UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE ANÁLISES CLÍNICAS E BIOMEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS E FISIOPATOLOGIA

JOÃO VÍTOR PEREZ DE SOUZA

Ação de sistemas de efluxo na resistência à isoniazida em *Mycobacterium tuberculosis*

MARINGÁ
2018

JOÃO VÍTOR PEREZ DE SOUZA

Ação de sistemas de efluxo na resistência à isoniazida em *Mycobacterium tuberculosis*

Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Fisiopatologia do Departamento de Análises Clínicas e Biomedicina, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Biociências e Fisiopatologia.
Área de concentração: Biociências e Fisiopatologia Aplicadas à Farmácia.

Orientadora: Prof.ª Dr.ª Rosilene Fressatti Cardoso
Coorientadora: Prof.ª Dr.ª Katiany R. Caleffi Ferracioli

Maringá

2018

Dados Internacionais de Catalogação na Publicação (CIP)
(Biblioteca Central - UEM, Maringá, PR, Brasil)

Souza, João Vítor Perez de
S729a Ação de sistemas de efluxo na resistência à isoniazida em Mycobacterium tuberculosis / João Vítor Perez de Souza. -- Maringá, 2018.
62 f. : il. color.

Orientador: Profª. Drª. Rosilene Fressatti Cardoso.
Co-orientador: Profª. Drª. Katiany R. Caleffi Ferracioli.
Dissertação (mestrado) - Universidade Estadual de Maringá, Centro de Ciências da Saúde, Departamento de Ciências Básicas da Saúde, Programa de Pós-Graduação em Biociências e Fisiopatologia, 2018.

1. Tuberculose (Mycobacterium tuberculosis) - Bombas de efluxo. 2. Tuberculose - Verapamil. I. Cardoso, Rosilene Fressatti, orient. II. Ferracioli, Katiany R. Caleffi, orient. III. Universidade Estadual de Maringá. Centro de Ciências da Saúde. Departamento de Análises Clínicas e Biomedicina. Programa de Pós-Graduação em Biociências e Fisiopatologia. IV. Título.

CDD 23.ed. 616.995

Márcia Regina Paiva de Brito – CRB-9/1267

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico esse trabalho para todas as pessoas que sofrem ou já sofreram de tuberculose. Espero que algum dia este trabalho possa contribuir de alguma forma para a vida dessas pessoas.

AGRADECIMENTOS

Em primeiro lugar aos meus pais, João e Renata, que forneceram a base para tudo que sou e serei na vida. Reconhecer em mim mesmo os traços, personalidade e caráter dos dois me faz muito feliz, uma vez que eles estarão sempre comigo, onde quer que eu esteja.

Aos familiares, que me mostraram exemplos a serem seguidos e valores a serem mantidos.

Às mentoras e mentores que me guiaram nessa jornada:

Todas as professoras do Laboratório de Bacteriologia Médica, por todos esses anos de orientação, atenção, carinho e paciência. Considero todas como amigas próximas e exemplos a serem seguidos pessoal e profissionalmente. Em especial às professoras Rosilene e Katiany, minhas mães científicas, por todo o conhecimento compartilhado e por acreditarem no meu potencial;

Às professoras Sandra Mara, Thaís Gomes e Maria Valdrinez pela orientação nos primeiros projetos de iniciação científica que participei na universidade. E por terem sido as que primeiro me direcionaram para a pesquisa;

Ao Paulo, Carla, dona Ivone e Marina do laboratório de imunologia do LEPAC por se mostrarem pacientes e interessados em ensinar conteúdos avançados para um aluno de primeiro ano durante meu estágio;

Aos professores Jorge Teixeira, Izabel Galhardo e Dennis Armando por me apresentarem o método científico, bem como o valor impar do sistema único de saúde brasileiro;

À toda equipe do Laboratório de Bacteriologia Médica do LEPAC, que são exemplos na prática da microbiologia. Graças ao carinho e atenção ao ensinar de todas as meninas, pude

aprender muito mais do que poderia esperar no breve período de estágio que passei no laboratório;

À professora Débora por mostrar como a ciência pode e deve ser compartilhada com a população para instigar, inspirar e plantar a semente da nova geração de cientistas. E juntamente a Luciane e professora Gessilda, agradeço pela paciência e disponibilidade para tirar dúvidas sobre a pós-graduação e o empenho em ajudar todos os alunos do PBF.

À minha namorada Letícia, por todo o amor, amizade, carinho e suporte. Não poderia por uma companheira melhor. Sinto que o nosso caminho será longo e cheio de obstáculos, porém repleto de frutos. Superaremos as dificuldades e colheremos os frutos um a um, estando lado a lado.

Ao grande amigo Jean por infinitas horas de conversas fiadas, alegrias, tristezas, comilanças e psicanálise. Além da grande amizade, agradeço pela parceria na pesquisa. Considerando os aspectos pessoais e profissionais, se algum dia eu me tornar um homem equivalente à metade do que ele é, estarei mais do que realizado.

Agradeço a todos os colegas da pesquisa do Laboratório de Bacteriologia Médica pela convivência e companheirismo do dia-a-dia.

Ter a oportunidade de realizar uma pós-graduação em uma universidade pública renomada é com certeza algo que serei grato para a vida toda. Assim, serei eternamente grato à Universidade Estadual de Maringá, ao Programa de Pós-graduação em Biociências e Fisiopatologia, ao Governo do Estado do Paraná, ao Governo Federal e aos órgãos de financiamento como o Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Fundação Araucária (FA).

Por fim, agradeço imensamente ao carinho e confiança que todas as pessoas e entidades depositaram em mim nessa jornada até aqui, e espero algum dia retribuir tudo isso de alguma forma.

Ação de sistemas de efluxo na resistência à isoniazida em *Mycobacterium tuberculosis*

RESUMO

A tuberculose (TB) pulmonar se mantém como uma ameaça global devido à alta transmissão e prevalência de humanos infectados por bacilos do complexo *Mycobacterium tuberculosis*. Características especificas da patogenia da doença e da estrutura do bacilo fazem com que o tratamento efetivo seja dificultado. Dentre elas, podemos citar: i) a presença de uma parede celular espessa e rica em lipídeos e ácidos micólicos; ii) a produção de enzimas hidrolíticas; iii) mutações aleatórias nos genes que codificam para os alvos dos fármacos; iv) e sistemas de efluxo capazes de reduzir a concentração intracelular de antimicrobianos. As bombas de efluxo (BEs) micobacterianas já são aceitas como mecanismos secundários de resistência a fármacos, mas ainda não há correlação entre efluxo e resistência à isoniazida (INH). Com o objetivo de estudar as BEs, pesquisadores vêm empregando moléculas com propriedades de inibidoras de bomba de efluxo (IBE). Verapamil (VP) é um dos fármacos mais utilizados com essa finalidade, uma vez que a redução da atividade de efluxo por ele exercida aumenta a suscetibilidade de *M. tuberculosis* a alguns medicamentos anti-TB. Assim, o objetivo deste estudo foi avaliar o papel das BEs na resistência à INH, bem como o efeito combinatório de VP e INH em *M. tuberculosis*. Os resultados obtidos neste estudo estão apresentados no manuscrito "Efflux pump gene expression and isoniazid resistance in *Mycobacterium tuberculosis"*. Primeiramente foi determinada a concentração inibitória mínima (CIM) e o efeito combinatório dos fármacos INH e VP, através dos métodos *resazurin microtiter assay plate* (REMA) e *resazurin drugs combination microtiter assay* (REDCA). Em posse desses resultados, quatro bacilos com diferentes perfis de resistência foram selecionados para ensaios de expressão gênica de BEs. Cada *M. tuberculosis* foi exposto separadamente a 0,5 x CIM de INH e INH+VP por 48 h. Por meio da RT-qPCR, foi determinada a expressão relativa de 10 genes que codificam para BEs em *M. tuberculosis*. Foi observada redução significativa da CIM de INH após combinação com VP para isolados suscetíveis aos fármacos anti-TB. Resumidamente, os ensaios de expressão gênica revelaram padrões de resposta contra a pressão de INH que se correlacionaram com cada perfil de resistência, presença ou ausência de mutações em genes de resistência e efeito combinatório com VP.

Palavras-chave: Tuberculose, bombas de efluxo, isoniazida, verapamil.

Efflux pump gene expression and isoniazid resistance in *Mycobacterium tuberculosis*

ABSTRACT

Pulmonary tuberculosis (TB) remains a global threat due to the high transmission and prevalence of humans infected with bacilli of the *Mycobacterium tuberculosis* complex. Specific characteristics of the pathogenesis of the disease and the structure of the bacillus make effective treatment difficult. Among them, we can mention: i) the presence of a thick cell wall rich in lipids and mycolic acids; ii) the production of hydrolytic enzymes; iii) random mutations in the genes that code for drug targets; iv) and efflux systems capable of reducing the intracellular concentration of antimicrobials. Mycobacterial efflux pumps (EPs) are already accepted as secondary mechanisms of drug resistance, but we still lack strong correlation between efflux and resistance to isoniazid (INH). In order to study the EPs, researchers have been using molecules with properties of efflux pump inhibitors (EPI). Verapamil (VP) is one of the drugs most used for this purpose, since the reduction of the efflux activity exerted by it increases the susceptibility of *M. tuberculosis* to some anti-TB drugs. Thus, the objective of this study was to evaluate the role of EPs in resistance to INH, as well as the combinatorial effect of VP and INH in *M. tuberculosis*. The results obtained in this study are presented in the manuscript "Efflux pump gene expression and isoniazid resistance in *Mycobacterium tuberculosis*". First, the minimum inhibitory concentration (MIC) and the combinatorial effect of INH and VP were determined by resazurin microtiter assay plate (REMA) and resazurin drugs combination microtiter assay (REDCA) methods. With these results, four bacilli with different resistance profiles were selected for EPs gene expression assays. Each *M. tuberculosis* was exposed separately to 0.5 x MIC of INH and INH + VP for 48 h. Through RT-qPCR, the relative expression of 10 genes coding for EPs in *M. tuberculosis* was determined. Significant reduction of INH MIC was observed after combination with VP for isolates susceptible to anti-TB drugs. Briefly, gene expression assays revealed INH pressure response patterns that correlated with each resistance profile, presence or absence of mutations in resistance and combinatorial genes with VP.

Keywords: tuberculosis, efflux pumps, isoniazid, verapamil.

LISTA DE ILUSTRAÇÕES

CAPÍTULO I

CAPÍTULO II

Dissertação elaborada e formatada conforme as normas da ABNT, com referências e citações seguindo a norma Vancouver (Capítulo I e III). O manuscrito (Capítulo II) segue as normas do periódico *Tuberculosis*, Disponível em: <https://www.elsevier.com/journals/tuberculosis/1472-9792/guide-for-authors>

SUMÁRIO

CAPÍTULO I

1.1 INTRODUÇÃO

1.1.1 Tuberculose: epidemiologia, transmissão e tratamento.

A tuberculose (TB) é uma doença infectocontagiosa conhecida há séculos e em humanos é causada por bacilos do complexo *Mycobacterium tuberculosis*. Recentemente, a TB ultrapassou HIV/AIDS e se tornou a doença causada por um único agente infeccioso que mais mata no mundo, com cerca de 1.6 milhões de mortes em 2017 (1).

A transmissão ocorre, em sua maioria, por inalação de gotículas expelidas por indivíduos com a forma ativa da doença. Após a inalação, a infecção segue para a forma latente ou subclínica sendo que em uma pequena proporção dos casos, pode evoluir para a TB doença, sendo a manifestação pulmonar a mais comum (2). Cerca de 15% dos casos de TB manifestam-se fora do parênquima pulmonar, logo são denominados de TB extrapulmonar (TBEP) (2).

A manifestação crônica da doença é a mais comum, gerando diversas consequências na vida do portador, muitas vezes o impedindo de ter uma vida de qualidade e privando-o de certas interações sociais. Estima-se que a TB seja responsável por gastos de até 6 bilhões de dólares anualmente, sem incluir gastos com pesquisa e desenvolvimento (1). Se levado em conta que frequentemente a doença afeta o indivíduo em sua idade mais produtiva, os prejuízos são ainda maiores.

Estima-se que cerca de um terço da população mundial esteja infectada e um número ainda maior tenha pelo menos entrado em contato com o bacilo (2). O Brasil se encontra no grupo de 30 países que concentram 87% dos casos de TB mundialmente, sendo que as maiores preocupações no país são o grande número de casos e coinfecção com o vírus HIV (1).

O primeiro tratamento para TB data de 1942, com o descobrimento do fármaco estreptomicina (3). Desde então, pesquisadores e entidades governamentais propuseram diferentes esquemas de tratamento, porém baixos índices de sucesso terapêutico e o

surgimento de resistência aos fármacos empregados inviabilizaram várias das estratégias propostas.

Na tentativa de aumentar os índices de cura e evitar o surgimento de casos de resistência, a Organização Mundial da Saúde (OMS) propõe um esquema básico de tratamento para a TB, que perdura por seis meses: dois meses de tratamento intensivo com o emprego de isoniazida (INH), rifampicina (RIF), etambutol (EMB) e pirazinamida (PZA); seguidos por uma fase de manutenção com quatro meses de INH e RIF (1). No Brasil, o tratamento completo é disponibilizado gratuitamente pelo sistema único de saúde e todos os anos as diretrizes de tratamento são atualizadas pelo plano nacional de controle à TB (4).

Mesmo no cenário em que *M. tuberculosis* seja suscetível a fármacos, vários obstáculos dificultam o tratamento da TB e fazem com que o número de opções terapêuticas seja extremamente limitado. Dentre esses podemos citar: i) resistência intrínseca a certos antimicrobianos (por exemplo, beta-lactâmicos) devido à baixa permeabilidade de sua parede celular ou produção de enzimas hidrolíticas (5); ii) seu tempo de multiplicação elevado que muitas vezes inviabiliza fármacos que tem um período de meia-vida sistêmico curto; iii) possibilidade de ocorrência de estado de dormência bacteriana durante a infecção, com bacilos metabolicamente menos ativos e consequente diminuição da ação antimicrobiana (6); iv) e sistemas de efluxo são capazes de reduzir as concentrações intracelulares de compostos tóxicos, tornando a bactéria tolerante a diversos fármacos (7).

Além dos mecanismos citados anteriormente, *M. tuberculosis* pode desenvolver resistência adquirida durante um tratamento inapropriado. Brevemente, ao abandonar o tratamento antes da conclusão, bacilos resistentes podem ser selecionados e assim linhagens resistentes são estabelecidas. De modo geral, a resistência para os principais fármacos anti-TB se da por erros na replicação do material genético e falhas nos sistemas de reparo de DNA (8). Mais especificamente, se essas mutações ocorrerem em sítios importantes para a função de um fármaco (ex. sítios catalíticos), esse bacilo se torna resistente (8).

A seleção de bacilos resistentes a qualquer um dos fármacos de primeira linha anti-TB reduz grandemente a eficácia do tratamento. Para INH, a resistência é particularmente importante, uma vez que este fármaco demonstra alta atividade bactericida contra bacilos em replicação ativa, reduzindo a carga bacilar pulmonar e as chances de transmissão em poucos dias (2).

Quando a resistência a INH é acompanhada de resistência a RIF, temos a denominação de resistência a múltiplos fármacos (MDR). Nestes casos, fármacos de segunda-linha têm que

ser empregados. Infelizmente, tais fármacos (ex. aminoglicosídeos) são mais tóxicos e frequentemente causam efeitos adversos, tornando o tratamento ainda mais difícil (9). Cerca de 160 mil novos casos de TB MDR foram reportados em 2017. Do total de casos de TB MDR, 87 % iniciaram o tratamento e apenas 55 % obtiveram cura (1).

Para *Mycobacterium tuberculosis* que são classificados como MDR e adicionalmente apresentam resistência a uma fluoroquinolona e a um medicamento injetável de segunda linha (amicacina, canamicina ou capreomicina) aplica-se a denominação de resistência extensiva a fármacos (XDR). Em situações de TB XDR, os índices de sucesso de tratamento caem para cerca de 30 % (1). Adicionalmente, já foram reportados casos de TB totalmente resistente a fármacos, na qual as chances de sucesso no tratamento são ainda menores (1,2).

1.1.2 Isoniazida: mecanismo de ação e resistência

A INH é um dos principais fármacos no esquema terapêutico contra a TB. Curiosamente, o primeiro uso da molécula de INH foi como um fármaco antidepressivo (10), sendo que sua atividade antimicobacteriana foi descrita posteriormente. Middlebrook e colaboradores (11) foram responsáveis pela primeira descrição de tal atividade, juntamente com a ocorrência de resistência à INH *in vitro*. Em 1970 e 1972 foi descrito que INH age inibindo a síntese de ácidos micólicos, um dos principais componentes da parede celular micobacteriana (12) e que essa inibição está correlacionada com a morte celular (13), respectivamente. Ainda antes da virada do século, os principais mecanismos de ação e resistência a INH foram elucidados (14–17). A Figura 1, adaptada de Islam et al. (2017), evidencia as principais etapas do descobrimento do fármaco INH, bem como seu mecanismo de ação (8).

Com base nessas e em outras publicações subsequentes, o mecanismo de ação de INH, bem como as principais causas da resistência foram entendidas. Resumidamente, INH precisa ser metabolizada pela enzima catalase-peroxidase micobacteriana (KatG) para iniciar sua ação no bacilo; passando do seu estado de pró-fármaco (ácido isonicotínico hidrazida) para fármaco propriamente dito (ácido isonicotínico). Logo, mutações no gene (*katG*) que codifica a enzima necessária para a ativação do fármaco, são as principais responsáveis pelo surgimento de resistência à INH. Mudanças no sítio ativo de KatG dificultam a acomodação da INH no sítio catalítico da enzima, levando a grande aumento na concentração inibitória mínima (CIM) de INH para *M. tuberculosis* (18).

Em seu estado ativo, a INH reage não enzimaticamente com o co-fator NAD^+ transformando-se em um aduto de ácido isonicotínico-NAD^+ (8). Após essa modificação, a molécula de INH tem alta afinidade pela enzima enoil-ACP redutase NADH dependente (InhA),, codificada pelo gene *inhA*. Essa enzima faz parte da via de síntese de ácidos graxos (FASII) e é responsável por adicionar carbonos na molécula de ácido micólico "pré-maturo" (15), sendo que inibição desta impede a síntese dos ácidos micólicos e leva a morte do bacilo (13). Para este gene, mutações estruturais e no promotor já foram reportadas como causadoras de resistência a INH. Enquanto mutações estruturais são responsáveis pela diminuição da afinidade da enzima pelo aduto, mutações no promotor gênico causam superexpressão da enzima, assim a inibição que seria causada por uma concentração baixa de INH ativada, se torna insuficiente, sendo necessárias concentrações maiores para um bloqueio completo (19).

O mecanismo de ação da INH está diretamente ligado a enzimas pertencentes às vias de estresse oxidativo e síntese de ácidos graxos. Assim, mutações que alterem essas vias metabólicas podem afetar a susceptibilidade à INH com maior ou menor magnitude (20,21).

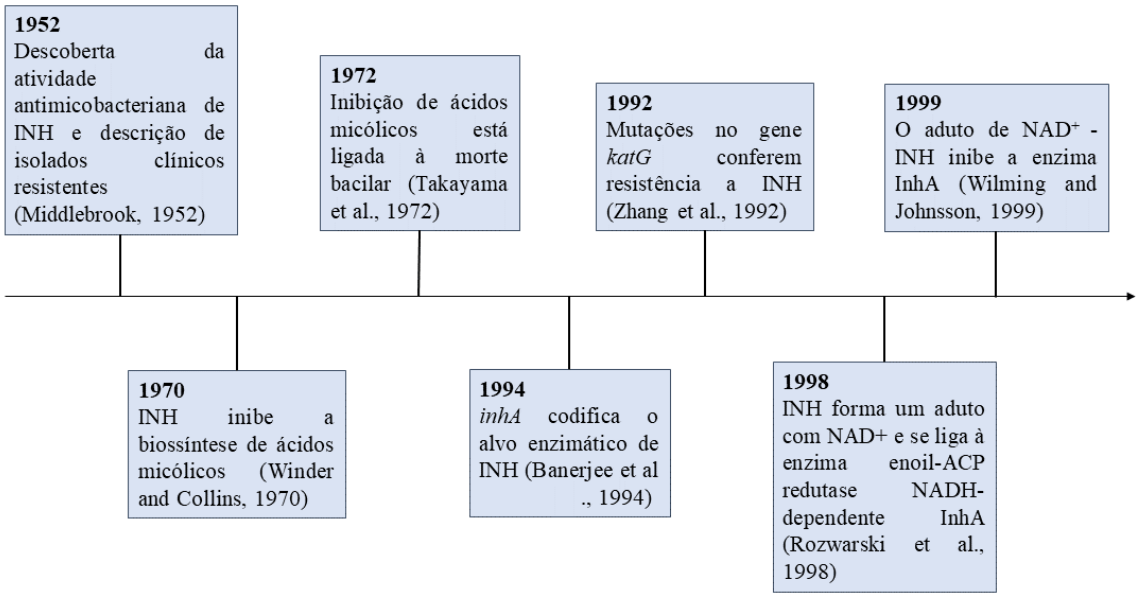


Figura 1. Histórico do descobrimento e elucidação dos principais mecanismos de resistência à isoniazida em *Mycobacterium tuberculosis.* Adaptada de Islam et al. (2017). INH, isoniazida; *inhA*, gene que codifica para a enzima enoil-ACP redutase NADH dependente; *katG*, gene que codifica para enzima catalase-peroxidase.

Apesar do avanço realizado no entendimento dos mecanismos moleculares de resistência em *M. tuberculosis*, muito ainda há a esclarecer. Em uma pequena parcela dos isolados clínicos, a resistência não pode ser explicada pela presença de mutações gênicas o

que leva a crer que mecanismos alternativos estão ativos. Dentre esses, destacam-se sistemas de efluxo bacterianos. Apesar de as bombas de efluxo (BEs) serem capazes de estruir uma grande variedade de compostos tóxicos ao microrganismo, é a sua habilidade de diminuir as concentrações intracelulares de fármacos na bactéria que gera maiores consequências no tratamento (22).

Desde 2003, publicações na literatura trazem dados de como os sistemas de efluxo micobacterianos impactam na resistência a fármacos (23,24) e atualmente o papel desses sistemas já é amplamente aceito (25–27). Além de colaborarem para a redução na susceptibilidade a um fármaco, comumente podem causar resistência cruzada a outros, uma vez que cada BE tem uma grande variedade de possíveis substratos (22).

Para estudar a fundo o papel de sistemas de efluxo na resistência micobacteriana, pesquisadores vem combinando moléculas capazes de inibir tais bombas com os antimicrobianos comumente utilizados na terapia anti-TB. Dentre os inibidores de bombas de efluxo (IBEs) mais utilizados, podemos citar verapamil (VP) por ser capaz de inibir BEs de diversas famílias (28) e ter características farmacológicas bem estabelecidas (29–31). Em humanos, VP é utilizado como um inibidor de canais de cálcio, para o tratamento de condições cardiovasculares, como arritmias e hipertensão arterial. Em mamíferos, já foi comprovado que VP inibe a glicoproteína P (P-gp), um transportador presente na maior parte do trato gastrointestinal, classificado na família ATP-*binding cassette* (ABC) que também é encontrada e micobactérias (7).

Proteínas das famílias *resistance nodulation division* (RND), *small multidrug resistance family* (SMR) e *major facilitator superfamily* (MFS) já foram relacionadas a algum grau de resistência a INH (32–34), porém o papel das BEs na resistência a este fármaco ainda é controverso, uma vez que a molécula do pró-fármaco INH é pequena, solúvel em água e sem carga iônica em pH fisiológico, características essas que garantem a difusão passiva por membranas (35).

1.1.3 Avaliação da susceptibilidade de *Mycobacterium tuberculosis* a fármacos

Avaliar os níveis de susceptibilidade á fármacos de *M. tuberculosis* fornece informações importantes que devem ser levadas em conta para o tratamento de um paciente com TB (36). Baseado na resposta indicada pelo teste realizado, o clínico pode optar por esquemas terapêuticos que sejam mais eficazes ou que apresentem maior possibilidade de cura.

Devido às características peculiares de *M. tuberculosis*, métodos convencionais de determinação de susceptibilidade *in vitro* apresentam algumas limitações. Dentre essas características, o tempo de crescimento elevado de bactérias do complexo *M. tuberculosis* e o alto nível de biossegurança necessário para manipular amostras clínicas possivelmente positivas e culturas desse microrganismo são as que se apresentam como os maiores obstáculos (37).

O método mais utilizado em centros de referência é o equipamento BD BACTET MGIT (Becton e Dickinson). O sistema baseia-se no método das proporções, ou seja, possui concentrações fixas dos fármacos de primeira linha anti-TB. Caso haja detecção de crescimento bacteriano no tubo contendo o fármaco em questão, o isolado clínico é considerado resistente [38].

O equipamento possui sensores que detectam pequenas mudanças na concentração de oxigênio presente na cultura a cada 60 minutos, assim possibilitando maior sensibilidade na detecção de crescimento. Apesar de prover agilidade, sensibilidade e confiança no diagnóstico de resistência micobacteriana, o sistema BD BACTEC MGIT tem limitações importantes como alto custo de implementação e falta de resultados quantitativos de susceptibilidade que indiquem qual é a concentração inibitória mínima (CIM) do fármaco em questão (37). Desta forma, pesquisadores lançam mão de métodos que sejam ao mesmo tempo eficientes e com melhor custo-benefício.

1.1.3.1 *Resazurin Microtiter Assay Plate* (REMA)

Dentre os métodos mais utilizados por pesquisadores para a avaliação de susceptibilidade a fármacos, destaca-se o método REMA, proposto por Palomino et al. (2002) (38). Brevemente, o método trata-se de uma microdiluição de fármacos em meio de cultura Middlebrook 7H9, com posterior adição de cultura micobacteriana padronizada na escala de turbidez de McFarland 1.0 e diluída 1:20 em Middlebrook 7H9. Por ser realizado em microplacas de 96 orifícios, uma variedade de fármacos pode ser testada, garantindo grande versatilidade ao ensaio.

Após término do preparo da placa, a preparação é incubada por sete dias em estufa em temperatura de 35 ± 2 ºC, à atmosfera normal. Decorrido o tempo de incubação, adiciona-se 30 µL de uma solução de 0,01 % de resazurina. Após 24 h de incubação nas mesmas condições, faz-se a leitura da microplaca. A mudança visual da coloração azul para rosa,

indica redução da resazurina e, portanto, crescimento bacteriano. A CIM é determinada como o último orifício que não demonstrou viragem da cor.

O método de REMA apresenta-se como uma alternativa interessante para o pesquisador, uma vez que não são necessários equipamentos específicos além dos já disponíveis em laboratórios que trabalham com bactérias do complexo *M. tuberculosis* (38).

Adicionalmente, a característica de microdiluição permite a avaliação quantitativa da susceptibilidade dos *M. tuberculosis* testados. Assim, possibilitando a classificação dos níveis de resistência de forma acurada.

1.1.4 Avaliação do efeito combinatório entre fármacos anti-TB

Tendo em vista o regime poliquimioterápico utilizado para o tratamento da TB, o estudo sobre a interação entre fármacos é de suma importância para a proposta de novas estratégias. Diversas metodologias podem ser utilizadas para estudar a interação entre fármacos, desde análises de atividade anti-micobacteriana *in vitro*, até ensaios *in vivo* com modelos de infecção em animais de laboratório (39).

Dentre as metodologias disponíveis *in vitro*, podemos destacar o método *rezasurin drugs combination microtiter assay* (REDCA), padronizado por Caleffi-Ferracioli et al. (2013) (40). Brevemente, o método tem preparo similar ao REMA mencionado anteriormente, porém permite a diluição simultânea de dois fármacos. No eixo x da microplaca de 96 orifícios, é adicionado fármaco A, que é diluído verticalmente em série; após, é adicionado no eixo y o fármaco B, que é então diluído horizontalmente. As condições de incubação, revelação e leitura do ensaio são as mesmas do método REMA (40).

Dependendo do tipo de combinação utilizado, pode-se aplicar diferentes cálculos para interpretar os efeitos combinatórios entre os fármacos. Se realizada combinação entre dois antimicrobianos, pode-se calcular índices como o FICI, do inglês índice de concentração inibitória fracional, por meio da equação "$FICI = CIM_{(\text{fármaco A combinado ao B})}/CIM_{(\text{fármaco A})} + CIM_{(\text{fármaco B combinado à A})}/CIM_{(\text{fármaco B})}$". A interpretação desse índice revela se a interação entre os fármacos foi sinérgica ($FICI \leq 0,5$); indiferente ($0,5 > FICI \leq 4$); e antagônica ($FICI > 4$) (41).

Alternativamente, quando os testes são feitos para avaliar a interação entre um antimicrobiano e um candidato a fármaco (ex. inibidor de bomba de efluxo), o uso do FIC, do inglês concentração inibitória fracional é mais adequado. Diferentemente do FICI, o FIC considera apenas o efeito do candidato sobre o antimicrobiano em questão. A caracterização

da interação entre os fármacos se dá como sinérgica (FIC ≤ 0,25); indiferente (0,25 > FIC ≤ 2); e antagônica (FIC > 2), de acordo o resultado obtido na equação "FIC = $CIM_{(fármaco\ A)}$ /$CIM_{(Fármaco\ A\ combinado\ ao\ B)}$"(42).

De modo similar ao FIC, pode-se calcular o fator modulador (FM) através da equação "FM = $CIM_{(Fármaco\ A\ combinado\ ao\ B)}$/$CIM_{(fármaco\ A)}$. Comparado aos outros índices, FM tem a vantagem de evidenciar diretamente qual foi o efeito combinatório que o fármaco B exerceu no fármaco A. Por exemplo, um valor de FM de oito, significa que a combinação foi capaz de reduzir a CIM do fármaco A em oito vezes (42).

1.2 JUSTIFICATIVA

O desenvolvimento de resistência a qualquer um dos fármacos de primeira linha contra a TB dificulta muito o tratamento da doença, podendo aumentar o tempo de tratamento para 24 meses ou mais. Apesar de os mecanismos mais clássicos de resistência em *M. tuberculosis* já estarem bem caracterizados, bombas de efluxo também têm mostrado papel importante neste fenômeno. Graças a tais sistemas de efluxo, a bactéria é capaz de resistir à pressão inicial do tratamento até surgirem mutações espontâneas no genoma que acarretam em reduzida susceptibilidade.

Considerando o impacto da resistência micobacteriana no tratamento da TB, o estudo destes mecanismos de resistência secundários são de suma importância para o controle da doença, bem como o desenvolvimento de novas estratégias que possam ser efetivas no enfretamento da TB.

1.3 OBJETIVOS

1.3.1 Geral

Avaliar o papel de bombas de efluxo na resistência ao fármaco isoniazida de *M. tuberculosis.*

1.3.2 Objetivos específicos

- Determinar a concentração inibitória mínima (CIM) dos fármacos isoniazida (INH) e verapamil (VP) para *M. tuberculosis*, através do método *Resazurin microtiter assay plate* (REMA);
- Avaliar o efeito combinatório entre INH e VP em *M. tuberculosis*, por meio do método *resazurin drugs combination microtiter assay* (REDCA);
- Selecionar *M. tuberculosis* com diferentes CIMs e efeitos combinatórios de INH e VP para estudos de expressão gênica de bombas de efluxo, de acordo com os resultados dos métodos anteriores;
- Analisar a expressão relativa de 10 genes que codificam bombas de efluxo nos *M. tuberculosis* selecionados.

REFERÊNCIAS

1. WHO. Global Tuberculosis Report 2018 [Internet]. Geneva, Switzerland.; 2018. Available from: http://www.who.int/tb/publications/global_report/en/

2. Grosset JH, Chaisson RE. Handbook of Tuberculosis [Internet]. Grosset JH, Chaisson RE, editors. Cham: Springer International Publishing; 2017. Available from: http://link.springer.com/10.1007/978-3-319-26273-4

3. Flechner I, Maruta K, Burkart V, Kawai K, Kolb H, Kiesel U. Streptomycin treatment of pulmonary tuberculosis. Br Med J [Internet]. 1948 Oct 30;2(4582):769–82. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2091872

4. Ministério da Saúde. Panorama da Tuberculose no Brasil [Internet]. Brasília, Distrito Federal; 2016 Jun. Available from: http://portalsaude.saude.gov.br/images/pdf/2014/novembro/24/panorama-tuberculose-brasil-out14-web.pdf

5. Nguyen L. Antibiotic resistance mechanisms in *M. tuberculosis*: an update. Arch Toxicol. 2017;90(7):1585–604.

6. Zhang Y. THE MAGIC BULLETS AND TUBERCULOSIS DRUG TARGETS. Annu Rev Pharmacol Toxicol [Internet]. 2005 Feb;45(1):529–64. Available from: http://www.annualreviews.org/doi/10.1146/annurev.pharmtox.45.120403.100120

7. Schmalstieg AM, Srivastava S, Belkaya S, Deshpande D, Meek C, Leff R, et al. The

Antibiotic Resistance Arrow of Time: Efflux Pump Induction Is a General First Step in the Evolution of Mycobacterial Drug Resistance. Antimicrob Agents Chemother [Internet]. 2012 Sep;56(9):4806–15. Available from: http://aac.asm.org/lookup/doi/10.1128/AAC.05546-11

8. Islam MM, Hameed HMA, Mugweru J, Chhotaray C, Wang C, Tan Y, et al. Drug resistance mechanisms and novel drug targets for tuberculosis therapy. J Genet Genomics [Internet]. 2017 Jan;44(1):21–37. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1673852716301497

9. Green KD, Garneau-Tsodikova S. Resistance in tuberculosis: what do we know and where can we go? Front Microbiol [Internet]. 2013;4:208. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2013.00208/abstract

10. Moncrieff J. The creation of the concept of an antidepressant: An historical analysis. Soc Sci Med [Internet]. 2008 Jun;66(11):2346–55. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0277953608000129

11. MIDDLEBROOK G. Sterilization of tubercle bacilli by isonicotinic acid hydrazide and the incidence of variants resistant to the drug in vitro. Am Rev Tuberc. 1952 Jun;65(6):765–7.

12. Winder FG, Collins PB. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J Gen Microbiol. 1970 Sep;63(1):41–8.

13. Takayama K, Wang L, David HL. Effect of Isoniazid on the In Vivo Mycolic Acid Synthesis, Cell Growth, and Viability of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother [Internet]. 1972 Jul 1;2(1):29 LP-35. Available from: http://aac.asm.org/content/2/1/29.abstract

14. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase—peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature [Internet]. 1992 Aug 13;358:591. Available from: http://dx.doi.org/10.1038/358591a0

15. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, et al. inhA, a Gene Encoding a Target for isoniazid and Ethionamide in *Mycobacterium tuberculosis*. Science (80-). 1993;263(September):227–30.

16. Rozwarski DA, Grant GA, Barton DHR, Jacobs WR, Sacchettini JC. Modification of

the NADH of the Isoniazid Target (InhA) from *Mycobacterium tuberculosis*. Science (80-) [Internet]. 1998 Jan 2;279(5347):98 LP-102. Available from: http://science.sciencemag.org/content/279/5347/98.abstract

17. Wilming M, Johnsson K. Spontaneous Formation of the Bioactive Form of the Tuberculosis Drug Isoniazid. Angew Chemie Int Ed [Internet]. 1999 Sep 6;38(17):2588–90. Available from: https://doi.org/10.1002/(SICI)1521-3773(19990903)38:17%3C2588::AID-ANIE2588%3E3.0.CO

18. Pimentel AL, de Lima Scodro RB, Caleffi-Ferracioli KR, Siqueira VLD, Campanerut-Sá PAZ, Lopes LDG, et al. Mutations in catalase-peroxidase KatG from isoniazid resistant *Mycobacterium tuberculosis* clinical isolates: insights from molecular dynamics simulations. J Mol Model [Internet]. 2017 Apr 16;23(4):121. Available from: http://link.springer.com/10.1007/s00894-017-3290-3

19. Vilchèze C, Jacobs JR. WR. Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis*: Genes, Mutations, and Causalities. Microbiol Spectr [Internet]. 2014 Aug 22;2(4):1–21. Available from: http://www.asmscience.org/content/journal/microbiolspec/10.1128/microbiolspec.MGM2-0014-2013

20. Nieto LM, Mehaffy C, Dobos KM. The Physiology of *Mycobacterium tuberculosis* in the Context of Drug Resistance: A System Biology Perspective. In: Mycobacterium - Research and Development [Internet]. InTech; 2018. Available from: http://www.intechopen.com/books/mycobacterium-research-and-development/the-physiology-of-mycobacterium-tuberculosis-in-the-context-of-drug-resistance-a-system-biology-pers

21. Miesel L, Weisbrod TR, Marcinkeviciene JA, Bittman R, Jacobs WR. NADH dehydrogenase defects confer isoniazid resistance and conditional- lethality in *Mycobacterium smegmatis*. J Bacteriol. 1998;180(9):2459–67.

22. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, et al. Multidrug efflux pumps: structure, function and regulation. Nat Rev Microbiol [Internet]. 2018;16(9):523–39. Available from: http://dx.doi.org/10.1038/s41579-018-0048-6

23. Li X, Zhang L, Nikaido H. Efflux Pump-Mediated Intrinsic Drug Resistance in

Mycobacterium smegmatis. Antimicrob Agents Chemother. 2004;48(7):2415–23.

24. Viveiros M. Mycobacterial efflux pumps and chemotherapeutic implications. Int J Antimicrob Agents [Internet]. 2003 Sep;22(3):274–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0924857903002085

25. te Brake LHM, de Knegt GJ, de Steenwinkel JE, van Dam TJP, Burger DM, Russel FGM, et al. The role of efflux pumps in tuberculosis treatment and their promise as a target in drug development: unraveling the black box. Annu Rev Pharmacol Toxicol [Internet]. 2018 Jan 6;58(1):271–91. Available from: http://www.annualreviews.org/doi/10.1146/annurev-pharmtox-010617-052438

26. Machado D, Coelho TS, Perdigão J, Pereira C, Couto I, Portugal I, et al. Interplay between Mutations and Efflux in Drug Resistant Clinical Isolates of *Mycobacterium tuberculosis*. Front Microbiol [Internet]. 2017 Apr 27;8. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00711/full

27. Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, et al. Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity against Drug Resistant *Mycobacterium tuberculosis*. Neyrolles O, editor. PLoS One [Internet]. 2016 Feb 26;11(2):e0149326. Available from: https://dx.plos.org/10.1371/journal.pone.0149326

28. Pule CM, Sampson SL, Warren RM, Black PA, van Helden PD, Victor TC, et al. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. J Antimicrob Chemother [Internet]. 2016 Jan;71(1):17–26. Available from: https://academic.oup.com/jac/article-lookup/doi/10.1093/jac/dkv316

29. McTavish D, Sorkin EM. Verapamil. An updated review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hypertension. Drugs. 1989 Jul;38(1):19–76.

30. Schwartz JB, Todd E, Abernethy DR, Mitchell JR. Steady state verapamil tissue distribution in the dog: differing tissue accumulation. Pharmacology. 1986;32(6):307–12.

31. Solans C, Bregante MA, Aramayona JJ, Fraile LJ, Garcia MA. Comparison of the pharmacokinetics of verapamil in the pregnant and non-pregnant rabbit: study of

maternal and foetal tissue levels. Xenobiotica. 2000 Jan;30(1):93–102.

32. Pang Y, Lu J, Wang Y, Song Y, Wang S, Zhao Y. Study of the Rifampin Monoresistance Mechanism in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother [Internet]. 2013 Feb 1;57(2):893–900. Available from: http://aac.asm.org/cgi/doi/10.1128/AAC.01024-12

33. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, et al. Microarray Analysis of Efflux Pump Genes in Multidrug-Resistant *Mycobacterium tuberculosis* During Stress Induced by Common Anti-Tuberculous Drugs. Microb Drug Resist [Internet]. 2010 Mar;16(1):21–8. Available from: http://www.liebertpub.com/doi/10.1089/mdr.2009.0054

34. Sriraman K, Nilgiriwala K, Saranath D, Chatterjee A, Mistry N. Deregulation of Genes Associated with Alternate Drug Resistance Mechanisms in *Mycobacterium tuberculosis*. Curr Microbiol. 2018 Apr;75(4):394–400.

35. Bardou F, Raynaud C, Ramos C, Laneelle MA, Lanrelle G. Mechanism of isoniazid uptake in *Mycobacterium tuberculosis*. Microbiology [Internet]. 1998 Sep 1;144(9):2539–44. Available from: http://mic.microbiologyresearch.org/content/journal/micro/10.1099/00221287-144-9-2539

36. Secretaria de Vigilância em Saúde. Programa Nacional de Controle da Tuberculose [Internet]. 2018. Available from: http://portalarquivos2.saude.gov.br/images/pdf/2018/marco/19/APRES-PADRAO-JAN-2018-REDUZIDA.pdf

37. Drobniewski F, Rüsch-Gerdes S, Hoffner S. Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* (EUCAST document E.DEF 8.1) – Report of the Subcommittee on Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis* of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Clin Microbiol Infect [Internet]. 2007 Dec 1 [cited 2018 Dec 3];13(12):1144–56. Available from: https://www.sciencedirect.com/science/article/pii/S1198743X14625817

38. Palomino J-C, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug

Resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother [Internet]. 2002 Aug 1;46(8):2720–2. Available from: http://aac.asm.org/cgi/doi/10.1128/AAC.46.8.2720-2722.2002

39. Parish T, Brown AC. Mycobacteria Protocols [Internet]. Parish T, Brown AC, editors. Totowa, NJ: Humana Press; 2009. (Methods in Molecular Biology; vol. 465). Available from: http://link.springer.com/10.1007/978-1-59745-207-6

40. Caleffi-Ferracioli KR, Maltempe FG, Siqueira VLD, Cardoso RF. Fast detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard resazurin method. Tuberculosis [Internet]. 2013 Nov;93(6):660–3. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1472979213001613

41. Pillai S, Moellering R, Eliopoulos GM. Antimicrobial Combinations. 5th ed. New York: Lippincott Williams & Wilkins; 2005.

42. Coelho T, Machado D, Couto I, Maschmann R, Ramos D, von Groll A, et al. Enhancement of antibiotic activity by efflux inhibitors against multidrug resistant *Mycobacterium tuberculosis* clinical isolates from Brazil. Front Microbiol [Internet]. 2015 Apr 28;6(APR):1–12. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00330/abstract

CAPÍTULO II

Efflux pump expression and isoniazid resistance in *Mycobacterium tuberculosis*

João Vítor Perez de Souza[a,*]; Katiany Rizzieri Caleffi-Ferracioli[a]; Letícia Sayuri Murase[a]; Carolina Trevisolli Palomo[a]; Regiane Bertin de Lima Scodro[a]; Vera Lúcia Dias Siqueira[a]; Fernando Rogério Pavan[b]; Rosilene Fressatti Cardoso[a];

[a] Laboratório de Bacteriologia Médica, Universidade Estadual de Maringá, Maringá, Paraná, Brasil.

b Faculdade de Ciências Farmacêuticas, Departamento de Ciências Biológicas, Universidade Estadual Paulista, Araraquara, São Paulo, Brasil.

***Corresponding author**. João Vítor Perez de Souza. Laboratório de Bacteriologia Médica, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. Colombo Avenue, 5790, Postal number: 87020-900, Maringa, Parana, Brazil. Phone: +55 44 3011-5375; Fax: +55 44 3011-4797; E-mail: jvperezsouza@gmail.com

Abstract

Mycobacterial efflux is now widely accepted as a secondary mechanism of resistance to anti-TB drugs, but much is unclear about the relation between efflux and INH resistance. Thus, the aim of this study was to evaluate the role of efflux pumps (EPs) in the INH resistance, as well as the combinatory effect of INH and VP in *M. tuberculosis*. Minimal inhibitory concentrations and combinatory effects of INH+VP were accessed through resazurin microtiter assay plate (REMA) and resazurin drugs combination microtiter assay (REDCA), respectively. From the results of REMA and REDCA, we selected four bacilli with different susceptibility profiles and assessed their expression of 10 EPs genes through RT-qPCR after exposure to INH and INH+VP for 48 h. Significant reduction of INH MIC upon combination with VP was observed for INH susceptible isolates. Briefly, gene expression assays revealed responses against INH pressure that correlated with each resistance profile, presence or absence of *katG* mutations and combinatory effect with VP. Although the blockage of EPs does not impart in increased susceptibility for INH in bacteria with *katG* mutations, the conjunct administration of an EPI could cause important results for isolates with a low degree of INH resisatnce and act in the prevention of resistance in susceptible bacilli.

Keywords: Drug resistance, efflux pump inhibitors, verapamil, efflux pump, isoniazid, tuberculosis.

1. Introduction

Despite the existence of standard therapeutic against susceptible *Mycobacterium tuberculosis*, first line anti-tuberculosis (TB) drugs such as isoniazid (INH) and rifampicin (RIF) are ineffective to treat patients infected by multidrug resistant (MDR) bacilli. Unfortunately, second line drugs (e.g. aminoglycosides) happen to be more toxic and often cause adverse effects, making the back-up treatment even harder [1].

INH is one of the most important drugs in TB's treatment and usually is the first drug resistance to emerge [2]. The development of resistance for INH is influenced by mutations in several genes [3,4]. Of these, the two most common are in *katG*, which codifies the catalase-peroxidase responsible for the activation of INH, and *inhA*, the target of the activated drug [3]. Although there are clear evidences that mutations on the *M. tuberculosis* genome are the main responsible for INH resistance, efflux pump systems seem to also be involved [4,5].

During stress caused by a drug, the bacilli increases the expression of genes that encode membrane transport proteins, as known as efflux pumps (EPs) in an attempt to protect itself [6]. In addition, several EPs have a multi-substrate (multidrug EPs) specificity and are able to extrude a wide variety of compounds [5].

Although there is clear evidence that EPs play important roles in resistance to many anti-TB drugs [5], the impact of these efflux systems in INH resistance is not yet fully understood. The INH pro-drug is a very small molecule, that has good dissolubility in water and is non-ionized in physiological pH. These characteristics render great membrane diffusibility to INH through cell membranes [7].

Verapamil (VP) is one of the molecules used to test EP activity, due to its properties as an efflux pump inhibitor (EPI) [8]. In addition, VP is also been studied as an important adjuvant for TB therapy, since the reduce of efflux activity increases the susceptibility of *M. tuberculosis* to many anti-TB drugs [9].

Taking into consideration the limited number of drugs available to treat TB, the high burden of the disease in the world and the fast emergence of resistance, the development of new strategies to deal with TB is a primal need. Thus, the aim of this study was to evaluate the role of EPs in the INH resistance, as well as the effect of VP in combination to INH in *M. tuberculosis* isolates harboring different resistance profiles.

2. Material and methods

2.1 Bacterial strains and growth conditions

Fifteen *M. tuberculosis* isolates and the H_{37}Rv (ATCC 27294) reference strain belonging to the Laboratory of Medical Bacteriology of the State University of Maringa, Brazil were selected based upon previous detection of INH resistance by the automated method BD BACTEC ™MGIT™960 (BD, Franklin Lakes – NJ, USA).

The clinical isolates selected had already been previously characterized by MIRU-VNTR, Spoligotyping and tested by the commercial kit GenoType MTBDRplus® (Hain Lifescience GmbH, Nehren, Germany) to detect mutations related to INH and RIF resistance [10]. All data of the previous characterization is available in Table 1.

Bacilli were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA), added with 0.2% glycerol (vol/vol), 0.025% tween 80 (vol/ vol) and supplemented with OADC (Oleic acid, albumin, dextrose and catalase) (BBL/Becton-Dickinson, Sparks, MD, USA) for 15 days at 35 ± 2 ºC.

2.2 Drugs

Isoniazid and verapamil were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The stock solutions were prepared following the manufacturer's instructions and further dilutions were prepared in OADC-supplemented Middlebrook 7H9.

2.3 Minimal inhibitory concentration (MIC) and combinatory assays

The MIC of each drug was determined by the means of Resazurin Microtiter Assay Plate (REMA) as described by Palomino et al. [11].

The combinatory effect of INH+ VP was determined by resazurin drugs combination microtiter assay (REDCA), as previously described by Calleffi-Ferracioli et al.[12]. Combinations of INH+VP were applied to all *M. tuberculosis* tested. To evaluate the combinatory effect of the association, the modulation factor (MF) was calculated by the means of: $MF = MIC_{(INH\ )}/ MIC_{(INH+VP)}$. MF reflects the MIC reduction of a given antimicrobial when combined to an EPI. A MF value ≥4 (four fold reduction) was considered significant [13].

2.4 Effux pump gene expression

Four *M. tuberculosis* were selected to access gene expression: $H_{37}Rv$ (reference strain); BRF 47 (susceptible isolate); BRF 14 (INH monoresistant isolate, harboring S315T *katG* mutation); and 3614, an MDR isolate resistant to INH, RIF and EMB (harboring c-15t and I21T mutations in the *inhA* gene) (Table 1). The bacilli were exposed to 0.5×MIC of INH or INH+VP for 48 h at 35 ± 2 °C. Additionally, pure bacterial culture was used as a control sample. RNA was extracted and purified using RNeasy Plus Mini Kit (Qiagen Biotechnology, Valencia, CA, USA) and quantified in Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA, USA). DNA contaminants were removed with RNase-free DNase I (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized using random primers of total RNA and SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The qPCR was performed using Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with specific primers for the EPs studied (Table 2) in the StepOne Real-Time PCR Systems (ThermoFisher Scientific, Waltham, MA, USA). Melting curves and negative controls were performed in all reactions. The 16S RNA gene (*rrs*) was used to normalize all reactions and samples were tested in triplicate. Relative quantification of target gene expression was calculated by the means of $2^{-\Delta\Delta}CT$ [14] method and rates above two (>2) and lower than 0.5 (<0.5) were considered as significantly up-regulated and down-regulated, respectively.

3. Results and Discussion

The major resistance mechanism to INH is due to spontaneous mutations on genes responsible either for the activation or which are the target of this drug [3]. Although that is widely known, alternative resistance mechanisms such as EP extrusion seem to also take part in reduced susceptibility for this drug. Thus, we sought to evaluate the role of EPs in INH resistance as well as the effect of the EPI VP in combination to INH in EP genes expression of *M. tuberculosis*. To our knowledge, this is the first study that assayed 10 EP genes in *M. tuberculosis* harboring different resistance profiles exposed to INH and INH+VP.

From the 15 clinical isolates tested, 12 (80%) were monoresistant to INH, two (13.33%) susceptible to first-line anti-TB drugs and one MDR (6.67%). Minimal inhibitory concentrations of VP and INH for the reference strain and the susceptible clinical isolates, ranged from 125 to 250 µg/mL and 0.06 to 0.125 µg/mL, respectively. For the INH resistant and MDR clinical isolates, MICs of VP and INH had a range from 62.5 to 250 µg/mL and 4 to 32 µg/mL, respectively (Table 3).

From these results, we tested INH and VP combined to access if the blockade of EPs would impart in increased susceptibility for INH (Table 3). Both susceptible bacilli tested (BRF 35 and 47) presented great reductions (MF= eight and 16) of INH MIC upon combination to VP, while the

reference strain did not (MF = 2). Regarding the INH monoresistant isolates, all of them (12) did not present great reductions of INH MIC upon combination with VP (MF= 1 or 2). One possible explanation for these results is the presence of *katG* (S315T) mutations (Table 1), which greatly hinder the access of INH to the catalytic site of the catalase-peroxidase enzyme [15]. In these isolates, INH is not metabolized to its active form (or at least less metabolized) and so will not cause damage to the bacteria. In this sense, the reduction of EP activity by VP would not act in a synergic way with INH. Similarly, the MDR isolate (3614) tested did not show significant reduction of INH MIC (*inhA* mutations). Differently from our results, a previous study revealed great modulatory effects of INH+VP in MDR and XDR isolates harboring similar *inhA* mutations [9].

Although at first look our modulatory results seem discouraging, the great reduction of INH MIC when combined to VP observed for the susceptible isolates provide interesting potential for future therapy. As it is now consensus [6,16], efflux systems act as one of the first defense mechanisms for bacteria to survive the early pressure of antibiotics. Additionally, these pumps act as the "middleman" until a random mutation in resistance genes appears and impart in a more definitive resistance phenotype for *M. tuberculosis* [16]. In this sense, the combination of INH+VP could aid in the initial clearance process of bacilli by INH and reduce the persistence of bacteria that one day might acquire *de novo* genetic resistance.

In our study, only susceptible isolates showed significant reductions of INH MIC upon combination to VP. Differently from our findings, Machado et al. [17] observed that after *in vitro* induction of resistance to INH, the conjunct administration of INH and VP reduced INH MIC at the same level of the completely susceptible predecessor strain. Agreeing with our results, no significant MIC reductions was observed in isolates who acquired *katG* mutations during the induction process.

In order to evaluate how the EPs of *M. tuberculosis* respond to INH pressure, we selected four *M. tuberculosis* with different susceptibility profiles to access EP gene expression (Table 3). For H_{37}Rv, an overall low response in gene expression was observed for the majority of exposures and genes studied (Figure 1), in comparison with the control. Exposure INH caused no significant overexpression and, in fact, three genes (*Rv1410c, Rv1458c* and *Rv1819c*) were found downregulated. Our results agree with a previous study that showed no significant overexpression of EPs genes for the H_{37}Rv strain, upon exposure to INH [4]. Considering the origin and the genetic background of the reference strain, this pattern could be explained by its pan-susceptibility and lack of some virulence genes [18]. Seemingly, the combination with VP was able to improve the pressure of INH on the strain. This is exemplified by two genes (*Rv2942* and *Rv3065)* that did not present differential expression when exposed to INH alone. Similar results were also found for these genes in the other isolates tested (Figures 1-4). From the cited genes, *Rv2942* has been related

to maintenance of virulence factors, lipid transport and efflux of INH in *M. tuberculosis* [17,19,20] and *M. smegmatis* [21]; and *Rv3065* has been associated with resistance to INH and RIF [16,22].

Although $H_{37}Rv$ (Figure 1) and BRF 47 (Figure 2) are both susceptible to first-line anti-TB drugs, the gene expression profiles observed were quite different. Here, INH exposure caused overexpression of seven genes versus none for the reference strain. All of them (*Rv2942, Rv2846, Rv3065, Rv1258, Rv1458, Rv1218* and *Rv1819)* have been related to some degree of INH resistance and/or found overexpressed in resistant isolates [16,23–27]. A previous report [4] has pointed out that *Rv2846*, one of such genes, is overexpressed in INH exposure mainly in susceptible isolates. This information agrees with our data, in which BRF 47 (Figure 2) isolate presented overexpression of this gene, whereas the monoresistant one (BRF 14) did not (Figure 3). The combination of the anti-TB drug + EPI caused a noticeable increase in the expression rates for the majority of genes tested. This overall increase in the gene expression rates of INH+VP when compared to INH alone, agrees with the results from REDCA, in which the combination of drugs was able to reduce INH MIC by as much as 16-fold for BRF 47 isolate (Table 3).

For the BRF 14 isolate, RT-qPCR revealed an overall low response in EP gene expression upon exposure to INH alone and in combination to VP (Figure 3). As discussed earlier, the S315T mutation in *katG* greatly hinders the activation of INH by the catalase enzyme (KatG), thus the drug probably will not cause any stress to the bacteria. In spite of limited to four genes (*Rv3065, Rv1456c*, *Rv1458c* and *Rv1218c*), the combination of INH+VP was able to improve the pressure of INH in this isolate. The limited action of the combination agrees with the lack of modulation seen in REDCA for this bacillus (Table 3). Unfortunately, BRF 14 did not show quantifiable levels of *Rv2942*, so its response remains unsure.

Although VP was not able to reduce INH MIC in a significant manner for the 3614 isolate (MF=2), the gene expression assays revealed patterns different from the other resistant organism. Here, exposure to INH caused overexpression of all genes studied (Figure 4) despite the fact that this isolate has c-15t and I21T mutations in the *inhA* gene. Mutations in the promoter region of *inhA* (e.g. c-15t) increase the expression of the NADH-dependent enoyl-ACP reductase (InhA), the target of activated INH, up to 20-fold, rendering the inhibitory activity of INH insufficient [28]. Differently from mutations in the *katG* gene that mostly nullify INH action, here the drug is still able to cause damage. In addition, it has been proposed that activated INH has secondary targets besides InhA [29]. Regarding the combination with VP, there was apparently no improvement for this isolate with the exception of *Rv3065*. In fact, most genes demonstrated a decrease in expression rates compared to INH alone.

Of all genes studied, *Rv1410c* stands out since it is associated with the transport of INH, lipids and important virulence factors from the cytosol to the cell wall of *M. tuberculosis* [30]. Of

note, this gene was found downregulated or without significant change for $H_{37}Rv$, BRF 47 and BRF 14, while upregulated for 3614 isolate under INH exposure. Agreeing with our results, previous reports [4,31] have shown that *Rv1410c* is found overexpressed in MDR/XDR isolates and without significant change for $H_{37}Rv$ and susceptible isolates exposed to INH. Additionally, the operon formed *Rv1410* and *Rv1411* have been proposed as a possible target for drug development, since deletion of this operon caused great attenuation of *M. tuberculosis* growth inside macrophages [32,33].

Although limited to one microorganism for each resistance profile, comparing each gene expression pattern provides us some data regarding the evolution of each bacilli as well as the emergence of resistance to INH. Taking a closer look to isolates BRF 47 and BRF 14 (Figures 2 and 3, respectively) a striking difference in the expression rates upon INH exposure can be noted. Possibly, BRF 47 is in an evolutionary stage that precedes the emergence of a mutation related to resistance and so relies solely in the expression of EPs to tolerate the damage caused by INH. Following this line of thought, an inappropriate treatment has led to the selection of BRF 14, an INH resistant *M. tuberculosis* with a mutation in the *katG* gene. Although BRF 14 went through similar pressures and is able to express EPs in defense, now INH will not be activated and will not cause damage. Previous report by Machado et al exemplify this line of thought showing how exposure to INH maintained a constant high-level EP gene expression in stages that preceded the appearance of mutations in *katG* [17].

4. Conclusions

To this date, many papers have shown that efflux pumps play pivotal roles in resistance to many anti-TB drugs. For isoniazid, these systems allow that susceptible bacillus survive the initial pressure of the drug until a mutation in resistance genes randomly appears. Although the blockage of EPs does not impart in increased susceptibility for bacteria with *katG* mutations, the conjunct administration of an EPI could cause important results for isolates harboring more permissive mutations (e.g. *inhA)* and act in the prevention of resistance in susceptible bacilli.

Financial disclosure

This study was supported by the National Council of Technological and Scientific Development (CNPq) (grant 2016/14957-5). The funding agency did not participate in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Acknowledgements

The authors are thankful to the staff of the Medical Bacteriology Laboratory from State University of Maringa - Parana, Brazil, Coordination of Superior Education Personnel Improvement (CAPES)

and National Council of Technological and Scientific Development (CNPq) for grant 2016/14957-5.

References

[1] WHO. Global Tuberculosis Report 2018. Geneva, Switzerland.: 2018.

[2] Manson AL, Cohen KA, Abeel T, Desjardins CA, Armstrong DT, Barry CE, et al. Genomic analysis of globally diverse *Mycobacterium tuberculosis* strains provides insights into the emergence and spread of multidrug resistance. Nat Genet 2017;49:395–402. doi:10.1038/ng.3767.

[3] Laurenzo D, Mousa SA. Mechanisms of drug resistance in Mycobacterium tuberculosis and current status of rapid molecular diagnostic testing. Acta Trop 2011;119:5–10. doi:10.1016/j.actatropica.2011.04.008.

[4] Narang A, Giri A, Gupta S, Garima K, Bose M, Varma-Basil M. Contribution of putative efflux pump genes to isoniazid resistance in clinical isolates of *Mycobacterium tuberculosis*. Int J Mycobacteriology 2017;6:177. doi:10.4103/ijmy.ijmy_26_17.

[5] Pule CM, Sampson SL, Warren RM, Black PA, van Helden PD, Victor TC, et al. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. J Antimicrob Chemother 2016;71:17–26. doi:10.1093/jac/dkv316.

[6] Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, et al. Microarray Analysis of Efflux Pump Genes in Multidrug-Resistant *Mycobacterium tuberculosis* During Stress Induced by Common Anti-Tuberculous Drugs. Microb Drug Resist 2010;16:21–8. doi:10.1089/mdr.2009.0054.

[7] Bardou F, Raynaud C, Ramos C, Laneelle MA, Lanrelle G. Mechanism of isoniazid uptake in *Mycobacterium tuberculosis*. Microbiology 1998;144:2539–44. doi:10.1099/00221287-144-9-2539.

[8] Machado D, Coelho TS, Perdigão J, Pereira C, Couto I, Portugal I, et al. Interplay between Mutations and Efflux in Drug Resistant Clinical Isolates of *Mycobacterium tuberculosis*. Front Microbiol 2017;8. doi:10.3389/fmicb.2017.00711.

[9] Machado D, Pires D, Perdigão J, Couto I, Portugal I, Martins M, et al. Ion Channel Blockers as Antimicrobial Agents, Efflux Inhibitors, and Enhancers of Macrophage Killing Activity against Drug Resistant *Mycobacterium tuberculosis*. PLoS One 2016;11:e0149326. doi:10.1371/journal.pone.0149326.

[10] de Almeida AL, Scodro RB de L, de Carvalho HC, Costacurta GF, Baldin VP, Santos NCS, et al. RDRio *Mycobacterium tuberculosis* lineage in the Brazil/Paraguay/Argentina triple border. Tuberculosis 2018;110:68–78. doi:10.1016/j.tube.2018.03.008.

[11] Palomino J-C, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin Microtiter Assay Plate: Simple and Inexpensive Method for Detection of Drug Resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2002;46:2720–2. doi:10.1128/AAC.46.8.2720-2722.2002.

[12] Caleffi-Ferracioli KR, Maltempe FG, Siqueira VLD, Cardoso RF. Fast detection of drug interaction in *Mycobacterium tuberculosis* by a checkerboard resazurin method. Tuberculosis 2013;93:660–3. doi:10.1016/j.tube.2013.09.001.

[13] Coelho T, Machado D, Couto I, Maschmann R, Ramos D, von Groll A, et al. Enhancement of antibiotic activity by efflux inhibitors against multidrug resistant *Mycobacterium tuberculosis* clinical isolates from Brazil. Front Microbiol 2015;6:1–12. doi:10.3389/fmicb.2015.00330.

[14] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the 2 □□ C T Method 2001;408:402–8. doi:10.1006/meth.2001.1262.

[15] Pimentel AL, de Lima Scodro RB, Caleffi-Ferracioli KR, Siqueira VLD, Campanerut-Sá PAZ, Lopes LDG, et al. Mutations in catalase-peroxidase KatG from isoniazid resistant *Mycobacterium tuberculosis* clinical isolates: insights from molecular dynamics simulations. J Mol Model 2017;23:121. doi:10.1007/s00894-017-3290-3.

[16] Sriraman K, Nilgiriwala K, Saranath D, Chatterjee A, Mistry N. Deregulation of Genes Associated with Alternate Drug Resistance Mechanisms in *Mycobacterium tuberculosis*. Curr Microbiol 2018;75:394–400. doi:10.1007/s00284-017-1393-9.

[17] Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, et al. Multidrug efflux pumps: structure, function and regulation. Nat Rev Microbiol 2018;16:523–39. doi:10.1038/s41579-018-0048-6.

[18] Machado D, Couto I, Perdigão J, Rodrigues L, Portugal I, Baptista P, et al. Contribution of Efflux to the Emergence of Isoniazid and Multidrug Resistance in *Mycobacterium tuberculosis*. PLoS One 2012;7:e34538. doi:10.1371/journal.pone.0034538.

[19] Pérez J, Garcia R, Bach H, de Waard JH, Jacobs WR, Av-Gay Y, et al. *Mycobacterium tuberculosis transporter* MmpL7 is a potential substrate for kinase PknD. Biochem Biophys Res Commun 2006;348:6–12. doi:10.1016/j.bbrc.2006.06.164.

[20] Bailo R, Bhatt A, Aínsa JA. Lipid transport in *Mycobacterium tuberculosis* and its implications in virulence and drug development. Biochem Pharmacol 2015;96:159–67. doi:10.1016/j.bcp.2015.05.001.

[21] Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G. *mmpL7* Gene of *Mycobacterium tuberculosis* Is Responsible for Isoniazid Efflux in *Mycobacterium smegmatis*. Antimicrob

Agents Chemother 2005;49:4775–7. doi:10.1128/AAC.49.11.4775-4777.2005.

[22] Rodrigues L, Villellas C, Bailo R, Viveiros M, Ainsa JA. Role of the Mmr Efflux Pump in Drug Resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2013;57:751–7. doi:10.1128/AAC.01482-12.

[23] Hao P, Shi-Liang Z, Ju L, Ya-Xin D, Biao H, Xu W, et al. The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from *Mycobacterium tuberculosis* clinical isolates in China. Folia Microbiol (Praha) 2011;56:549–53. doi:10.1007/s12223-011-0080-7.

[24] Wang K, Pei H, Huang B, Zhu X, Zhang J, Zhou B, et al. The Expression of ABC Efflux Pump, Rv1217c–Rv1218c, and Its Association with Multidrug Resistance of *Mycobacterium tuberculosis* in China. Curr Microbiol 2013;66:222–6. doi:10.1007/s00284-012-0215-3.

[25] Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M. Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol 2012;12:695–700. doi:10.1016/j.meegid.2011.08.009.

[26] Li G, Zhang J, Guo Q, Jiang Y, Wei J, Zhao LL, et al. Efflux pump gene expression in multidrug-resistant Mycobacterium tuberculosis clinical isolates. PLoS One 2015;10:1–12. doi:10.1371/journal.pone.0119013.

[27] te Brake LHM, de Knegt GJ, de Steenwinkel JE, van Dam TJP, Burger DM, Russel FGM, et al. The role of efflux pumps in tuberculosis treatment and their promise as a target in drug development: unraveling the black box. Annu Rev Pharmacol Toxicol 2018;58:271–91. doi:10.1146/annurev-pharmtox-010617-052438.

[28] Vilchèze C, Jacobs JR. WR. Resistance to Isoniazid and Ethionamide in *Mycobacterium tuberculosis*: Genes, Mutations, and Causalities. Microbiol Spectr 2014;2:1–21. doi:10.1128/microbiolspec.MGM2-0014-2013.

[29] Campanerut-Sá PAZ, Ghiraldi-Lopes LD, Meneguello JE, Fiorini A, Evaristo GPC, Siqueira VLD, et al. Proteomic and morphological changes produced by subinhibitory concentration of isoniazid in Mycobacterium tuberculosis. Future Microbiol 2016;11:1123–32. doi:10.2217/fmb-2016-5000.

[30] Bigi F, Gioffré A, Klepp L, de la Paz Santangelo M, Alito A, Caimi K, et al. The knockout of the lprG-Rv1410 operon produces strong attenuation of *Mycobacterium tuberculosis*. Microbes Infect 2004;6:182–7. doi:10.1016/j.micinf.2003.10.010.

[31] Kardan Yamchi J, Haeili M, Gizaw Feyisa S, Kazemian H, Hashemi Shahraki A, Zahednamazi F, et al. Evaluation of efflux pump gene expression among drug susceptible and drug resistant strains of *Mycobacterium tuberculosis* from Iran. Infect Genet Evol 2015;36:23–6. doi:10.1016/j.meegid.2015.08.036.

[32] Bianco M V., Blanco FC, Imperiale B, Forrellad MA, Rocha R V., Klepp LI, et al. Role of

P27-P55 operon from *Mycobacterium tuberculosis* in the resistance to toxic compounds. BMC Infect Dis 2011;11:195. doi:10.1186/1471-2334-11-195.

[33] Ramón-García S, Stewart GR, Hui ZK, Mohn WW, Thompson CJ. The mycobacterial P55 efflux pump is required for optimal growth on cholesterol. Virulence 2015;6:444–8. doi:10.1080/21505594.2015.1044195.

[34] Demitto FDO, do Amaral RCR, Maltempe FG, Siqueira VLD, Scodro RB de L, Lopes MA, et al. In Vitro Activity of Rifampicin and Verapamil Combination in Multidrug-Resistant *Mycobacterium tuberculosis*. PLoS One 2015;10:e0116545. doi:10.1371/journal.pone.0116545.

[35] Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, et al. Assessment of Efflux Pump Gene Expression in a Clinical Isolate *Mycobacterium tuberculosis* by Real-Time Reverse Transcription PCR. Microb Drug Resist 2008;14:7–11. doi:10.1089/mdr.2008.0772.

Table 1. Molecular characterization of the *Mycobacterium tuberculosis* selected by resistance-related mutation detection by GenoType MTBDRplus®, 12/24 *loci* mycobacterial interspersed repetitive unit (MIRU) and Spoligotyping.

Strain/Clinical isolate	Susceptibility profile	12/24 *loci* MIRU	Spoligotyping	Mutations			Reference
				katG	*inhA*	*rpoB*	
$H_{37}Rv$	S	-	777777477760771	WT	WT	WT	[34]
BRF 04	INH^R	124326143324224234434132	776177400000171	S315T	WT	WT	[10]
BRF 14	INH^R	223326143324224234414132	777777607760731	S315T	WT	WT	[10]
BRF 16	INH^R	223326143324224234414132	777777607760731	S315T	WT	WT	[10]
BRF 23	INH^R	125326143323224234324132	777777607760731	S315T	WT	WT	[10]
BRF 26	INH^R	224326143324224234314132	777777607760731	S315T	WT	WT	[10]
BRF 35	S	224326143226224283334132	777737606560771	WT	WT	WT	[10]
BRF 47	S	225215163323323474324333	777777200000771	WT	WT	WT	[10]
BRF 57	INH^R	124326143324224234334132	777777607760731	S315T	WT	WT	[10]
BRF 70	INH^R	124326133323224264434232	777777607760731	S315T	WT	WT	[10]
BRF 75	INH^R	124326143424224214214132	777777607760731	S315T	WT	WT	[10]
BRF 81	INH^R	125326153122224263332132	777637607400031	S315T	WT	WT	[10]
BRF 84	INH^R	125326153122224263332132	777637607400031	S315T	WT	WT	[10]
BRF 100	INH^R	124326143324224234333132	777777607760731	S315T	WT	WT	[10]
BRF 101	INH^R	124326143324224234333132	777777607760731	S315T	WT	WT	[10]
3614	$INH^R/RIF^R/EMB^R$	224225163321	677737607769771	WT	c-15t I21T	S531L	[34]

VP: verapamil; INH: isoniazid; RIF: rifampicin; MF: modulation factor; S: susceptible to first-line anti-TB drugs; ^{R}: resistant to the indicated drug; WT: wild type; -: not performed.

Table 2. Primers used to assess relative quantification of the selected efflux pump gene in *Mycobacterium tuberculosis* by RT-qPCR

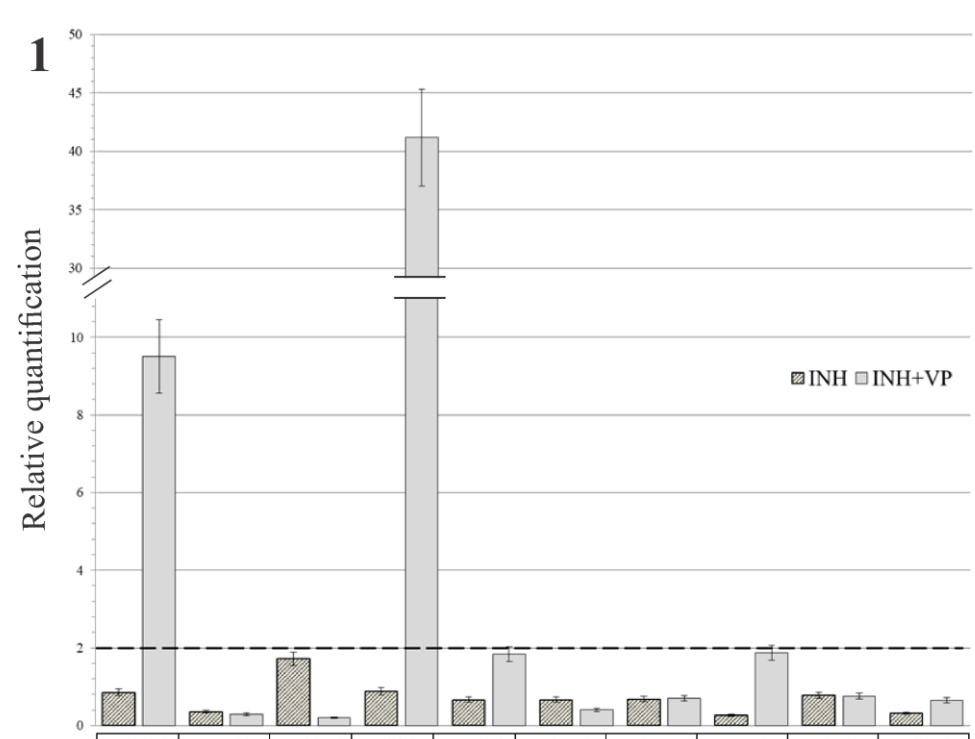
Efflux pump gene	Transporter family	Sequences (5'- 3')	Amplicon size (bp)	Reference
Rv2942	RND	Fw-TACCCAAGGTGGAAACAA Rv-CGTCAGAATAGAGGAACCAG	214	[25]
Rv1410c	MFS	Fw-AGTGGGAAATAAGCCAGTAA Rv-TGGTTGATGTCGAGCTGT	198	[25]
Rv2846	MFS	Fw-ATGGTAATGCCTGACATCC Rv-CTACGGGAAACCAACAAAG	131	[25]
Rv3065	SMR	Fw-AACCAGCCTGCTCAAAAG Rv-CAACCACCTTCATCACAGA	221	[25]
Rv1258c	MFS	Fw-AGTTATAGATCGGCTGGATG Rv-GTGCTGTTCCCGAAATAC	268	[25]
Rv1456c	ABC	Fw-GAGTCGCACCAGAATCGC Rv-TCGCTGTTGGTTGCCTAC	90	[23]
Rv1457c	ABC	Fw-GTAGCACCGAGTCGTTTG Rv-ATCTCCACCGCATTCACC	80	[23]
Rv1458c	ABC	Fw-CAGTCCAAGTACCTCAATG Rv-GCGATACGGGTCAATAAC	163	[23]
Rv1218c	ABC	Fw- CCGCAAGGCGTCTAGTGAA Rv- TGGACCCGTTGATGGAAAA	173	[35]
Rv1819c	ABC	Fw- CGGTGATTTCTTTCACAGC Rv- CCGACAGATTCCATCCATT	351	[35]
16s RNA		Fw- CAAGGCTAAAACTCAAAGGA Rv- GGACTTAACCCAACATCTCA	197	[25]

Fw, sense primer; Rv, antisense primer; bp, base pairs; MFS, major facilitator superfamily; SMR, small multidrug resistance family; ABC, ATP-binding cassette; RND, resistance-nodulation-cell division superfamily

Table 3. Minimal inhibitory concentration of verapamil and isoniazid and modulation factor of the tested combinations in *Mycobacterium tuberculosis*.

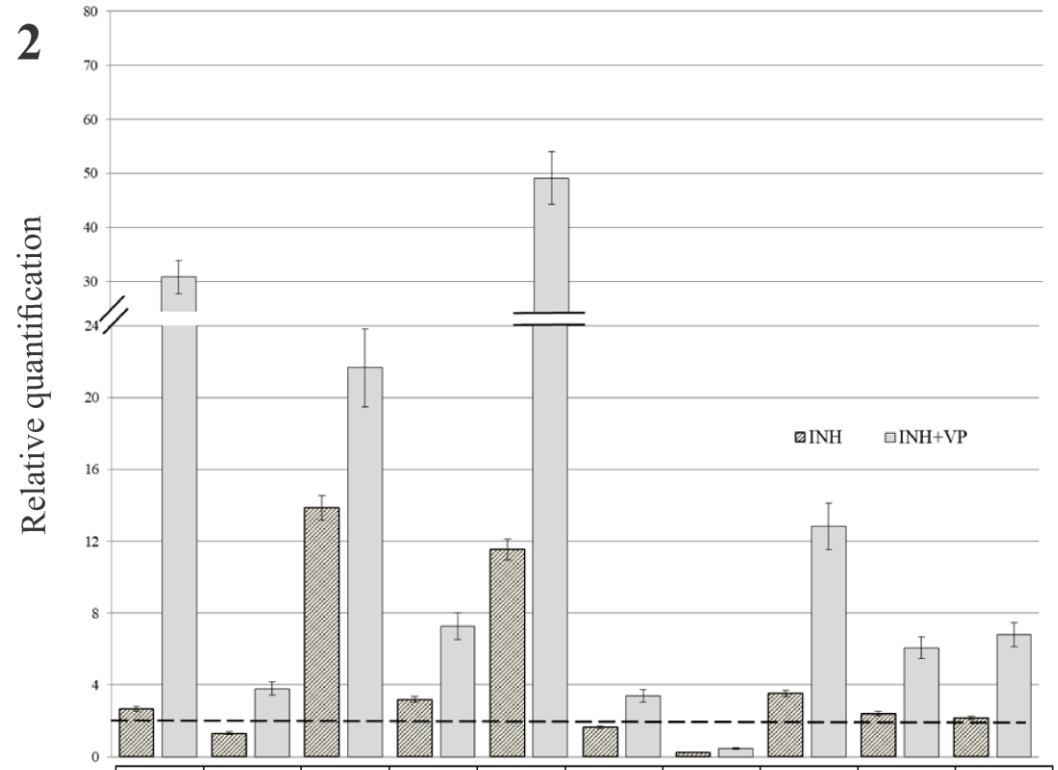
Strain/Clinical isolate	Susceptibility profile	MIC (µg/mL)			MF
		VP	INH	INH+VP	
$H_{37}Rv$*	S	125	0.06	0.03	2
BRF 35	S	250	0.06	**0.0078**	**8**
BRF 47*	S	250	0.125	**0.0078**	**16**
BRF 04	INH^R	125	4	2	2
BRF 14*	INH^R	125	32	16	2
BRF 16	INH^R	125	8	4	2
BRF 23	INH^R	125	8	8	1
BRF 26	INH^R	125	16	16	1
BRF 57	INH^R	125	8	4	2
BRF 70	INH^R	250	8	8	1
BRF 75	INH^R	250	16	8	2
BRF 81	INH^R	125	8	4	2
BRF 84	INH^R	250	8	4	2
BRF 100	INH^R	125	32	16	2
BRF 101	INH^R	125	32	16	2
3614*	$INH^R/RIF^R/EMB^R$	62.5	6.25	3.125	2

VP: verapamil; INH: isoniazid; RIF: rifampicin; MF: modulation factor; *: isolates selected for gene expression assays; S: susceptible to first-line anti-TB drugs; ^{R}: resistance to the indicated drug; Results in bold indicate synergism.



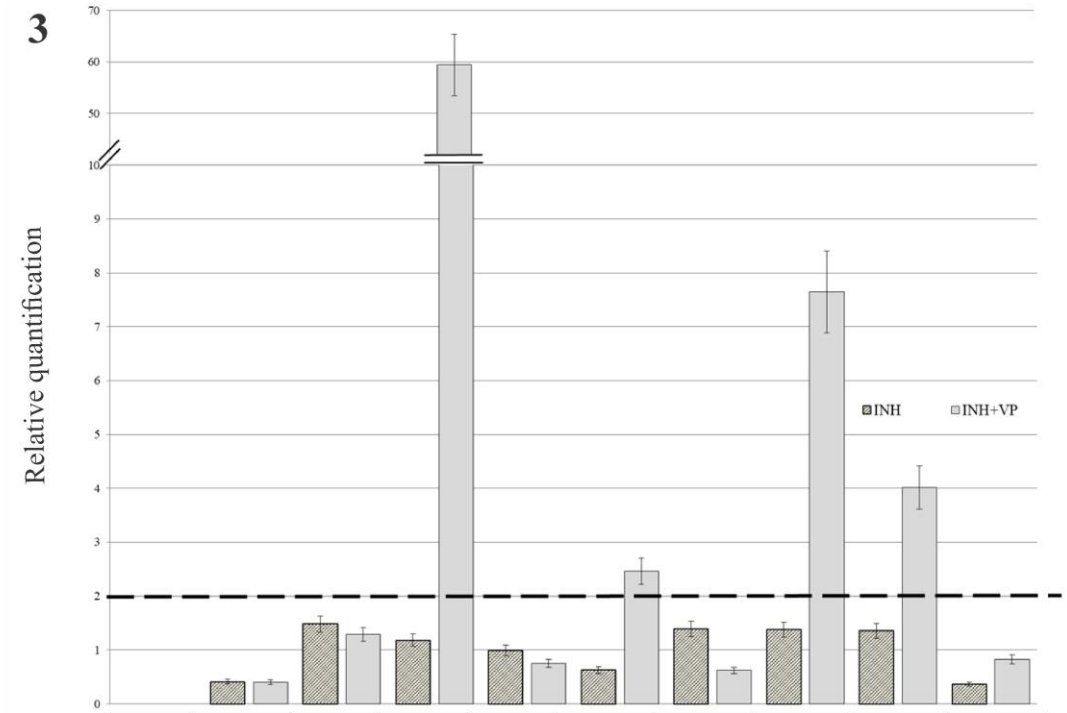
	Rv2942	*Rv1410c*	*Rv2846*	*Rv3065*	*Rv1258*	*Rv1456c*	*Rv1457c*	*Rv1458c*	*Rv1218c*	*Rv1819c*
INH	0,855	0,351	1,722	0,880	0,665	0,663	0,680	0,264	0,782	0,311
INH+VP	9,507	0,290	0,206	41,157	1,839	0,403	0,702	1,870	0,755	0,649

Figure 1. Relative quantification of 10 efflux pump genes in *Mycobacterium tuberculosis* $H_{37}Rv$, accessed by RT-qPCR after 48 h exposure to 0.5xMIC of isoniazid (INH) and the combination INH+VP. Relative quantification was determined by the means of $2^{-\Delta\Delta CT}$. Relative quantification rates above two (> 2) and lower than 0.5 (< 0.5) were considered as significantly overexpressed and underexpressed, respectively. Dashed line represents the cutoff of significant overexpression. Results were normalized using 16S RNA gene and presented in linear scale.



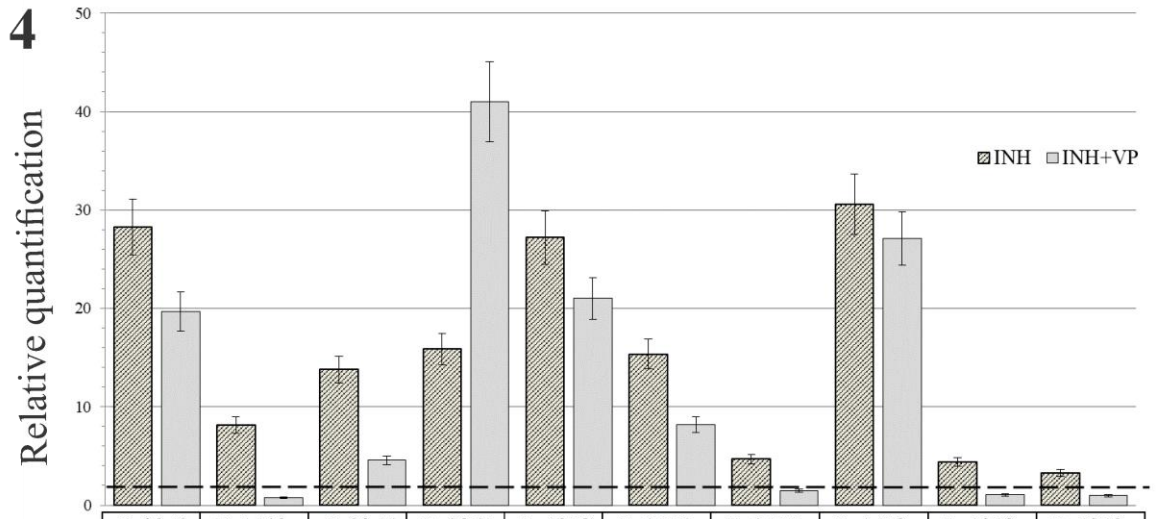
	Rv2942	*Rv1410c*	*Rv2846*	*Rv3065*	*Rv1258*	*Rv1456c*	*Rv1457c*	*Rv1458c*	*Rv1218c*	*Rv1819c*
INH	2,676	1,311	13,867	3,192	11,544	1,638	0,222	3,513	2,406	2,158
INH+VP	30,847	3,802	21,660	7,270	49,141	3,397	0,455	12,830	6,065	6,795

Figure 2. Relative quantification of 10 efflux pump genes in *Mycobacterium tuberculosis* BRF 47, accessed by RT-qPCR after 48 h exposure to 0.5xMIC of isoniazid (INH) and the combination INH+VP. Relative quantification was determined by the means of $2^{-\Delta\Delta CT}$. Relative quantification rates above two (> 2) and lower than 0.5 (< 0.5) were considered as significantly overexpressed and underexpressed, respectively. Dashed line represents the cutoff of significant overexpression. Results were normalized using 16S RNA gene and presented in linear scale.



	Rv2942	*Rv1410c*	*Rv2846*	*Rv3065*	*Rv1258*	*Rv1456c*	*Rv1457c*	*Rv1458c*	*Rv1218c*	*Rv1819c*
INH	-	0,409	1,481	1,181	0,989	0,626	1,390	1,373	1,353	0,363
INH+VP	-	0,400	1,286	59,403	0,752	2,459	0,619	7,647	4,012	0,825

Figure 3. Relative quantification of 10 efflux pump genes in *Mycobacterium tuberculosis* BRF 14, accessed by RT-qPCR after 48 h exposure to 0.5xMIC of isoniazid (INH) and the combination INH+VP. Relative quantification was determined by the means of $2^{-\Delta\Delta CT}$. Relative quantification rates above two (> 2) and lower than 0.5 (< 0.5) were considered as significantly overexpressed and underexpressed, respectively. Dashed line represents the cutoff of significant overexpression. Results were normalized using 16S RNA gene and presented in linear scale. -, relative transcription levels not detected.



	Rv2942	*Rv1410c*	*Rv2846*	*Rv3065*	*Rv1258*	*Rv1456c*	*Rv1457c*	*Rv1458c*	*Rv1218c*	*Rv1819c*
INH	28,258	8,143	13,789	15,879	27,207	15,353	4,690	30,594	4,429	3,278
INH+VP	19,677	0,747	4,557	40,999	21,009	8,187	1,484	27,111	1,045	0,969

Figure 4. Relative quantification of 10 efflux pump genes in *Mycobacterium tuberculosis* 3614, accessed by RT-qPCR after 48 h exposure to 0.5xMIC of isoniazid (INH) and the combination INH+VP. Relative quantification was determined by the means of $2^{-\Delta\Delta CT}$. Relative quantification rates above two (> 2) and lower than 0.5 (< 0.5) were considered as significantly overexpressed and underexpressed, respectively. Dashed line represents the cutoff of significant overexpression. Results were normalized using 16S RNA gene and presented in linear scale.

CAPÍTULO III

3.1 CONCLUSÕES

Os sistemas de efluxo têm se mostrado como mecanismos importantes no desenvolvimento de resistência aos antimicrobianos em *Mycobacterium tuberculosis*, visto que cada proteína transportadora é capaz de expulsar uma grande variedade de substratos. Com base nos resultados obtidos no presente estudo e que corroboram com publicações disponíveis na literatura, podemos concluir que estas bombas de efluxo têm papel fundamental na resistência à isoniazida, principalmente em isolados sensíveis.

3.2 PERSPECTIVAS

Dando continuidade aos resultados aqui obtidos, nosso grupo de pesquisa buscará identificar novas estratégias que possam superar os mecanismos de resistência à isoniazida mediados por sistemas de efluxo. Dentre essas estratégias, buscaremos comparar a taxa de surgimento de mutantes expostos somente a INH e compara-las à exposição conjunta de INH+VP.

ANEXO I: INSTRUÇÃO PARA AUTORES DO PERIÓDICO TUBERCULOSIS

TUBERCULOSIS

AUTHOR INFORMATION PACK

5. TABLE OF CONTENTS

ISSN: 1472-9792

6. DESCRIPTION

Tuberculosis is a speciality journal focusing on basic experimental research on tuberculosis, notably on bacteriological, immunological and pathogenesis aspects of the disease. The journal publishes original research and reviews on the host response and immunology of tuberculosis and the molecular biology, genetics and physiology of the organism, however discourages submissions with a metaanalytical focus (for example, articles based on searches of published articles in public electronic databases, especially where there is lack of evidence of the personal involvement of authors in the generation of such material).

Areas on which submissions are welcomed include:

Immunology

Immunogenetics

Pathogenetics

Microbiology

Microbial physiology

Pathogenesis

Pathology

Molecular epidemiology

Diagnostics

Vaccine developmentDrug resistanceThe resurgence of interest in tuberculosis has accelerated the pace of relevant research and Tuberculosis has grown with it, as the only journal dedicated to experimental biomedical research in tuberculosis.

7. IMPACT FACTOR

2017: 2.727 © Clarivate Analytics Journal Citation Reports 2018

8. ABSTRACTING AND INDEXING

Scopus

ADONIS

Cambridge Scientific Abstracts

Excerpta Medica

EMBASE

Biological Abstracts

BIOSIS

MEDLINE®

Current Awareness in Biological Sciences

Medical Documentation Service

CAB International

NMLUS

UMI Microfilms

Academy of Sciences Russia

9. EDITORIAL BOARD

10. GUIDE FOR AUTHORS

Article types

The following types of manuscripts are routinely accepted for consideration by Editors (please note that word count is from abstract to references but excluding references):

Original Articles: The form of these articles is discussed fully below under manuscript preparation; an abstract is required.

Letters: Headings should not be used in a letter; no abstract or keywords are required. The text should be no more than 800 words; there should be a maximum of 5 references and one table or figure may be included.

Reviews: An abstract and keywords are required. The text should be divided into sections by suitable headings. Tables and figures may be used as appropriate for the text.

Comments: Editorial comments are generally invited by the Tuberculosis Editorial team. They are 1,500 words in length with no abstract or keywords .

Short Communications: These should be no more than 2,500 words, with up to 15 references and a maximum of 3 figures or tables.

It is strongly advised that Authors provide a list of 4 or 5 potential reviewers (e-mail, phone and fax numbers) who are knowledgeable in the subject matter, have no conflict of interest, and are likely to agree to review the manuscript. When submitting your manuscript please specify under which of the following categories your paper should be included if accepted for publication. This will be of great assistance to the Editors with the review process:

Reviews

Comments

Molecular Aspects

Immunological Aspects

Mechanisms of Pathogenesis

Model Systems

Bovine Tuberculosis

Diagnostics

Drug Discovery and Resistance

Host Genetics of Susceptibility

Non-tuberculous Mycobacteria

This Journal does not accept Case Reports.

Books for review may be sent to either Editor at the addresses given below.

Editorial correspondence should go to the Editors-in-Chief at one of the following addresses:

Professor Warwick J. Britton

Centenary Institute, and Disciplines of Medicine, Immunology and Infectious Diseases, Sydney Medical School, The University of Sydney, NSW 2006 Australia.

Tel: +61 2 9515 5210 (work)Email: wbritton@med.usyd.edu.au

Brian D. Robertson, PhD

Imperial College London

Department of Microbiology

Centre for Molecular Microbiology and Infection

Flowers Building

London SW7 2AZ

UK

Tel: +44 (0)20 7594 3198

Fax: +44 (0)20 7594 3095

E-mail: b.robertson@imperial.ac.uk

Reviews' EditorPatrick J. BrennanDepartment of Microbiology, Immunology and PathologyCollege of Veterinary Medicine and Biomedical SciencesColorado State UniversityFort CollinsCO 80523-1682USA

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address

All necessary files have been uploaded:

Manuscript:
- Include keywords
- All figures (include relevant captions)
- All tables (including titles, description, footnotes)
- Ensure all figure and table citations in the text match the files provided
- Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations

- Manuscript has been 'spell checked' and 'grammar checked'
- All references mentioned in the Reference List are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including theInternet)
- A competing interests statement is provided, even if the authors have no competing interests todeclare
- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Center.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Ethics

Work on human beings that is submitted to Tuberculosis should comply with the principles laid down in the Declaration of Helsinki; Recommendations guiding physicians in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, and the 41st World Medical Assembly, Hong Kong, September 1989. The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed and that subjects gave informed consent to the work. Studies involving experiments with animals must state that their care was in accordance with institution guidelines.

Studies on patients or volunteers require ethics committee approval and informed consent which should be documented in your paper. Patients have a right to privacy. Therefore identifying information, including patients, images, names, initials, or hospital numbers, should not be included in videos, recordings, written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and you have obtained written informed consent for publication in print and electronic form from the patient (or parent, guardian or next of kin where applicable). If such consent is made subject to any conditions, Elsevier must be made aware of all such conditions. Written consents must be provided to

Elsevier on request. Even where consent has been given, identifying details should be omitted if they are not essential. If identifying characteristics are altered to protect anonymity, such as in genetic pedigrees, authors should provide assurance that alterations do not distort scientific meaning and editors should so note. If such consent has not been obtained, personal details of patients included in any part of the paper and in any supplementary materials (including all illustrations and videos) must be removed before submission.

Randomised controlled trials

All randomised controlled trials submitted for publication in Tuberculosis should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart. Please refer to the CONSORT statement website at http://www.consort-statement.org for more information. Tuberculosis has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) which require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of the article. For this purpose, a clinical trial is defined as any research project that prospectively assigns human subjects to intervention or comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Studies designed for other purposes, such as to study pharmacokinetics or major toxicity (e.g. phase I trials) would be exempt. Further information can be found at www.icmje.org.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places: 1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted. 2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyrightholder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Preprints

Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

Use of inclusive language

Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Authorship

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Reporting clinical trials

Randomized controlled trials should be presented according to the CONSORT guidelines. At manuscript submission, authors must provide the CONSORT checklist accompanied by a flow diagram that illustrates the progress of patients through the trial, including recruitment, enrollment, randomization, withdrawal and completion, and a detailed description of the randomization procedure. The CONSORT checklist and template flow diagram are available online.

Registration of clinical trials

Registration in a public trials registry is a condition for publication of clinical trials in this journal in accordance with International Committee of Medical Journal Editors recommendations. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of the article. A clinical trial is defined as any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects of health outcomes. Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example drugs, surgical procedures, devices, behavioural treatments, dietary interventions, and process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including

pharmacokinetic measures and adverse events. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) will not require registration.

Copyright

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

Author rights

As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing

Find out how you can share your research published in Elsevier journals.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the gold open access publication fee. Details of existing agreements are available online.

After acceptance, open access papers will be published under a noncommercial license. For authors requiring a commercial CC BY license, you can apply after your manuscript is accepted for publication.

Open access

This journal offers authors a choice in publishing their research:

Subscription

- Articles are made available to subscribers as well as developing countries and patient groups throughour universal access programs.
- No open access publication fee payable by authors.

- The Author is entitled to post the accepted manuscript in their institution's repository and make this public after an embargo period (known as green Open Access). The published journal article cannot be shared publicly, for example on ResearchGate or Academia.edu, to ensure the sustainability of peerreviewed research in journal publications. The embargo period for this journal can be found below.

Gold open access

- Articles are freely available to both subscribers and the wider public with permitted reuse.
- A gold open access publication fee is payable by authors or on their behalf, e.g. by their researchfunder or institution.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For gold open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

The gold open access publication fee for this journal is **USD 2500**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access

Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our green open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. Find out more.

This journal has an embargo period of 12 months.

Elsevier Researcher Academy

Researcher Academy is a free e-learning platform designed to support early and mid-career researchers throughout their research journey. The "Learn" environment at Researcher Academy offers several interactive modules, webinars, downloadable guides and resources to guide you through the process of writing for research and going through peer review. Feel free to use these free resources to improve your submission and navigate the publication process with ease.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Original articles and suggestions for review articles should be submitted by online submission at: https://www.evise.com/profile/api/navigate/TUBE.

During the submission process, the author should submit the manuscript file, figures, tables, acknowledgements, supplements and any other files, which need to be reviewed, in one file. The cover letter and any additional documents should be submitted as a separate file.

Referees

Please submit the names and institutional e-mail addresses of several potential referees. For more details, visit our Support site. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

PREPARATION

Papers should be submitted in journal style and must be written in good English. Failure to do so may lead to significant delays in publication. Spelling may be British or American, but this must be consistent throughout the paper. It is highly recommended that authors use the "spell-checking" facility on their word processing software.

Format the entire manuscript using 1.5 double spacing and 2 cm margins. Your paper should include page numbers and consecutive line numbering. Ensure that each new paragraph is clearly indicated. Present tables and figure legends on separate pages at the end of the manuscript. If possible, consult a recent issue of the journal to become familiar with layout and conventions. Number all pages consecutively.

Authors should note that upon submission of their manuscripts to the Editor, they must ensure that they have adhered to the following checklist:

One author is designated as the corresponding author and provides the following information:

- E-mail address
- Full postal address
- Telephone and fax numbers

Papers should be set out as follows, with each section beginning on a separate sheet: title page, summary, keywords, text, acknowledgements, references, tables, captions to illustrations.

- Title page: The title page should give the following information (in the order stated):
- Title. Concise and informative. Titles are often used in information-retrieval systems. Avoidabbreviations and formulae where possible.

- Author names and affiliations. Where the family name may be ambiguous (e.g., a double name),please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and the e-mail address of each author.

- Corresponding author. Clearly indicate who is willing to handle correspondence at all stages ofrefereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.

- Present/permanent address. If an author has moved since the work described in the article wasdone, or was visiting at the time, a 'Present address' (or 'Permanent address') may be

indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes. Abstract: Original articles should include an abstract of no more than 200 words. Abstracts for review articles may be up to 300 words, and for short communications 150 words. A recent copy of the journal should be consulted as a guide. An abstract is often presented separate from the article, so it must be able to stand alone.
Keywords: Immediately after the abstract, provide a minimum of 3maximum of 6 keywords, avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

DNA sequences and GenBank Accession numbers: Many Elsevier journals cite "gene accession numbers" in their running text and footnotes. Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Elsevier authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should type this information in the following manner:

For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text.** Letters in the accession number should always be capitalised. (See Example below). This combination of letters and format will enable Elsevier's typesetters to recognise the relevant texts as accession numbers and add the required link to GenBank's sequences.

Example: "GenBank accession nos. **AI631510** , **AI631511** , **AI632198** , and **BF223228**), a Bcell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**)".

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.** In the final version of the **printed article**, the accession number text will not appear bold or underlined. In the final version of the **electronic copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article. Text: Headings should be appropriate to the nature of the paper. In general those for experimental papers should follow the usual conventions. Other papers can be subdivided as the author desires; the use of headings enhances readability. Normally only two categories of headings should be used: major ones should be typed in capital letters; minor ones should be typed in lower case (with an initial capital letter) at the left hand margin and underlined. Do not use "he", "his", etc where the sex of the person is unknown; say "the patient", etc. Avoid inelegant alternatives such as "he/she". Patients should not be automatically designated as "she", and doctors as "he".

SHORT COMMUNICATIONS

Editors/Section Editors may recommend conversion of longer manuscripts to shorter versions, or short communications may be submitted as such. We recommend the format described by ASM publications (asm.journals.org).

Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper.

The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

- ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lowercase superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
- ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork

Electronic artwork General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Embed the used fonts if the application provides that option.
- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, oruse fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Size the illustrations close to the desired dimensions of the printed version.
- Submit each illustration as a separate file.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/ halftone combinations given below): At this time the following file types are not supported in Evise and would need to be sent outside of Evise to the JM to process after acceptance EPS, CSV, Video, KML/KMZ, mol, Newick or NeXML, MATLAB, PDB, PSE or MOL/MOL2, or NlfTl).

• EES: For supported file types in EES, please visit our Support site for EES • Evise: For supported file types in Evise, please visit ourSupport site for Evise. **Note**: Both lists will be expanded as more file types are supported.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), PDF or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) in addition to color reproduction in print.

For supported file types in EES, please visit our Support site for EES.

Evise: For supported file types in Evise, please visit our Support site for Evise. **Note**: Both lists will be expanded as more file types are supported.

Illustration services

Elsevier's WebShop offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Role of the funding source

All sources of funding should be declared as an acknowledgement at the end of the text. Authors should declare the role of study sponsors, if any, in the study design, in the

collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. If the study sponsors had no such involvement, the authors should so state.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:

http://open.mendeley.com/use-citation-style/tuberculosis

When preparing your manuscript, you will then be able to select this style using the Mendeley plugins for Microsoft Word or LibreOffice.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

[2] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon.2018;19:e00205. https://doi.org/10.1016/j.heliyon.2018.e00205 Reference to a book:

[3] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.

Reference to a chapter in an edited book:

[4] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, SmithRZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.

Reference to a website:

[5] Cancer Research UK. Cancer statistics reports for the UK, http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/; 2003 [accessed 13 March 2003].

Reference to a dataset:

[dataset] [6] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. https://doi.org/10.17632/ xwj98nb39r.1.

Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by 'et al.' For further details you are referred to 'Uniform Requirements for Manuscripts submitted to Biomedical Journals' (J Am Med Assoc 1997;277:927–34) (see also Samples of Formatted References).

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations.

Data visualization

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Supplementary and multimedia data

Elsevier accepts electronic supplementary and multimedia data to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, movies, animation sequences, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please ensure that data are provided in one of our recommended file formats. At this time the following file types are not supported in Evise and would need to be sent outside of Evise to the JM to process after acceptance EPS, CSV, Video, KML/ KMZ, mol, Newick or NeXML, MATLAB, PDB, PSE or MOL/MOL2, or NlfTl).

- EES: For supported file types in EES, please visit our Support site for EES.
- Evise: For supported file types in Evise, please visit our Support site for Evise.

Note: Both lists will be expanded as more file types are supported.

Research data

This journal requires and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your

software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. When sharing data in one of these ways, you are expected to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.

Data linking

If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Mendeley Data

This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the opportunity to upload your relevant datasets directly to *Mendeley Data*. The datasets will be listed and directly accessible to readers next to your published article online.

For more information, visit the Mendeley Data for journals page.

Data statement

To foster transparency, we require you to state the availability of your data in your submission if your data is unavailable to access or unsuitable to post. This may also be a requirement of your funding body or institution. You will have the opportunity to provide a data statement during the submission process. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

AFTER ACCEPTANCE

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

Proprietary names

Proprietary names of drugs, instruments, etc. should be indicated by the use of initial capital letters.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch.

You can also check the status of your submitted article or find out when your accepted article will be published.