity.

Conflict of interests

The authors state that they have no conflict of interests.

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Ethical approval

Ethical approval was received from the Ethical Committee of the *Hospital Clínic de Barcelona* (Barcelona, Spain) [HCB/2016/0344 and HCB/2018/0275].

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Table 1
MICs, MBECs and MBICs of the antibiotics and potential antibiofilm agents tested against planktonic and biofilm forming forms of *M. avium* and *M. intracellulare* clinical isolates.

Isolates	MICs of planktonic forms (µg/mL)									
	CLR	AMK	MXF	EMB	ASA	IBP	PCM	NAC	DADS	Tween 80
M. avium										
MIC_{50}	4	16	1	4	>64	>64	>64	>64	>64	>64
MIC_{90}	4	16	2	8	>64	>64	>64	>64	>64	>64
M. intracellulare										
MIC_{50}	1	8	1	8	>64	>64	>64	>64	>64	>64
MIC_{90}	2	16	2	8	>64	>64	>64	>64	>64	>64

Isolates	MBECs and MBICs of biofilm forming forms (µg/mL)									
	CLR	AMK	MXF	EMB	ASA	IBP	PCM	NAC	DADS	Tween 80
M. avium										
$MBEC_{50}$	2048	2048	256	2048	4096	4096	4096	4096	4096	4096
$MBEC_{90}$	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096
$MBIC_{90}$	4096	2048	4096	2048	4096	4096	4096	4096	4096	4096
M. intracellulare										
$MBEC_{50}$	4096	4096	4096	2048	4096	4096	4096	4096	4096	4096
$MBEC_{90}$	4096	4096	4096	4096	4096	4096	4096	4096	4096	4096
$MBIC_{90}$	4096	4096	1024	2048	4096	4096	4096	4096	4096	4096

MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; CLR, clarithromycin; AMK, amikacin; MXF, moxifloxacin; EMB, ethambutol; ASA, acetyl-salicylic acid; IBP, ibuprofen; PCM, paracetamol; NAC, N-acetyl-L-cysteine; DADS.

Table 2
MICs, MBECs and MBICs of the antibiotic combinations against planktonic and biofilm forming forms of *M. avium* and *M. intracellulare* clinical isolates

Isolates	MICs of planktonic forms (μg/mL)					
	CLR/AMK	∑FIC CLR/AMK	AMK/MXF	∑FIC AMK/MXF	CLR/EMB	∑FIC CLR/EMB
M. avium						
MIC_{50}	4	1.25	2	2.125	4	2
MIC_{90}	4	1.25	4	2.25	4	2
M. intracellulare						
MIC_{50}	2	2.25	2	2.25	2	2.25
MIC_{90}	4	2.25	4	2.125	4	2.5

Isolates	MBECs and MBICs of biofilm forming forms (μg/mL)					
	CLR/AMK	∑FIC CLR/AMK	AMK/MXF	∑FIC AMK/MXF	CLR/EMB	∑FIC CLR/EMB
M. avium						
$MBEC_{50}$	256	0.25	128	0.5625	256	0.25
$MBEC_{90}$	256	0.5	256	1.25	512	0.625
$MBIC_{90}$	1024		2048		2048	
M. intracellulare						
$MBEC_{50}$	256	0.125	256	0.125	128	0.09375
$MBEC_{90}$	512	0.25	512	2	512	0.25
$MBIC_{90}$	512		2048		512	

MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; CLR, clarithromycin; AMK, amikacin; FIC, fractional inhibitory concentration; MXF, moxifloxacin; EMB, ethambutol.

Table 3
MBECs and MBICs of the combinations including antibiotics and potential antibiofilm agents tested against biofilm forming forms of *M. avium* and *M. intracellulare* clinical isolates

Isolates	MBIC (µg/mL)											
	CLR/AMK /ASA	∑FIC CLR/AMK /ASA	CLR/AMK /IBP	∑FIC CLR/AMK /IBP	CLR/AMK /PCM	∑FIC CLR/AMK /PCM	CLR/AMK /NAC	∑FIC CLR/AMK /NAC	CLR/AMK /DADS	∑FIC CLR/AMK /DADS	CLR/AMK /TWEEN 80	∑FIC CLR/AMK /TWEEN 80
M. avium												
$MBEC_{50}$	128	0.15625	128	0.15625	128	0.15625	256	0.375	128	0.15625	256	0.1875
$MBEC_{90}$	128	0.40625	128	0.40625	128	0.40625	256	0.8125	128	0.40625	256	0.8125
$MBIC_{90}$	128		128		128		256		128		256	
M. intracellulare												
$MBEC_{50}$	128	0.09375	128	0.09375	128	0.09375	128	0.09375	128	0.09375	128	0.09375
$MBEC_{90}$	128	0.09375	256	0.1875	256	0.1875	128	0.09375	128	0.09375	256	0.1875
$MBIC_{90}$	128		256		256		128		128		256	

MBEC, minimum biofilm eradication concentration; MBIC, minimum biofilm inhibitory concentration; CLR, clarithromycin; AMK, amikacin; ASA, acetyl-salicylic acid; FIC, fractional inhibitory concentration; IBP, ibuprofen; PCM, paracetamol; NAC, N-acetyl-L-cysteine; DADS, diallyl disulphide.

8. DISCUSSION

At the moment, almost 200 species of the *Mycobacterium* genus have been described, most of them during the last 10-15 years due to the improvements in molecular taxonomy (74). The most important mycobacteria, in terms of virulence and clinical outcome, is *M. tuberculosis*, the causative agent of tuberculosis (TB), responsible for 8-9 million cases and 1.2 million deaths/year worldwide (75). The emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) has led to the need for new treatment options and strategies (76). The incidence of TB is directly related to HIV incidence and economic development since more than 80% of the cases are reported from developing countries (75).

However, the infections caused by nontuberculous mycobacteria (NTM) have increased in the past years. These infections are of increasing interest among two populations, immunosuppressed patients such as those with HIV, often causing disseminated disease in them, and patients with chronic pulmonary diseases (18, 77, 78). The origin of NTM is environmental and their geographical distribution differs among the regions (18, 78), influenced by the climate, soil, water and vegetation (79). The development of infection by NTM may be associated with the immunological status of the patients. In both groups of patients, the most frequently isolated species are *M. avium* complex (*M. avium*, *M. intracellulare, M. chimaera*) and *Mycobacterium abscessus,* followed by *M. chelonae*, *M. kansasii*, *M. fortuitum* and *M. xenopi* (18, 78). Recently, taxonomical changes have occurred in several bacterial genera, including Mycobacteria, due to new evidences in molecular epidemiology. Mycobacterial species have been divided into five new genera: *Mycobacterium, Mycolicibacter, Mycolicibacterium, Mycolicibacillus and Mycobacteroides* (1). Over the last years, infections caused by NTM have significantly increased (80). The increase in the prevalence of NTM infections is related to different causes such as a reduction in the incidence of TB, the aging of the population, and

the increase in the life expectancy of patients with immunosuppression or chronic pulmonary disease, especially bronchiectasis and other obstructive disorders. In addition, improvements in microbiological diagnostic techniques have also contributed to the higher prevalence of these mycobacteria along the last years (28, 29). Bronchiectasis is the third most frequent inflammatory lung disease. Although its real incidence is unknown, in Spain it is estimated that it affects from 40-550 patients/10^5 inhabitants, with an annual treatment cost of 4700€/patient (81). Currently, the clinical management of pulmonary infections produced by NTM in patients with previous diseases, such as bronchiectasis, is complex, especially regarding treatment, due to the scarce response to antibiotic regimens and the tendency to chronicity in these patients due to the pathogenic mechanism of this disease.

In the clinical diagnosis is difficult to differentiate between colonisation and infection. The decision to treat should be well founded since the treatment is often lengthy and relapses are frequent.

The recommended treatment for these infections is based on the administration of different antibiotics, according to the species isolated (34, 82). The most recent guideline is from the British Thoracic Society (BTS) (34). NTM are resistant to many of the common antimicrobials as well as to most of the antituberculous drugs, making the treatment difficult (32, 83). The antibiotics used to treat NTM infections are aminoglycosides, cephalosporins, macrolides, quinolones, linezolid, carbapenems, rifamycins, tetracyclines and glycylcyclines (tigecycline). The molecular mechanisms associated with resistance are not well-known. In the meantime, new drugs have been discovered and implemented, some authors have suggested the use of drugs usually used against MDR-TB, such as bedaquiline and clofazimine or delamanid and pretomanid, among others (84). These would be implemented in future studies.

NTM-pulmonary infections in patients with chronic lung diseases and disseminated NTM infections in immunosuppressed patients are the most difficult to treat. In general, NTM pulmonary infections are less aggressive than TB, but their management is complex, since the evolution may be long and torpid, cure is not always obtained and recurrence is frequent. Different reasons might explain why the management of these patients is problematic: 1. No new efficient drugs to treat these infections; 2. no effective antibiotic-combinations recognized as the standard treatment, opposite to what happens with current TB treatment; 3. pulmonary infections in patients suffering from chronic diseases that act as a barrier between drugs and the microorganisms, since mucus and secretions hide bacteria, being difficult for antibiotics to reach them; 4. mycobacteria can develop biofilm, which hampers the access of antibiotics to the biofilm matrix, and indirectly protect the mycobacteria preventing the action of drugs against mycobacterial cells inside the biofilm structure.

The lack of new drugs is a strategy that should be urgently addressed, intensifying the research on new drugs, but also exploring all the possibilities offered by existing drugs, but combining them in new schemes. However, little is known about the effect of schedules including antibiotic combinations. Some studies have found unsuspected synergisms in combinations against NTM including old antibiotics as clofazimine and bedaquiline or synergistic effect between rifampicin and imipenem, among others (85, 86). The high percentage of resistance to drugs observed among the most common NTM, causes difficulty for a unified treatment schedule, valid for most of the species, such as stablished for TB. However, most of the authors agree that the schedule should include 3-4 drugs, if possible macrolides and aminoglycosides among them.

On the other hand, the results of the drug susceptibility tests are often not correlated with clinical outcome (87). This is probably due to a much higher drug concentration needed at

lung level than reached in serum. This is because the presence of secretions in obstructive disease make it difficult the interaction between mycobacteria and antibiotics (31).

In addition to incorporating other drugs and studying new combinations, other forms of drug-administration such as nebulization or inhalation should be studied. This is a less invasive procedure that locally increases the bioavailability, allowing a reduction in the dosage and undesired side effects. There are several formulations which allow the administration of drugs directly to the lung by inhalation, directly from the drug in powder form, and even the use of encapsulation formulas of the drugs, such as the use of nanoparticles, microparticles or liposomes, which enable drug penetration into the cells. Liposomes have been studied in macrophage cell cultures and in animal models, showing good tolerance and no immunogenicity (88). There are previous experiences for other pathologies as asthma, COPD and treatment of *Pseudomonas aeruginosa* in cystic fibrosis. Several studies have demonstrated that inhaled particles are incorporated by alveolar macrophages, increasing their activity (25). The most experienced is amikacin in liposomal formulation, used in the treatment of *M. avium* lung infection with promising results. Other antibiotics, such are ciprofloxacin, levofloxacin, gentamicin, capreomicin, tobramycin, azithromycin, and clofazimine can also be prescribed for inhaled administration. In TB treatment, combinations including rifampicin and isoniazid have been also applied by inhalation, although more evidence is needed. New strategies such as nebulisation, the administration of antibiotics attached to nanoparticles or encapsulated in liposomes, have recently been proposed (25, 89). Inhaled antibiotics are a reliable option for future treatments.

Among the new strategies proposed, the use of antimicrobial peptides (AMPs) is a new line that should be studied. AMPs are natural antibiotics produced by several forms of life, microorganisms and humans. Usually AMPs have 20-60 amino-acids, are amphipathic,

charged positively and divided in four classes. AMPs have an important role in innate immune response and in modulating the host response. Most of them have as a target the bacterial membrane by, for instance, promoting the disruption of the membrane. We studied the activity of 12 different AMPs against clinical isolates of *M. tuberculosis* and *M. avium*, using 96-well plates and the resazurin assay. Although all of them needed high doses for inhibiting the growth of mycobacteria, mastoparan, melittin and indolicidin reached the better results for *M. tuberculosis* and indolicidin for *M. avium*. There are very few studies that tested AMPs in mycobacteria. AMPs disrupt the bacterial membrane, impairing the permeability and allowing a better entrance of the antibiotics. The differences observed in the response to AMPs, in our study and in the literature, are possibly due to the composition of the bacterial wall, since the AMPs merge to this structure. From this point of view, the AMPs should be tested against every species of interest (90). Future studies should be addressed to analyse the response in different species and also combine AMPs with antibiotics in order to boost their effect or find synergism, aiming to overcome resistance and also reduce side effects. This has been observed for isoniazid and rifampicin (91). The place of AMPs in the treatment should be established in future studies, determining how to combine them with common drugs, the dose and the method of administration, for instance using delivery systems such as nanoparticles or liposomes.

Pulmonary infections caused by NTM are the most challenging to treat among the diseases caused by these microorganisms. An important proportion of the clinical isolates causing these infections belong to the *Mycobacterium avium* complex (MAC). The most representative members are *M. avium* and *M. intracellulare.* Recently, a growing proportion also corresponds to *M. chimaera*. MAC are largely present in the environment, mainly in water and soil. Although in most guidelines *M. avium* and *M. intracellulare* are commonly included in the same complex, some differences in the clinical presentation as well as in the antibiotic susceptibility pattern have been observed among them. In general, *M. intracellulare* shows less severe clinical

manifestations and lower antibiotic resistance in comparison with *M. avium*. At the moment, the frequency of both species as pathogenic agents is similar. For this reason, it is important to differentiate them, since not considering these differences may affect the patient outcome, not only regarding the drugs administered but also because long treatments lead to considerable side effects. Colonization by MAC is not always indicative of treatment and differentiating between colonization and infection can be very difficult. In patients requiring treatment, combination regimens including a macrolide (*i.e.* clarithromycin) are the most advised in guidelines due to the high levels of natural resistance to many drugs among MAC isolates (34). The other antibiotics included in therapy regimens are clofazimine, rifampicin or rifabutin, ethambutol, moxifloxacin or levofloxacin, linezolid and aminoglycosides (*i.e.* amikacin) (9, 92). There is a real need to develop novel treatment regimens against MAC, including new and old antibiotics, searching for unknown synergies which would result in improved patient outcome.

As in TB treatment, MAC lung infections are treated with drugs administered in combination. However, the microbiological method to determine the efficacy of antibiotics is standardized for individual testing, but not for antibiotic combinations. In addition, there is little knowledge of the pharmacodynamics and interactions of drug combinations. The time-kill assay allows establishing the pharmacodynamics of different antibiotics by observing the rate at which an antibiotic concentration kills bacteria over a period of time (54). Furthermore, models based on macrophage infection allow determining the activity of antibiotics against bacteria localized inside the human cells (93). Both methods allow interfering the dynamics of antibiotic combinations in a real scenario. From this point of view, we investigated the activity of amikacin, ethambutol and clarithromycin, alone and in combination, against *M. avium* and *M. intracellulare* clinical isolates using the time-kill assay and the THP-1 macrophage infection model and we also compared the antibiotic activities between both MAC species, *M avium* and *M. intracellulare*.

To our knowledge, few studies have been published about the effect of antibiotics against *M. avium* and *M. intracellulare*. Time-kill assay and macrophage model are excellent approaches to study the pharmacodynamics of antibiotics as well as their ability to act against bacteria inside human cells. In this study, we used methodology previously developed by our group and applied to TB (55, 58). The results were interesting since the combination of amikacin/ethambutol/clarithromycin, showed significantly better activity against *M. intracellulare* than against *M. avium* in the time-kill assay. Using the macrophage infection model, no significant differences between *M. avium* and *M. intracellulare* clinical isolates were detected, in contrast to the time-kill assay. However, our *ex vivo* model had a duration of 4 days while the time-kill assay was incubated for up to 14 days. Possibly a time extension of the macrophage model would show differences between these two species, as observed in the time-kill assay. Our results demonstrated a better activity of the studied combination and of the individual antibiotics in *M. intracellulare*, being possible that other drugs would show similar results.

In addition to MAC, infections caused by *Mycobacterium abscessus* are common among patients suffering chronic obstructive lung diseases. Recently, due to taxonomical changes, a new name for *Mycobacterium abscessus* has been proposed, *Mycobacteroides abscessus*. This species is a rapidly growing mycobacteria (RGM) that causes lung and skin infections which are difficult to treat due to resistance to most classes of antibiotics (11, 80). It is classified into three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* (1, 11, 94). The identification to subspecies level is important since their profiles of resistance to macrolides are different. Macrolides, as clarithromycin and azithromycin, are among the most active drugs against *M. abscessus*. The resistance to macrolides can be constitutive (*rrl* gene) or inducible (*erm*41 gene). The most common is the inducible resistance, due to the presence of a functional *erm*41 gene that codes for a methylase. Accordingly, *M. abscessus* subsp. *massiliense* shows an *erm*41 gene truncated, not functional,

being susceptible to macrolides. All the isolates of *M. abscessus* subsp. *bolletii* are resistant to macrolides. Most of *M. abscessus* subsp. *abscessus* isolates exhibit inducible macrolide resistance, although a few proportion of isolates have a non-functional *erm*41 gene, remaining susceptible. The most commonly isolated is *M. abscessus* subsp. *abscessus*, with slightly geographical differences (80, 95).

M. abscessus lung infections are common in patients with underlying respiratory diseases (94). The management of these infections is difficult due to high levels of resistance to most of the antibiotics commonly used. Depending on the macrolide resistance profile, the last guidelines of the BTS recommend the administration of different intravenous (iv) and oral antibiotics (amikacin, tigecycline, imipenem and oral clarithromycin) during the initial phase followed by oral and inhaled antibiotics (oral clofazimine, oral moxifloxacin and nebulised amikacin) for at least 12 months. The use of macrolides is recommended even in the presence of inducible resistance, but not when constitutive resistance associated to the *rrl* gene is found. It should be noted that most of the isolates show inducible resistance, not constitutive (34, 95). Moreover, side effects are common and treatment outcomes are very often poor (11). To develop new antibiotics and antibiotic combinations is a very urgent need.

The treatment for *M. abscessus* lung infections consists of the administration of different antibiotics in combination. Therefore, it is necessary to determine the efficacy of these combinations. We compared the *in vitro* activity of four different antibiotics, amikacin, tigecycline, imipenem and clarithromycin alone and in two different three-drug combinations (amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin) against *M. abscessus* subsp *abscessus* clinical isolates using the *in vitro* time-kill assay.

To our knowledge, there are few studies on the effect of these antibiotics against *M. abscessus* clinical isolates using the time-kill assay and most of them are focused on two-drug combinations (96, 97). This method has been largely used in clinical microbiology and has

demonstrated to be consistent in the study of different antibiotics and microorganisms (55, 98-100).

The majority of patients with lung infections caused by *M. abscessus* receive combinations of two or more antibiotics during the treatment, although there is no standard combination broadly accepted and most of the treatments are based on empirical experience and experts opinions. In addition, most of the published studies only analyse two-drug combinations.

In this study, we used isolates of *M. abscessus* subsp. *abscessus*, the most isolated subspecies in our region. From a strain collection, we selected seven clinical isolates susceptible to macrolides, with the aim to compare the two three-drug combinations. The results show that the reduction in $\log_{10}$ CFU ml^{-1} with the combination including imipenem was lower than of that including clarithromycin, although both combinations had good activity and did not show statistically significant differences. This fact and the intravenous administration of imipenem, leads to the conclusion that combination including amikacin/tigecycline/imipenem can be recommended against macrolide resistant isolates. This is especially interesting in the treatment of lung infections caused by isolates showing resistance to macrolides. This is important, since at least fourteen months of treatment are necessary and often clinical outcomes remain very poor, with success rates of only 30-50% (101). In addition, in most countries isolates showing inducible resistance to macrolides are predominant, highlighting the interest of studying treatment combinations which do not include macrolides.

Apart from studying new combinations against NTM causing lung infections, there are relevant factors associated to the pathogeny of this infection that need to be taken into consideration, such as the ability to form biofilm in the respiratory three. This is specially favored by the presence of secretions, cellular debris, mucus and others, present in bronchiectasis, cystic fibrosis and obstructive lung diseases. Mycobacteria, as many other

bacteria, have the potential to form biofilm (60). Biofilms offer protection to bacteria from the environment, immune system and antibiotics (61). For this reason, the antibiotic concentrations needed to treat biofilm forming NTM lung infections can be several thousand times higher, in comparison with planktonic forms (64). From this point of view, common antibiotic susceptibility testing is not useful, since the interpretation is based on serum availability. There are several methods to analyze the biofilm production, although none is standardized for mycobacteria. Ideally, this method should be easy to perform and read, without requiring highly specialized equipment (102, 103).

We report *in vitro* biofilm formation in MAC clinical isolates by using a protocol based on the adherence to 96-well plates and the crystal violet method, modified from Christensen et al. and Kumar et al. (67, 104). This protocol was developed using 6 isolates each of *M. avium* and *M. intracellulare*. The optimal conditions stablished for biofilm formation were incubation at 42ºC for 4 weeks for *M. avium* and at 37ºC during 4 weeks for *M. intracellulare*. According to that, three categories of biofilm formation (strong, moderate and week) were stablished. Although not significant, slight differences between both species were observed since *M. avium* produced stronger biofilm than *M. intracellulare*. A total of 38 isolates were tested with this modification to consolidate the method. The rest of experiments performed by our group were based on this methodology of biofilm formation.

At the moment, there is little knowledge about the proportion of NTM isolates that can form biofilm and about its association to virulence. It has been suggested that non biofilm forming isolates have less ability to colonize the human mucosa and tissues (105). Biofilm formation makes it necessary to develop novel treatments that not only target the planktonic forms but also MAC biofilm. However, at the moment there are few or limited clinical options. One of the strategies is targeting the biofilm matrix (106). For example, agents such as Tween 80

and N-acetyl-L-Cysteine (NAC) have proved to be effective in disrupting the biofilm matrix, improving antibiotic penetration at the site of infection (107).

In the present study we studied the *in vitro* activity of 4 antibiotics, alone and in combination, against planktonic and biofilm forming forms of *M. avium* and *M. intracellulare* clinical isolates. We also study several chemical compounds, that we named potential antibiofilm agents, to test their activity against biofilm. The selection of these compounds was based on the previously observed activity (*i.e.* NAC and Tween 80), the anti-inflammatory activity of drugs which modulate tissue destruction or produce clinical improvement in TB (*i.e.* ibuprofen) (69). Consequently, acetyl-salicylic acid and paracetamol were also included. Diallyl disulphide is an agent derived from garlic and was included due to its antibacterial activity (108).

The main finding is the synergic activity of the three combinations of clarithromycin-amikacin, amikacin-moxifloxacin and clarithromycin-ethambutol against biofilm forming forms, as can be observed with the MBECs. In addition, we observed a reduction in the MBICs when potential antibiofilm agents were added to the antibiotic combinations, which could be associated to the disruption of the biofilm matrix. Differences in the antibiofilm activity of antibiotics have been observed in rapidly growing mycobacteria (109). For instance, a higher activity of ciprofloxacin when combined to NAC or Tween 80, than to clarithromycin or amikacin, in *Mycobacterium smegmatis* and *Mycobacterium fortuitum*. These differences could be related to the chemical properties of each drug. Moreover, in the presence of biofilm the concentrations required to eradicate mycobacteria can be up to 100 to 100.000 times higher than the MICs of the planktonic forms. The addition of detergents and mucolytics such as Tween 80 or NAC facilitated the activity of antibiotics and reduced the MBECs in two dilutions (109).

In our study, three combinations were studied, showing indifferent activity against the planktonic forms. Nonetheless, against biofilm forming forms the three combinations showed synergistic activity since they reduced the MBEC in more than 2 dilutions and with FICs lower than 0.5. This is the most relevant finding of the study given that it shows that these combinations are synergic against MAC biofilm. Although these concentrations are not low enough to be achieved in the bloodstream, they could potentially be used for inhaled administration.

When the potential antibiofilm agents were added to the antibiotic combination of clarithromycin-amikacin, an increase in the previous synergistic activity in the MBEC was not observed, but a significant reduction in the MBIC (*i.e.* A_{580} values) was measured. This suggests that these 6 agents could have activity against the biofilm matrix. Accordingly, they would have a role in enabling the activity of antibiotics. Further studies are needed to confirm this hypothesis. New combinations including the antibiotics more used in clinical practice should be tested, since the results obtained in this study open an interesting line to treat these patients with combinations.

On the other hand, a strategy to be implemented and developed in the next years is the use of new forms of drug administration, such as inhaled or nebulized antibiotics using different types of nanoparticles. This local administration will allow reaching higher concentrations in the lungs than the observed in serum after oral or iv administration, without causing side effects. This is an interesting line of investigation to be further developed in the future.

9. CONCLUSIONS

1. Although AMPs are promising alternatives in the treatment of mycobacterial infections, of the 12 AMPs studied only one showed moderate activity against clinical isolates of *M. avium* and three against *M. tuberculosis*. More studies should be performed to potentiate their activity and determine their place in the mycobacterial treatment.

2. Amikacin, ethambutol, clarithromycin and particularly the combination of amikacin/ethambutol/clarithromycin showed good activity against *M. avium* and *M. intracellulare* clinical isolates as well as no antagonistic activity using the time-kill assay and the THP-1 macrophage infection model.

3. The combination of amikacin/ethambutol/clarithromycin showed significantly higher activity against clinical isolates of *M. intracellulare* than against *M. avium* with the time-kill assay, highlighting the importance of performing specific identification for each MAC species.

4. The three-drug combinations, amikacin/tigecycline/imipenem and amikacin/tigecycline/clarithromycin, showed similar *in vitro* activity against *M. abscessus* subsp. *abscessus* clinical isolates as well as no antagonistic activity.

5. The combination of amikacin/tigecycline/imipenem can be an alternative to the combination of amikacin/tigecycline/clarithromycin against *M. abscessus* subsp. *abscessus* lung infections caused by clinical isolates resistant to clarithromycin.

6. A significant proportion of *M. avium* and *M. intracellulare* clinical isolates have the ability to produce biofilm.

7. An easy and reproducible method for determining the biofilm production in *M. avium* and *M. intracellulare* clinical isolates has been standardized.

8. A method for analyzing the antibiotic activity against *M. avium* and *M. intracellulare* clinical isolates producing biofilm has been described and implemented.

9. The minimal biofilm eradication concentrations (MBECs) of four antibiotics against *M. avium* and *M. intracellulare* clinical isolates increased up to one thousand times in the presence of biofilm compared to MICs in planktonic forms.

10. Three antibiotic combinations (clarithromycin/amikacin, clarithromycin/ethambutol and amikacin/moxifloxacin) showed synergism against *M. avium* and *M. intracellulare* clinical isolates producing biofilm in comparison with the antibiotics alone.

11. The addition of chemical agents with potential antibiofilm activity to the antibiotic combinations, showed synergism in *M. avium* and *M. intracellulare* clinical isolates producing biofilm compared to the antibiotics alone, and would have a lytic activity against the biofilm matrix.

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