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NATHALLY CLAUDIANE DE SOUZA SANTOS

Ação combinatória, *in vitro*, de linezolida e levofloxacino com fármacos antituberculose em *Mycobacterium tuberculosis*

Maringá
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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 Biociência e Fisiopatologia.

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"*Em vosso caminho, anunciai: 'O Reino dos Céus está próximo'. "*

(Mt 10,7)

Ação combinatória, *in vitro*, de linezolida e levofloxacino com fármacos antituberculose em *Mycobacterium tuberculosis*

RESUMO

A tuberculose (TB) é um problema de saúde pública mundial e seu tratamento padrão utiliza isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB). Entretanto, o crescente aumento no surgimento de linhagens de *Mycobacterium tuberculosis* multirresistentes (MDR) e extensivamente resistente (XDR) a fármacos elevou a busca por outras opções de tratamento, com fármacos mais recentes que incluem fluoroquinolonas (levofloxacino (LVX), moxifloxacino), linezolida (LNZ) entre outros. Neste sentido, o objetivo do presente estudo foi avaliar a atividade *in vitro* das combinações INH/RIF/LVX e INH/RIF/LNZ em isolados clínicos de *M. tuberculosis* por *checkerboard* tridimensional, utilizando resazurina como revelador de crescimento bacilar. Para as combinações INH/RIF/LVX e INH/RIF/LNZ foram avaliados na cepa de referência *M. tuberculosis* H_{37}Rv (ATCC 27294), um isolado clínico sensível aos fármacos de primeira linha e 10 isolados clínicos resistentes (um monorresistente a estreptomicina, 3 monorresistentes a INH e 6 isolados clínicos MDR-TB). Para tal, os fármacos LVX e LNZ foram utilizados em concentrações fixas de ½ e ¼ da concentração inibitória mínima (CIM) e INH e RIF em concentrações variando de 0,0015 mg/L a 100 mg/L e 0,00012 mg/L a 1.600 mg/L, respectivamente. Índice de concentração inibitória fracionada da sigla inglesa FICI de valores $\leq$ 0,75; 0,75 - 4 e $\geq$ 4 foram considerados sinérgico, indiferente e antagônico, respectivamente. O presente estudo resultou na elaboração do manuscrito "Ação combinatória, *in vitro*, de linezolida e levofloxacino com fármacos antituberculose em *mycobacterium tuberculosis*" que apresenta os resultado obtidos. Na concentração fixa de ½ da CIM de LVX e LNZ adicionado à INH e RIF foi observado sinergismo somente no isolado de *M. tuberculosis* 309. Quando a concentração fixa de LVX e LNZ foi de ¼ da CIM foi observado sinergismo em três isolados testados com INH/RIF/LVX e três com INH/RIF/LNZ. Embora não foram observados sinergismo em todos isolados nas combinações deste estudo, uma redução da CIM de INH e RIF em todos os isolados foram observadas. O presente resultado chama a atenção para a continuidade dos estudos e uso destas combinações no tratamento de casos de isolados clínicos multirresistentes.

Palavras-chave: *Mycobacterium tuberculosis*. Multirresistentes. *Checkerboard* tridimensional.

Combinatory action, in vitro, of linezolide and levofloxacin with antibuberculosis drugs in *Mycobacterium tuberculosis*

ABSTRACT

Tuberculosis (TB) is a worldwide public health problem and its standard treatment uses isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB). However, the increasing increase in the emergence of multidrug resistant (MDR) and extensively resistant (XDR) to *Mycobacterium tuberculosis* strains has increased the search for other treatment options, with newer drugs including fluoroquinolones (levofloxacin (LVX), moxifloxacin), linezolid (LNZ) among others. In this sense, the objective of the present study was to evaluate the in vitro activity of the combinations INH/RIF/LVX and INH/RIF/LNZ in clinical isolates of *M. tuberculosis* by three-dimensional checkerboard, using resazurin as developer of bacillary growth. For the INH/RIF/LVX and INH/RIF/LNZ combinations, the *M. tuberculosis* strain H37Rv (ATCC 27294), one clinical isolate sensitive to first-line drugs and 10 resistant clinical isolates (one monorresistant to streptomycin, 3 monorresistentes to INH and 6 multidrug-resistant). For this purpose, LVX and LNZ were used in fixed concentrations of ½, ¼, of the minimum inhibitory concentration (MIC), INH, and RIF in concentrations ranging from 0.0015 mg/L to 100 mg/L and 0.00012 mg/L To 1,600 mg/L, respectively. FICI fractional inhibitory concentration index of values ≤ 0.75; 0.75 - 4 and ≥ 4 were considered synergistic, indifferent and antagonistic, respectively. The present study resulted in the elaboration of the manuscript "Combinatory action, in vitro, of linezolide and levofloxacino with antibuberculosis drugs in *Mycobacterium tuberculosis*", which presents the results obtained. At the fixed concentration of ½ MIC of LVX and LNZ added to INH and RIF synergism was observed only in the *M. tuberculosis* 309 isolate. When the fixed concentration of LVX and LNZ was ¼ of MIC, synergism was observed in three isolates tested with INH/RIF/LVX and four with INH/RIF/LNZ. Although no synergism was observed in all isolates in the combinations of this study, a reduction of the MIC of INH and RIF in all isolates was observed. The present result draws attention to the continuity of studies and use of these combinations in the treatment of cases of multiresistant clinical isolates.

Keywords: *Mycobacterium tuberculosis*. Multiresistants. Three-dimensional checkerboard

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SUMÁRIO

CAPÍTULO I

1.1 INTRODUÇÃO

1.1.1 **A bactéria**

O gênero *Mycobacterium* compreende mais de 100 espécies[1], a maioria saprófitas de vida livre. É constituído por bacilos curvos ou retos, com diâmetro de 0,2 a 0,7 µm e 1 a 7 µm de comprimento[2].

São classificadas como Gram positivas, mas não é possível sua observação pelo método de Gram. As micobactérias compartilham uma característica que as distingue das demais bactérias que é a retenção de fucsina básica quando tratadas com álcool- ácido, conferindo-lhes a designação de bacilos álcool-ácido resistentes (BAAR) quando da utilização do método de coloração Ziehl-Neelsen[3,4]. Esta propriedade é devido à grande quantidade de lipídeos presentes em sua parede celular, especialmente os ácidos graxos de cadeia longa constituída pelos ácidos micólicos. São bactérias aeróbias estritas, não esporuladas e classificadas de acordo com seu tempo de crescimento em micobactérias de crescimento rápido, quando requerem menos de sete dias, e de crescimento lento quando requerem mais de sete dias para detecção das colônias em meio sólido[3].

Entre as espécies, *Mycobacterium tuberculosis* que foi identificado por Robert Koch em 1882, é a principal espécie do gênero e faz parte de um complexo constituído das seguintes espécies: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum, M. canetti, Mycobacterium microti*, *Mycobacterium pinnipedii* e *Mycobacterium caprae*[5,6], recentemente foram incluídos a este grupo *Mycobacterium orygis e Mycobacterium mungii*[7,8].

1.1.2 **A doença tuberculosa**

A tuberculose (TB) é uma doença bacteriana crônica, infectocontagiosa, de distribuição universal causada, principalmente, por *Mycobacterium tuberculosis*. A doença é conhecida há séculos, passível de prevenção, tratamento e mesmo de cura, porém continua sendo um importante problema de saúde pública mundial[1,2].

Dentre os vários fatores envolvidos na patogênese da TB, verificam-se variáveis ambientais, socioeconômicas e comportamentais, típicas da sociedade contemporânea. A TB acomete principalmente indivíduos na faixa etária correspondente a plena capacidade produtiva o que acarreta enorme prejuízo econômico ao país[2,9,10]. É estimado que 64 % dos casos de TB acometam homens e principalmente a população de menor renda11. Os fatores que intensificam o crescimento dessa doença são: aglomerados humanos, desnutrição, deterioração das condições socioeconômicas e medidas de controle ineficazes[9,11] e também a disseminação de práticas, tais como o etilismo, o tabagismo, o consumo de drogas ilícitas, o uso indiscriminado e, por vezes, abusivo de antimicrobianos e o abandono do tratamento[9,10].

Algumas condições inerentes ao indivíduo contribuem para a redução da resposta imunológica à infecção. Esta condição aumenta a probabilidade de TB ativa como ocorre em indivíduos em uso de corticóides e outros fármacos imunossupressores bem como desenvolvimento de algumas comorbidades como o diabetes, a hipertensão arterial e principalmente a HIV/AIDS[2,10].

A TB pode acometer uma série de órgãos e/ou sistemas, como por exemplo os casos de TB pleural, TB ganglionar periférica, TB meningoencefálica, TB pericárdica, TB óssea e, a mais frequente, TB pulmonar. A TB pulmonar também é a forma mais relevante da doença para a saúde publica. Esta forma além de apresentar tosse, com ou sem expectoração, por mais de três semanas, apresenta outros sintomas comuns, tal como a falta de apetite, perda de peso, cansaço, dor no peito, febre no fim do dia e acompanhada de suores noturnos, e, às vezes, hemoptise (escarro com gotículas de sangue)[11,12].

A TB pulmonar é transmitida de pessoa a pessoa por via aérea, por gotículas de 1,0 a 5,0 µm de diâmetro produzidas pelo indivíduo portador da forma clínica pulmonar ou laríngea da doença, ao tossir, espirrar ou falar[2,6,12]. Estas gotículas que possuem o bacilo de *M. tuberculosis*, ao serem inaladas pelo indivíduo saudável e atingirem os alvéolos, quando estes bacilos sobrevivem às defesas primárias contra agentes infecciosos, eles se multiplicam no interior do macrófago alveolar, onde iniciará o processo patológico[2,9]. O bacilo, embora não produza toxinas, tão logo se encontre envolvido pelos macrófagos alveolares, ativa um mecanismo que inibe, por meio dos sulfolipídeos, a fusão das vesículas fagocíticas com os lisossomos, impedindo, desse modo, sua eliminação. A bactéria induz, então, a produção de citocinas e de quimiocinas, provocando, nos pulmões, o início de uma resposta inflamatória, que faz com que macrófagos, linfócitos, monócitos e outras células do sistema imunológico migrem para o local da infecção e constituam um granuloma[13,14]. Em sua vasta maioria, o

sistema imunológico do hospedeiro competente impõe-se à infecção primária, levando à formação do granuloma caseoso, o qual não só contém o bacilo, como controla sua proliferação no organismo recém-infectado[15,16]. Os bacilos podem permanecer viáveis, por mecanismos próprios, porém em um estado de dormência no interior dos granulomas formados pela reação imunológica, durante toda a vida do indivíduo, e podem reativar quando as condições são favoráveis ao seu desenvolvimento[17,18].

As medidas de controle global da doença não foram suficientes para interromper a transmissão e erradicar a TB, e o risco de progressão entre os contatos infectados tem permitido a perpetuação da doença entre a população mundial[9,10]. Tal ineficácia faz não só subsistirem o risco, tanto de progressão da doença entre os contatos infectados, quanto o risco de perpetuação da TB entre a população mundial[17].

O diagnóstico laboratorial da TB faz-se principalmente por exame direto, por cultura e identificação do bacilo. Quanto mais cedo for diagnosticada a doença mais se estará contribuindo para a interrupção do seu ciclo de transmissão do bacilo e para o controle da TB[19,20].

1.1.3 **Epidemiologia**

Em 2015, a TB ficou em primeiro lugar no ranking mundial de morte por doenças infecciosas, ultrapassando pela primeira vez, o número de morte causado pelo vírus da imunodeficiência humana (HIV)[9]. Estima-se que, a cada ano no Brasil, ocorrem aproximadamente 70 mil casos novos e 4,6 mil mortes em decorrência desta doença[2,9]. Ocorreram 480.000 novos casos de TB-MDR no mundo, em 2015, 80 % dos casos de TB estão situados em um grupo de 20 países do mundo. Inserir dados epidemiológicos mundiais de resistência[9].

Em 1993, a OMS declarou que a TB se encontrava em estado de emergência mundial e foi recomendado o tratamento realizado pela estratégia DOTS *(Direct Observed Treatment Short-Course*). Este projeto da OMS preconiza a adesão política por parte das autoridades governamentais, estabelecimento de uma rede laboratorial de baciloscopia, garantia de medicação gratuita e de livre acesso, um sistema de informação adequado e a oferta de um tratamento supervisionado por profissional de saúde[21,22].

No continente americano, o Brasil e o Peru foram responsáveis por notificar 49 % do total de casos da doença em 2011[22,23]. Internamente, dos 67.966 casos novos de TB

diagnosticados no Brasil, em 2014, com um coeficiente nacional de incidência estimado em 33,5 para cada 100 mil habitantes, verificou-se que a maior parte das notificações era nos grandes centros urbanos nacionais, e, de forma heterogênea, de diferentes partes do país, dentre as quais as regiões Norte, Sudeste e Nordeste foram as que apresentaram os mais altos coeficientes de incidência[24].

O país, desde o início do século XX, vem adotando políticas públicas formuladas e implantadas pelos estados e organismos internacionais para o controle da TB. Em 1941 criou-se o Serviço Nacional de Tuberculose, marco efetivo do controle pelo Estado; fato relevante também foi a Campanha Nacional Contra a Tuberculose, em 1946[25]. Porém, apenas no início da década de 80 houve significativa redução da mortalidade e da incidência após introdução do esquema de longa duração com rifampicina, isoniazida e pirazinamida administrada ambulatoriamente. No entanto com o surgimento da AIDS causada pelo HIV em 1977, o quadro da TB teve um retrocesso[25].

Já, em termos de Estados da Federação, aqueles com as maiores taxas de incidência da doença são: Rio de Janeiro (RJ), Pernambuco (PE), Acre (AC) e o Rio Grande do Sul (RS)[26]. No país, a doença tem como aliados predisponentes, em geral, a má alimentação, abuso de álcool, tabaco e de outras drogas. Aliado a estes fatores podemos relacionar a deficiência de infraestrutura pública dos espaços urbanos mais periféricos, onde se encontram moradias precárias e redes de abastecimento de água e de esgoto sanitário inexistentes, situação típica de aglomerados urbanos não atendidos pelo poder público[23]. Tal contexto social faz com que, no Brasil, a TB seja considerada a quarta causa de morte, por doenças infecciosas, e a primeira *causa mortis,* entre indivíduos portadores de HIV/AIDS[23,27].

No Estado do Paraná, a incidência da TB é de 18,7 casos/100.000 habitantes no ano de 2015, porém em algumas regiões essa taxa é maior, como por exemplo, em Paranaguá (cidade portuária) e Foz do Iguaçu (fronteira com Paraguai e Argentina)[28].

1.1.4 **O tratamento da tuberculose**

A TB é uma doença curável em praticamente 100 % das novas ocorrências, desde que o bacilo seja sensível aos medicamentos anti-TB e sejam obedecidos os princípios básicos da terapia medicamentosa[1]. Atualmente, o tratamento recomendado para os novos casos de TB consiste no uso por dois meses de isoniazida (INH), rifampicina (RIF), etambutol (EMB) e pirazinamida (PZA), seguido de quatro meses com INH e RIF[12,28]. Situações como

monoterapia, prescrição imprópria dessa associação ou falta de colaboração do paciente para o uso desse esquema terapêutico podem levar ao surgimento de linhagens de *M. tuberculosis* resistentes a um ou mais fármacos[3,6].

A estreptomicina foi introduzida no mercado em 1943, porém em curto período de tempo surgiram as linhagens de *M. tuberculosis* resistentes a esta monoterapia. Em 1952 propôs-se a associação do ácido p-aminosalicílico (PAS) e isoniazida, por um período de 24 meses. Em 1960, o PAS foi substituído pelo etambutol (EMB), e o período de tratamento para reduzindo para 18 meses. No final desta década, o maior avanço no tratamento da TB foi alcançado associando-se a RIF com EMB e INH, que proporcionou estimativa de cura da doença em 95 %, num período de 9 a 12 meses de tratamento[29]. Somente na década de 80 foi realizada nova alteração da terapêutica compreendida por um período de 6 a 8 meses de tratamento, associando-se o fármaco PZA à INH e RIF nos meses iniciais seguidos pela combinação de INH e RIF ou INH/EMB[16].

No tratamento da TB resistente, os pacientes devem receber tratamento individualizado, dependendo da sensibilidade aos fármacos e das interações e da toxicidade para o paciente[12]. Apesar de os testes de sensibilidade aos fármacos serem feitos individualmente, os medicamentos utilizados no tratamento da TB atuam em combinação. Porém há poucos relatos na literatura realizados para avaliar a eficácia e sinergia das combinações desses medicamentos[21,25].

O controle da TB é severamente comprometido pelo surgimento de linhagens de *M. tuberculosis* multirresistente (MDR) e extensivamente resistente (XDR) aos fármacos. TB-MDR é reconhecida como doença causada por linhagens de *M. tuberculosis* resistentes a INH e a RIF, e TB-XDR como doença causada por linhagens que alem da resistência à INH, RIF é também resistente a uma fluoroquinolona e um dos três fármacos injetáveis de segunda linha (amicacina, canamicina ou capreomicina)[28].

O longo período de tratamento e a ocorrência de efeitos adversos no paciente têm levado a grande incidência de abandono à terapêutica e contribuído significativamente para o aparecimento da TB-MDR e especialmente TB-XDR[30]. Assim, nestes casos é necessário o prolongamento do tempo de tratamento e o emprego de fármacos de maior toxicidade, acentuando a incidência de abandono da terapêutica por parte dos pacientes[31]. Os fármacos que têm sido utilizados para o tratamento de TB-MDR e TB-XDR, incluem fluoroquinolona (levofloxacino (LVX), moxifloxacino), linezolida (LNZ), amoxicilina-clavulanato, claritromicina, tioridazina e clofazimina[32-36].

1.1.5 **A utilização de linezolida e levofloxacino para o tratamento de TB resistente**

A resistência às fluoroquinolonas foi um fator determinante na definição XDR[33]. Elas são comumente usadas para o tratamento de pacientes doentes por linhagens de *M. tuberculosis* resistentes e possui potencial de eficácia e rapidez no tratamento quando comparadas a outros fármacos[34,35]. Essa classe de fármacos, mostram atividade dose-dependente, quando utilizadas para tratar TB e também outras infecções bacterianas. Eles têm efeito bactericida em bactérias Gram negativas e Gram positivas, além disso sua dosagem pode ser otimizada individualmente pelo monitoramento terapêutico, evitando a resistência contra estes fármacos[36,37]. O LVX (Figura 1) é uma nova geração de fluoroquinolonas, que possui maior atividade e segurança contra linhagens de *M. tuberculosis* comparado a outros medicamentos da mesma classe[38,39]. Estudos mostram que a utilização de LVX no tratamento de pacientes com TB-MDR resultou em maior eficácia bem como os estudos *in vitro* e *in vivo* mostraram atividade contra *M. tuberculosis* de duas a três vezes maior que a de ofloxacino (CIM 1 µg/mL)[33,38-41]. As fluoroquinolonas inibem a atividade da DNA girase ou topoisomerase II, enzima essencial à sobrevivência bacteriana, assim a molécula de DNA passa a ocupar grande espaço no interior da bactéria e suas extremidades livres determinam síntese descontrolada de RNA mensageiro e de proteínas, determinando a morte das bactérias[34,35,37,38].

A oxazolidinona é uma nova classe de fármacos inibidores da síntese de proteínas bacteriana[42,43], e a LNZ (Figura 2) é o primeiro membro desta classe[44]. Este antimicrobiano tem um espectro de ação contra micro-organismos Gram-positivos, incluindo multirresistentes, pois inibe o início da síntese de proteínas pela ligação à subunidade 50S do ribossomo bacteriano[44-46]. A LNZ mostrou ter atividade bactericida precoce em pacientes com TB pulmonar, incluindo TB-MDR e TB-XDR em pesquisas realizadas *in vivo* combinado a fármacos de primeira linha em *M. tuberculosis*[38,47,48].

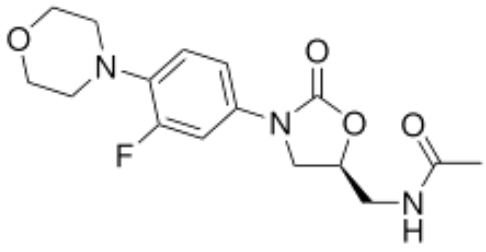

Figura 1: Estrutura química do levofloxacino

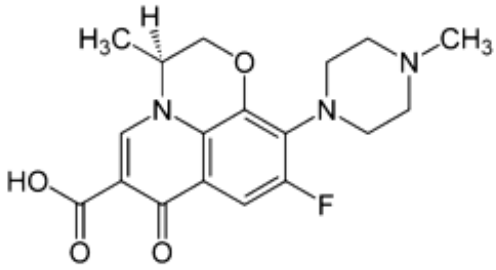

Figura 2: Estrutura química do linezolida

1.1.6 **Estudos de sinergismo entre fármacos - Método de *Checkerboard* tridimensional**

O método *checkerboard* tridimensional para estudos de sinergismo de fármacos foi descrito por Berenbaum (1978)[49], e aplicado por outros pesquisadores em patógenos Gram negativos[50,51]. Nesta linha de estudo, Bhusal et al. (2005) observou a interação de INH e RIF com a adição de um terceiro fármaco (sitafloxacino, gatifloxacino, claritromicina, minociclina, estreptomicina e EMB) em isolados de *M. tuberculosis*[51].

Os métodos de microdiluição em caldo por *checkerboard* são realizados para avaliar a combinações de dois ou três agentes antimicrobianos contra determinado micro-organismo[52]. É baseado no método de diluição e susceptibilidade em caldo segundo o *Clinical and Laboratory Standards Institute* (CLSI), para avaliar inibição bacteriana de concentrações específicas em um determinado tempo[53]. As interações *in vitro* são calculadas matematicamente e interpretadas como sinérgicas, indiferentes e antagônicas dependendo da atividade antibacteriana dos agentes combinados e individuai[49,54].

Este método avalia novas combinações, considerando aquelas em que não é previsto sinergismo ou que o mesmo seja desconhecido, como no caso de um novo agente antimicrobiano, ou quando os resultados ainda não são confiáveis devido ao desenvolvimento de fatores de resistência bacteriana ou falhas de tratamento[54]. Métodos para determinação de atividade sinérgica não foram padronizados completamente, necessitando de mais estudos[49].

Para avaliar possíveis efeitos sinérgicos, o índice de concentração inibitória fracionada da sigla em inglês FICI+ é calculado pela fórmula: FICI = (CIM A + B + C / CIM A) + (CIM B + C +A / CIM B) + (CIM C + A +B / CIM C), em que CIM A + B + C é a CIM do fármaco A combinado com os fármacos B e C, CIM B + C + A é a CIM do fármaco B combinado com os fármacos C e A, CIM C + A + B é o CIM do fármaco C combinado com os fármacos A e B, CIM A é o CIM do fármaco A testado isoladamente, CIM B é o CIM do fármaco B testado isoladamente, e CIM C é o CIM do fármaco C testado isoladamente. A técnica e a interpretação dos resultados das interações antimicrobianas foram feitas de acordo com Bhusal et al. (2005)[51], classificados como sinérgico (FICI $\leq$ 0,75), aditivo ou indiferente (FICI > 0,75 a 4,0) e antagonista (FICI > 4.0)[51].

1.2 JUSTIFICATIVA

Anteriormente, a TB era considerada uma moléstia infecciosa sob controle em muitos países. Porém, na década de 90, esta doença ressurgiu como um problema de saúde pública mundial, consequência da ausência de recursos e emprego de programas de controle inadequados que mantiveram os índices de incidência e prevalência elevados.

Atualmente, a terapêutica é composta por fármacos, introduzidos a tempo considerável no mercado, apresentando poucas inovações. O aumento da resistência a medicamentos contra *M. tuberculosis*, durante os últimos anos, apresenta um desafio terapêutico para a área da saúde na seleção de novos fármacos para o tratamento da TB. Existem poucos fármacos seguros e efetivos para o tratamento e, ainda, com a co-infecção por HIV, o problema torna-se maior, considerando a necessidade de associação de outros fármacos.

As fluoroquinolonas e oxazolidinonas parecem ter um papel útil no tratamento clínico, *in vivo*, combinado com INH ou RIF ou outro fármaco anti-TB contra as formas resistentes da TB. No entanto, são poucas as informações disponíveis, na literatura, sobre a ação de LVX e LNZ em associações com fármacos clássicos anti-TB contra isolados clínicos de *M. tuberculosis*. Neste sentido, um estudo que avalie a ação das combinações RIF/INH/LNZ e RIF/INH/LVX contra *M. tuberculosis* pode trazer benefícios para entender a ação destes fármacos no bacilo.

1.3 OBJETIVOS

1.3.1 **Objetivo Geral**

Avaliar a atividade, *in vitro*, das combinações RIF/INH/LNZ e RIF/INH/LVX contra a cepa padrão de *Mycobacterium tuberculosis* $H_{37}Rv$ e isolados clínicos de *M. tuberculosis* sensível e multirresistentes.

1.3.2 **Objetivos Específicos**

- Determinar a CIM de LNZ, LVX, INH e RIF na cepa padrão de *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) por *Resazurin Microtiter Assay Plate* (REMA).

- Determinar a CIM de LNZ, LVX, INH e RIF em isolados clínicos de *M. tuberculosis* sensível e resistentes por *Resazurin Microtiter Assay Plate* (REMA).
- Avaliar a interação das combinações RIF/INH/LNZ e RIF/INH/LVX na cepa padrão de *M. tuberculosis* H_{37}Rv (ATCC 27294) por *checkerboard* tridimensional.
- Avaliar a interação das combinações RIF/INH/LNZ e RIF/INH/LVX em isolados clínicos de *M. tuberculosis* sensível e resistentes por *checkerboard* tridimensional.

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CAPÍTULO II

MANUSCRITO: AÇÃO COMBINATÓRIA, *in vitro*, DE LINEZOLIDA E LEVOFLOXACINO COM FÁRMACOS ANTITUBERCULOSE EM *Mycobacterium tuberculosis*

AÇÃO COMBINATÓRIA, *in vitro*, DE LINEZOLIDA E LEVOFLOXACINO COM FÁRMACOS ANTITUBERCULOSE EM *Mycobacterium tuberculosis*

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ABSTRACT

Introduction: Tuberculosis (TB) is a worldwide public health problem and it is necessary to use polytherapy with a period of at least six months for treatment. Standard treatment uses isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (ETH). With the increasing increase in the emergence of multidrug resistant (MDR) and extensively resistant (XDR) *Mycobacterium tuberculosis* drug lines, the search for other treatment options has been great. In this sense, the objective of the present study was to evaluate the in vitro activity of INH / RIF / levofloxacin (LVX) and INH / RIF / linezolid (LNZ) combinations in clinical isolates of *M. tuberculosis*.

Methods: The INH / RIF / LNZ and INH / RIF / LVX combinations were evaluated in the reference strain *M. tuberculosis* H_{37}Rv (ATCC 27294) in a clinical isolate sensitive to first-line drugs (INH, RIF, PZA and EMB) and 10 isolates Resistant by a three-dimensional checkerboard, using resazurin as a bacillary growth developer. For this, the drugs LVX and LNZ were used in fixed concentrations of ½ and ¼ of the minimum inhibitory concentration (MIC). INH and RIF were tested at concentrations ranging from 0.0002 mg / L to 400 mg / L to 0.0005 mg / L to 3,000 mg / L, respectively. MIC was defined as the lowest concentration of INH / RIF / LVX and INH / RIF / LNZ combinations in which no visible bacillary growth was observed. Inhibitory concentration fractions (FICs) were determined after seven days incubation of the microplates at 35-37 ° C. The fractional inhibitory concentration indexes (ICIF) with values $\leq$ 0.75, 0.75 - 4 and $\geq$ 4 were considered synergistic, indifferent and antagonistic, respectively.

Results: At the fixed concentration of ½ of MIC of LVX and LNZ added to INH and RIF, synergism was observed only in isolate 309. When the fixed concentration of LVX and LNZ was ¼ of MIC, synergism was observed in 3 isolates tested with INH/RIF/LVX and 3 with INH/RIF/LNZ.

Conclusion: Although no synergism was observed in the other isolates when subjected to the combinations of this study, there was a reduction of the MIC of INH and RIF for all isolates. The present result calls attention to the potential use of these combinations in the treatment of cases of multidrug-resistant.

Keys words: *Mycobacterium tuberculosis*, multidrug-resistant, three-dimensional checkerboard

RESUMO

Introdução: A tuberculose (TB) é um problema de saúde pública mundial e é necessário o uso de politerapia de no mínimo seis meses para o tratamento. O tratamento padrão utiliza isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB). Com o crescente aumento no surgimento de linhagens de *Mycobacterium tuberculosis* multirresistentes (MDR) e extensivamente resistente (XDR) a fármacos, a busca por outras opções de tratamento tem sido grande. Neste sentido, o objetivo do presente estudo foi avaliar a atividade *in vitro* das combinações INH/RIF/LVX e INH/RIF/LNZ em isolados clínicos de *M. tuberculosis.*

Métodos: As combinações INH/RIF/LNZ e INH/RIF/LVX foram avaliadas na cepa de referência *M. tuberculosis* $H_{37}Rv$ (ATCC 27294), em um isolado clínico sensível aos fármacos de primeira linha (INH, RIF, PZA e EMB) e dez isolados clínicos resistentes por *checkerboard* tridimensional, utilizando resazurina como revelador de crescimento bacilar. Para tal, os fármacos LVX e LNZ foram utilizados em concentrações fixas de ½ e ¼ da concentração inibitória mínima (CIM). INH e RIF foram testadas nas concentrações variando de 0,0002 mg/L a 400 mg/L 0,0005 mg/L a 3.200 mg/L, respectivamente. A CIM foi definida como a menor concentração das combinações INH/RIF/LVX e INH/RIF/LNZ em que não se observou crescimento bacilar visível com a utilização do agente revelador de resazurina. As frações de concentração inibitórias (FICs) foram determinadas após sete dias de incubação das microplacas a 35-37ºC. Os índices de concentração inibitória fracionada da sigla em inglês FICI com valores $\leq 0{,}75$; 0,75 - 4 e ≥ 4 foram considerados sinérgicos, indiferentes e antagônicos, respectivamente.

Resultados: Na concentração fixa de ½ da CIM de LVX e LNZ adicionado à INH e RIF foi observado sinergismo somente no isolado 309. Quando a concentração fixa de LVX e LNZ foi de ¼ da CIM foi observado sinergismo em três isolados testados com INH/RIF/LVX e três com INH/RIF/LNZ.

Conclusão: Embora não foram observados sinergismos em todos isolados nas combinações deste estudo, houve uma redução da CIM de INH e RIF para todos os isolados foram observados. O presente resultado chama a atenção para continuidade dos estudos e uso destas combinações no tratamento de casos de isolados clínicos multirresistentes.

Palavras-chave: *Mycobacterium tuberculosis*, multirresistentes, c*heckerboard* tridimensional

1. INTRODUÇÃO

A tuberculose (TB) é uma doença infecto-contagiosa causada, principalmente, pelo bacilo *Mycobacterium tuberculosis*; conhecida há séculos e que continua sendo um importante problema de saúde pública mundial.[1,2] Em 2015, a TB ficou em primeiro lugar no ranking mundial de morte por doenças infecciosas, ultrapassando pela primeira vez, o número de mortes causado pelo vírus da imunodeficiência humana (HIV).[3] Ocorreram 480.000 novos casos de TB-MDR no mundo, em 2015, 80 % dos casos de TB estão situados em um grupo de 20 países do mundo.[3]

O tratamento recomendado para os novos casos de TB consiste na associação dos fármacos: isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB), nos primeiros 2 meses, e 4 meses de INH e RIF.[4,5] Situações como monoterapia, prescrição imprópria dessa associação ou falta de colaboração do paciente para o uso desse esquema terapêutico podem levar ao surgimento de linhagens de *M. tuberculosis* resistentes a um ou mais fármacos.[6,7]

O recente aumento de isolados clínicos multirresistentes de *M. tuberculosis* determinou uma necessidade urgente para a descoberta e desenvolvimento de novos fármacos anti-TB.[8] Nas últimas décadas, o controle da TB tornou-se severamente comprometido pelo surgimento de isolados clínicos multirresistentes (MDR) e extensivamente resistentes (XDR). TB-MDR é reconhecida como doença causada por *M. tuberculosis* resistentes à INH e RIF, e TB-XDR causada por bacilo resistente à INH, RIF, uma fluoroquinolona e um dos três injetáveis de segunda linha (amicacina, canamicina ou capreomicina).[9,10]

No tratamento da TB resistente, os pacientes devem receber tratamento individualizado, dependendo da sensibilidade aos fármacos, toxicidade e as interações para o paciente.[11] Apesar de os testes de sensibilidade aos fármacos serem feitos individualmente, os medicamentos utilizados no tratamento da TB atuam em combinação. Porém há poucos relatos na literatura realizados para avaliar a eficácia e sinergia das combinações desses medicamentos.[12,13]

As opções de tratamento para pacientes com TB-MDR e especialmente com TB-XDR são, atualmente, muito limitadas.[10] Na busca por novas opções de tratamento e com menor toxicidade, diferentes fármacos estão em estudo para uso na terapia da TB como

levofloxacino (LVX), moxifloxacina, linezolina (LNZ), amoxicilina-clavulanato, claritromicina, tioridazina e clofazimina.[4,10]

A LVX constitui uma nova geração de fluoroquinolonas, que possui atividade acentuada contra *M. tuberculosis* comparada a outros fármacos da mesma classe.[14]A LNZ, que é um fármaco pertencente a classe de oxazolidinonas, também mostrou ter atividade bactericida precoce em *M. tuberculosis* e é sugerido seu uso no tratamento de pacientes com TB pulmonar resistente aos fármacos convencionais anti-TB.[15]

A TB continua sendo uma das maiores doença infectocontagiosa crônica, em numero de novos casos, possui poucas inovações em seu tratamento e novos fármacos nos últimos tempos, continuando a ser um desafio terapêutico para a saúde mundial. A utilização de LVX e LNZ nas combinações deste trabalho não foram relatadas anteriormente na literatura, sendo assim este estudo é pioneiro na avaliação destas combinações.

Diante do exposto, o presente estudo propôs avaliar a atividade extracelular, *in vitro,* das combinações INH/RIF/LNZ e INH/RIF/LVX em isolados clínicos de *M. tuberculosis* resistentes a fármacos anti-TB.

2. MATERIAL E MÉTODOS

2.1. Cepa de referência e isolados clínicos de *M. tuberculosis* sensível e resistentes

M. tuberculosis H_{37}Rv (ATCC 27294) e onze isolados clínicos de *M. tuberculosis* (um isolado sensível, seis MDR, três monorresistentes a INH e um resistente a SM) pertencentes ao laboratório de bacteriologia médica da Universidade Estadual de Maringá, foram semeados em Middlebrook 7H9 (DifcoLaboratories, Detroit, USA) enriquecido com Acido Oléico, Albumina, Dextrose e Catalase - OADC (BBL/Becton-Dickinson, Sparks, MD, USA) e incubados por 15 dias a 35 ºC. As suspensões bacterianas foram padronizadas com escala de McFarland nº 1 e diluída 1:20 em Middlebrook 7H9 suplementado com OADC.

2.2. Agentes Antimicrobianos

Os antimicrobianos INH, RIF, LVX e LNZ (Sigma-Aldrich, St. Louis, MO) foram diluídos de acordo com instruções do fabricante. Foram realizadas diluições na razão dois com variação de 0,0009 a 0,25 mg/L; 0,125 a 32,0 mg/L; 0,125 a 4,0 a mg/L e 0,0156 a 16,0 mg/L para INH, RIF, LNZ, LVX, respectivamente.[16,17]

2.3. Determinação da Concentração Inibitória Mínima (CIM)

A CIM de cada fármaco para os isolados de *M. tuberculosis* foi determinada pelo método *Resazurin Microtiter Assay Plate* (REMA), em microplacas de 96 orifícios, como descrito por Palomino et al. (2002).[18] Os fármacos foram seriadamente diluídos em Middlebrook 7H9 suplementado com OADC. Em seguida, foi adicionado 100 µL do inóculo bacteriano previamente padronizado e diluido. As microplacas foram seladas e incubadas a 35 °C em atmosfera normal por sete dias. Após esse período, 30 µL de solução de resazurina 0,02 % (Acros, Morris Plains, NJ, USA) recém-preparada, foram adicionados a cada orifício e as placas foram re-incubadas a 35 ºC por 24 h - 48 h para posterior leitura visual. A mudança da cor azul para rósea, pela redução da resazurina, foi considerada como presença de crescimento bacteriano. A CIM foi definida como a menor concentração do fármaco capaz de inibir o crescimento bacteriano. Foram considerados sensíveis isolados com CIM $\leq$ 0,25 mg/L para INH,[18,19] $\leq$ 0,5 mg/L para RIF, [16,18] $\leq$ 1 mg/L para LVX e $\leq$ 1 mg/L para LNZ,[17,19] (em duplicata).

2.4. Avaliação da combinação extracelular de RIF/INH/LVX e RIF/INH/LNZ em *M. tuberculosis* resistentes

A interação extracelular entre os fármacos INH e RIF com LNZ e entre INH e RIF com LVX em *M. tuberculosis* foi avaliada, em duplicata, pelo método *checkerboard* tridimensional,[9] empregando resazurina como agente revelador de crescimento micobacteriano. INH e RIF foram diluídas seriadamente na razão dois diretamente nas cavidades das microplacas. LVX e LNZ foram adicionadas a cada orifício, contendo as diluições de INH (verticalmente) e RIF (horizontalmente), nas concentrações de ½ e ¼ da CIM previamente determinadas. O inóculo bacteriano foi adicionado após previa

padronização pela escala de McFarland nº 1. As placas foram incubadas a 35-37 ºC por sete dias e após inserido o agente revelador resazurina, foram novamente incubadas e realizou-se leituras 24 e 48 horas após a revelação das mesmas. Os seguintes controles foram utilizados em cada experimento: Middlebrook 7H9-OADC sem fármacos e sem inóculo bacteriano (Controle negativo), Middlebrook 7H9-OADC sem fármacos e com inóculo bacteriano (Controle positivo). A mudança de azul para rósea da resazurina foi considerada como a presença de crescimento bacteriano.

Os possíveis efeitos sinérgicos foram avaliados utilizando o modelo matemático pela determinação do índice de concentração inibidora fracionada (FICI) pela fórmula: FICI = (CIM A + B + C / CIM A) + (CIM B + C +A / CIM B) + (CIM C + A +B / CIM C), em que CIM A + B + C é a CIM do fármaco A combinado com os fármacos B e C, CIM B + C + A é a CIM do fármaco B combinado com os fármacos C e A, CIM C + A + B é o CIM do fármaco C combinado com os fármacos A e B, CIM A, CIM B e CIM C é a CIM do fármaco A, B ou C testado isoladamente, respectivamente. A técnica e a interpretação dos resultados das interações antimicrobianas foram realizadas de acordo com Bhusal et al. (2005).[9] Os efeitos das combinações antimicrobianas foram classificados como sinérgico (FICI $\leq$ 0,75), aditivo ou indiferente (FICI > 0,75 a 4,0) e antagonista (FICI > 4.0).

3. RESULTADOS

Os valores das CIMs dos fármacos INH, RIF, LVX e LNZ isoladamente e das combinações INH/RIF/LVX e INH/RIF/LNZ em concentrações sub-inibitórias fixas (½ e ¼ CIM) encontram-se na Tabela I e II. A CIM variou entre 0,03 - 6,25 mg/L para INH; 0,008 - 100 mg/L para RIF; 0,12 – 0,25 mg/L para LVX e 0,25 - 0,5 mg/L para LNZ.

A associação INH/RIF/LVX apresentou resultado equivalentes para INH e melhor resultado para RIF quando LVX foi utilizada na concentração sub-inibitória de ¼ CIM, com diminuições de até três vezes na CIM da INH e de RIF para alguns isolados clínicos resistentes. Dentre os 10 isolados clínicos resistentes, cinco (50 %) apresentaram redução de pelo menos duas vezes no valor da CIM da INH na associação com LVX (½ da CIM) e cinco (50 %) diminuição de pelo menos duas vezes no valor da CIM da INH quando LVX foi associada na concentração de ¼ da CIM; seis (60 %) isolados apresentaram redução de pelo menos duas vezes o valor da CIM da RIF na associação com ½ da CIM de LVX e quando ¼

da CIM de LVX foi utilizado na tripla combinação; oito (80 %) isolados apresentaram redução de pelo menos duas vezes no valor da CIM de RIF.

A associação INH/RIF/LNZ apresentou melhor resultado quando foi utilizada a concentração sub-inibitória de ¼ CIM de LNZ para INH, com diminuição de até três vezes na CIM da INH e quando foi utilizada a concentração sub-inibitória de ½ CIM de LNZ diminuição de até quatro vezes na CIM de RIF para alguns isolados clínicos resistentes. Dentre os dez isolados clínicos resistentes, cinco (50 %) apresentaram diminuição de pelo menos duas vezes na CIM da INH em associação com ½ da CIM de LNZ e em sete isolados (70 %) diminuição de duas vezes da CIM da INH quando ¼ CIM de LNZ foi associada. Sete (70 %) isolados apresentaram redução de duas a quatro vezes o valor da CIM da RIF quando da associação com ½ CIM de LNZ. Foi observado redução, no valor de duas vezes ou três vezes no valor da CIM da RIF em sete (70 %) isolados quando ¼ da CIM de LNZ foi utilizado.

4. DISCUSSÃO

A terapia recomendada para o tratamento de TB é embasada no uso de combinações de fármacos e em tempo prolongado para atingir o bacilo em suas diferentes etapas no desenvolvimento da doença. O esquema básico recomendado para casos recém-diagnosticados utiliza a combinação de quatro fármacos, INH, RIF, PZA e EMB. Porém, em algumas situações como nos casos de monoresistência, TB-MDR ou TB-XDR este esquema terapêutico não alcança o efeito desejado.

Em situações de TB-MDR e TB-XDR vem sendo utilizado, na clínica médica, LNZ ou LVX para o tratamento de pacientes com esta forma grave da doença. A utilização destes novos fármacos, assim como outras novas combinações, para o tratamento da TB proporcionou aos pacientes com TB novas chances de cura para as formas resistentes da doença. Os fármacos utilizados no presente estudo, LNZ ou LVX, já foram utilizados em alguns pacientes, com TB resistente, como alternativa de tratamento, porém, não administrados em combinação com os fármacos utilizados em nosso estudo.[14, 20-26]

O método *checkerboard* tridimensional foi descrito por Berenbaum[20], e aplicado por outros autores para estudos de combinações de três fármacos em patógenos Gram negativos.[21] Bhusal et al. (2005),[9] estudou a interação de INH e RIF com a adição de um terceiro fármaco

(sitafloxacina, gatifloxacino, claritromicina, minociclina, estreptomicina e EMB) em *M. tuberculosis* e também observou sinergismo entre INH e RIF com fluoroquinolonas em alguns isolados clínicos de *M. tuberculosis* corroborando os dados obtidos no presente trabalho. Pode-se observar nos estudos realizados *in vitro*, no presente trabalho, que combinações de INH/RIF com LVX ou LNZ apresentaram efeito satisfatório levando a redução da CIM dos dois fármacos anti-TB em todos os isolados de *M. tuberculosis* testados. Efeito sinérgico entre os fármacos INH/RIF/LVX foi observado em quatro dos dez isolados de *M. tuberculosis* resistentes. De acordo com Rodriguez Diaz et al. (2003),[14] a combinação, realizada *in vitro* pelo método de *checkerboard* bidimensional, de LVX com INH e RIF, apresentou também sinergismo em isolados de *M. tuberculosis* sensíveis e resistentes a INH. Ação sinérgica entre RIF e INH combinados com LVX foi observada nos isolados clínicos sete (resistente a EMB), 71A (MDR), 109 (MDR) quando INH e RIF foram combinadas a ¼ da CIM de LVX. Para o isolado 309 (MDR) a combinação apresentou ação sinérgica somente quando ½ da CIM de LVX.

O sinergismo encontrado em nosso estudo, com a adição de LVX na combinação, é corroborada por alguns estudos com a utilização de fluoroquinolonas de nova geração.[22-24] Nestes estudos, observou-se alta atividade anti-*M. tuberculosis* das fluoroquinolonas contra isolados de *M. tuberculosis* resistentes, reduzindo o tempo de tratamento dos pacientes. O estudo de Rastogi et al. (1996),[25] *in vivo*, mostra que com a adição de LVX ao tratamento padrão apresenta efeitos satisfatórios com conversão de cultura positiva para negativa durante o tratamento de mais de 80 % dos pacientes com TB-XDR em um prazo menor que o padrão. Ainda vale ressaltar que, em nosso estudo, a adição de LVX levou uma diminuição no valor da CIM dos fármacos em todos os ensaios com os isolados testados o que em circunstâncias *in vivo* pode contribuir na cura do paciente com TB.

No caso da combinação *in vitro* de INH/RIF/LNZ, foi observado efeito sinérgico em cinco dos dez isolados de *M. tuberculosis* resistentes. Destes, quatro dos isolados foram os mesmos para os quais a combinação INH/RIF/LVX também apresentou sinergismo (7, 71A, 109 e 309). A ação sinérgica observada nestes isolados foi detectada quando INH e RIF foram combinadas a ¼ da CIM de LNZ, exceto para o isolado 309 que foi com ½ da CIM de LNZ. A combinação de INH e RIF com ¼ da CIM de LNZ também mostrou ação sinérgica no isolado 3614 (MDR). Estudos *in vitro*, de combinação bidimensional entre INH ou RIF com LNZ realizada por Rodriguez Diaz et al. (2003)[14] detectou efeito sinérgico com LNZ, principalmente em isolados de *M. tuberculosis* monorresistentes à INH.

No caso das oxazolidinonas, a LNZ tem se mostrado promissora no tratamento de TB-MDR e TB-XDR.[26,27] Estudo realizado no Paquistão com LVX, LNZ e amoxicilina em isolados de pacientes recém-tratados com o tratamento padrão, avaliou a interação extracelular destes fármacos *in vitro* e demonstrou que a LNZ pode ser eficaz para o tratamento de casos de TB-MDR e TB-XDR. [28]

Nosso estudo chama atenção de interações entre fármacos, pois as combinações INH/RIF/LVX e INH/RIF/LNZ apresentaram melhor ação contra *M. tuberculosis* quando o terceiro fármaco testado, LVX ou LNZ, foram utilizados na concentração de ¼ da CIM no teste trimdimensional. Neste caso, ocorreu uma redução de 2 a 3 vezes na CIM dos fármacos de primeira linha com resultados sinérgicos melhores do que o uso de ½ da CIM. Não foram observados antagonismo entre os fármacos deste estudo em nenhum dos ensaios realizados.

Esta diferença pode ser, de certo modo, explicada considerando que uma ação sinérgica ou mesmo antagônica de uma combinação específica de fármacos, contra determinado micro-organismo, não é meramente uma propriedade de cada fármaco e sim dependente também da dose de cada um na combinação.[29] A farmacodinâmica de INH e RIF diferem entre si, pois INH é concentração dependente enquanto RIF é tempo dependente. Assim quando um paciente é exposto ao tratamento padrão, os bacilos sensíveis são mortos primeiramente pela ação rápida de INH, em contrapartida a RIF elimina os demais bacilos conforme o tempo de tratamento vai passando. A adição de LVX e LNZ contribui para que o tempo de tratamento seja reduzido e para que haja redução das concentrações de INH e RIF utilizadas na terapêutica do paciente, sendo assim alguns estudos já mostram atividade bactericida da classe de fluoroquinolonas,[30,31] e também de LNZ,[15] *in vivo* e *in vitro*, contra *M. tuberculosis*.

Estudos *in vitro* de sinergismo de fármacos com o objetivo de determinação quantitativa das colônias de *M. tuberculosis*, ou seja, métodos para avaliar a concentração bactericida de fármacos em *M. tuberculosis*, mostra sua importância em serem conduzidos devido à complexidade de experimentos clínicos. A variação na população de pacientes participantes é grande e inclui idade, sexo, história de tratamento prévio entre outras variáveis que pode levar a falhas ou resultados inconclusivos.[32,33] Isto chama a atenção para que os estudos *in vitro* de combinação de fármacos, sejam realizados previamente ao *in vivo* para determinação da concentração adequada de cada fármaco a ser aplicado.

Deve ser considerado ainda que, o tratamento da TB, devido à patogenia de *M. tuberculosis* exige regime de quimioterapia multifármacos e o sucesso do mesmo não depende

somente da ação individualizada ou da combinação de fármacos, mas também da adesão do paciente ao tratamento. É importante recordar que a importância da redução dos efeitos colaterais dos fármacos pela redução da dosagem destes pode levar a uma maior adesão do paciente a tratamento. Também, a adição de um fármaco que possa reduzir o tempo de tratamento pode ampliar ainda mais este efeito positivo dos fármacos utilizados neste estudo no tratamento da TB.

Em conclusão, vale ressaltar que houve uma redução da CIM de INH e RIF em todos os isolado estudados, mesmo naqueles que não apresentaram sinergismo entre os fármacos. Sendo assim, o uso de LVX e LNZ pode contribuir na terapêutica do paciente com TB, principalmente em casos MDR. O presente resultado chama a atenção para continuidade dos estudos e uso destas combinações no tratamento de casos de isolados clínicos multirresistentes.

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Tabela I. Ação combinatória de isoniazida, rifampicina e levofloxacino, pelo método de *checkerboard* tridimensional, em isolados clínicos de *Mycobacterium tuberculosis* e cepa de referência $H_{37}Rv$

Isolados	Perfil de Sensibilidade	CIM (mg/L) Individual			***Checkerboard* INH/RIF com ½ da CIM de LVX**				***Checkerboard* INH/RIF com ¼ da CIM de LVX**			
		INH	**RIF**	**LVX**	INH	RIF	LVX	**FICI**	INH	RIF	LVX	**FICI**
					CIM (mg/L)				CIM (mg/L)			
$H_{37}Rv$*	Sensível	0,03	0,008	0,25	0,016	0,004	0,12	1,5	0,016	0,002	0,062	1
27	Sensível	0,03	0,001	0,5	0,012	0,0002	0,25	1,25	0,012	0,0002	0,125	1
7	SM^R	0,03	0,008	0,25	0,016	0,004	0,125	1,5	0,007	0,002	0,062	**0,75**
14 P	INH^R	2	0,06	0,25	1	0,03	0,125	1,5	1	0,016	0,062	1
23	INH^R	2	0,06	0,12	0,5	0,03	0,062	1,25	1	0,016	0,031	1
26	INH^R	2	0,06	0,25	1	0,03	0,125	1,5	0,5	0,03	0,062	1
309	INH^R/RIF^R	6,25	100	0,25	0,78	25	0,25	**0,75**	1,56	50	0,125	1,25
45	INH^R/RIF^R	4	64	0,25	2	16	0,125	1,25	2	8	0,062	0,875
71 A	INH^R/RIF^R	6,25	50	0,25	1,56	3,125	0,125	0,81	1,56	6,25	0,062	**0,625**
109	INH^R/RIF^R	2	100	0,25	1	25	0,125	1,25	0,25	25	0,062	**0,625**
19 RP	$INH^R/RIF^R/EMB^R$	3,12	25	0,25	0,78	6,25	0,125	1	1,56	6,25	0,062	1
3614	$INH^R/RIF^R/EMB^R/SM^R/ETH^R$	6,25	25	0,25	1,56	3,125	0,125	0,875	3,125	3,125	0,062	0,875

* Cepa de referência *Mycobacterium tuberculosis* (ATCC 27294); R: resistente; FICI: Índice de Concentração da Fração Inibitória; CIM: Concentração Inibitória Mínima; INH: isoniazida; RIF: rifampicina; LVX: levofloxacino; EMB: etambutol; SM: Estreptomicina; ETH: Etionamida.

Tabela II. Ação combinatória de isoniazida, rifampicina e linezolida, pelo método de *checkerboard* tridimensional, em isolados clínicos de *Mycobacterium tuberculosis* e cepa de referência $H_{37}Rv$

Isolados	**Perfil de Sensibilidade**	CIM (mg/L) Individual			***Checkerboard* INH/RIF com ½ da CIM de LNZ**				***Checkerboard* INH/RIF com ¼ da CIM de LNZ**			
		INH	**RIF**	**LNZ**	INH	RIF	LNZ	**FICI**	INH	RIF	LNZ	**FICI**
					CIM (mg/L)				CIM (mg/L)			
$H_{37}Rv$*	Sensível	0,03	0,008	0,5	0,016	0,004	0,25	1,5	0,016	0,002	0,125	1
27	Sensível	0,03	0,001	0,5	0,012	0,0002	0,25	1,25	0,012	0,0002	0,125	1
7	SM^R	0,03	0,008	0,25	0,016	0,004	0,125	2	0,007	0,002	0,062	**0,75**
14	INH^R	2	0,06	0,25	1	0,03	0,125	1,5	1	0,016	0,062	1
23	INH^R	2	0,06	0,25	0,5	0,03	0,125	1,25	1	0,008	0,062	0,875
26	INH^R	2	0,06	0,25	1	0,03	0,125	1,5	0,5	0,03	0,062	1
309	INH^R/RIF^R	6,25	100	0,5	0,78	25	0,25	**0,75**	1,56	50	0,125	1,25
45	INH^R/RIF^R	4	64	0,25	2	16	0,125	1,25	2	16	0,062	1
71 A	INH^R/RIF^R	6,25	50	0,25	1,56	12,5	0,125	1	1,56	12,5	0,062	**0,75**
109	INH^R/RIF^R	2	100	0,25	1	25	0,125	1,25	0,25	25	0,062	**0,625**
19 RP	$INH^R/RIF^R/EMB^R$	3,12	25	0,25	0,78	1,56	0,125	0,812	1,56	12,5	0,062	1,25
3614	$INH^R/RIF^R/EMB^R/SM^R/ETH^R$	6,25	25	0,25	1,56	1,56	0,125	0,812	1,56	3,125	0,062	**0,625**

*Cepa de referência *Mycobacterium tuberculosis* (ATCC 27294); R: resistente; FICI: Índice de Concentração da Fração Inibitória; CIM: Concentração Inibitória Mínima; INH: isoniazida; RIF: rifampicina; LNZ: linezolida; EMB: etambutol; SM: Estreptomicina; ETH: Etionamida.

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CAPÍTULO III

4.1. CONCLUSÕES

- O efeito sinérgico da combinação INH/RIF/LVX, foi observado em três isolados utilizando a combinação de com ¼ da CIM de LVX e em um isolado observou-se o efeito sinérgico entre os farmacos quando a combinação foi utilizada com ½ da CIM de LVX.
- No caso da combinação entre INH/RIF/LNZ, quatro isolados clínicos apresentaram efeito sinérgico dos fármacos com ¼ da CIM de LNZ e um com ½ da CIM de LNZ.
- Este estudo demonstrou que o uso das combinações com LVX e LNZ no tratamento de casos TB-MDR apresentam-se favoráveis contribuindo com o tratamento padrão.
- Os ensaios que não apresentaram sinergismo obtiveram uma redução da CIM de todos os fármacos. Portanto, o uso de LVX e LNZ pode contribuir na terapêutica do paciente com TB e reduzir as concentrações dos fármacos de primeira linha utilizados no tratamento quando comparado ao tratamento para TB-MDR.

4.2. PERSPECTIVAS

Estudos das combinações de INH/RIF/LNZ e INH/RIF/LVX, *in vitro*, não foram descritos na literatura até o presente momento, portanto são necessários estudos mais abrangentes sobre o efeito desses fármacos combinados. O desenvolvimento de novos estudos utilizando estas combinações e envolvendo maior número de isolados clínicos de *M. tuberculosis*, e a inclusão de outras técnicas utilizando a combinação proposta em isolados submetidos à dormência, e a macrófagos infectados em cultura celular, assim como avaliar o efeito bactericida destes fármacos isoladamente e em combinação. Esses estudos poderão ajudar a elucidar os efeitos destas combinações contra o bacilo de *M. tuberculosis*.

Anexo

INSTRUCTIONS FOR AUTHORS

BACKGROUND AND SCOPE OF THE JOURNALEDITORIAL OFFICE CONTACT INFORMATIONPROCESSING OF PAPERS

OPTIONAL OPEN ACCESSJOURNAL STYLEONLINE SUBMISSION DETAILS

BACKGROUND AND SCOPE OF THE JOURNAL

Background

The *Journal of Antimicrobial Chemotherapy* was founded in 1975 by the British Society for Antimicrobial Chemotherapy (BSAC) as part of its mission to facilitate the acquisition and dissemination of knowledge in the field of antimicrobial chemotherapy. Proceeds from the Journal are used by the BSAC to further these objectives. Articles are published continuously online in JAC Advance Access and assembled into monthly printed and online issues. The Journal has an Impact Factor of 5.313 in 2014.

Aims

The Journal publishes articles that further knowledge and advance the science and application of antimicrobial chemotherapy with antibiotics and antifungal, antiviral and antiprotozoal agents. The Journal publishes primarily in human medicine, and articles in veterinary medicine likely to have an impact on global health.

Scope

The Journal particularly welcomes manuscripts on:

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• the action of antimicrobial agents and the mechanisms, genetics and epidemiology of antimicrobial resistance

• antimicrobial stewardship

• the genetic basis of antimicrobial resistance

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• consolidate our knowledge of antimicrobial agents and their use

• consider the future of antimicrobial chemotherapy

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• drug stability studies

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Authors must state their compliance with relevant institutional and national standards for animal care and experimentation, together with the details of any authorities that licensed the experiments.

JAC supports the use of the ARRIVE Guidelines (https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/NC3Rs%20ARRIVE%20Guidelines%20Checklist%20(fillable).pdf) and articles reporting research in animals must include a completed ARRIVE checklist (https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/NC3Rs%20ARRIVE%20Guidelines%20Checklist%20(fillable).pdf) which must be uploaded with the article so it is available for the scrutiny of the Editor and referees.

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ALL papers submitted to *JAC* reporting original research MUST include a ‘Funding’ section. This section should appear after the 'Acknowledgements' section.

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If a professional medical writer or similar service was involved in the origin or preparation of a manuscript and this support was funded, the source must be declared in the Funding section.

Sources of funding may of course still be thanked in the Acknowledgements section, but should not be listed again in the Transparency declarations (see below), unless there is an important reason for doing so. For example if the funder played any decision-making role in the research this must be stated.

The following rules should be followed:

• The sentence should begin: ‘This work was supported by …’

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• Grant numbers should be complete and accurate and provided in brackets as follows: ‘(grant number ABX CDXXXXXX)’

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An example is given here: ‘This work was supported by the National Institutes of Health (P50 CA098252 and CA118790 to R. B. S. R.) and the Alcohol & Education Research Council (HFY GR667789).

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If only some authors need to make a declaration it must be made clear that the remaining authors have nothing to declare, for example:

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Other useful information

In some instances (often when the authors themselves have no interests to declare) it may be helpful to readers as background information to give brief details of organizations that do have an interest but do not appear elsewhere in the article, for example 'Fantastazole is owned by Wonder Pharmaceuticals'.

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Contributions

The contribution of each author must be clearly stated in the Transparency declarations section, after the information on conflicts of interest.

Reporting standards

All involved in the publication of health intervention research have a duty to patients and society at large to ensure that this research is reported in a complete, accurate and transparent fashion. This includes authors, referees, Editors and Journals. *JAC* takes this responsibility seriously and endorses the work of organizations such as the EQUATOR network (http://www.equator-network.org/), an international initiative that seeks to improve the reliability and value of the medical research literature.

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Randomized controlled trials

Authors should comply with the Consolidated Standards of Reporting Trials (CONSORT) statement (www.consort-statement.org/) and use the resources within it (for example the checklist and flow diagram) to ensure they have addressed potential criticisms and provided all necessary information. Authors should include a CONSORT flow diagram in their article, and provide a copy of the completed checklist.

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For systematic reviews and meta-analyses of randomized controlled trials authors should comply with the PRISMA statement (which replaces the QUORUM statement), which consists of a checklist and flow diagram (http://www.prisma-statement.org/index.htm). Authors should include a PRISMA flow diagram in their article, and provide a copy of the completed checklist.

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Authors should comply with the ORION statement (www.idrn.org/orion.php), which is the CONSORT equivalent for infection control studies. Its purpose is to increase the quality of research and reporting in the area of nosocomial infection.

Economic evaluations

Authors of articles describing economic evaluations of antimicrobial interventions are encouraged to make use of the following resources, where applicable, in order to ensure that their work is both optimal and adequately described.

International Society of Pharmacoeconomics and Outcomes Research (ISPOR) Checklist for retrospective database studies, which can be accessed at: http://www.ispor.org/workpaper/healthscience/ret_dbTFR0203.asp

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General

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Where possible SI units should be used.

Please ensure that characters with a similar appearance are consistent throughout the document and not from different Unicode sub ranges as with the Greek Delta.

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Spelling

British spelling should be used. Spelling should follow that of the *Oxford Dictionary for Scientific Writers and Editors* and where this gives no guidance the *Concise Oxford Dictionary*. Spelling of drug names should conform with that given in the latest edition of the *British National Formulary* (published by the British Medical Association and the Royal Pharmaceutical Society of Great Britain and available online at http://www.bnf.org/bnf), but please note that *JAC* will continue to use methicillin (not meticillin).

Abbreviations

Non-standard abbreviations should be defined at the first occurrence and introduced only where multiple use is made. See here for abbreviations that may be used without definition, as well as antimicrobial abbreviations (which may be used in Tables and Figures).

Dosage frequencies and routes of administration

Latin dosage frequency abbreviations are not permitted (qd, bd, bid, tds etc.), however, constructions q12h, q8h and so on are permitted as there is less likelihood of confusion. . Routes of administration other than intramuscular (im) and intravenous (iv), which may be abbreviated after definition, should be given in full in English.

MICs

Please note that all MIC data in *JAC* must be expressed in terms of mg/L (not μg/mL).

Nomenclature

Authors are required to check and ensure that in all instances the most up to date nomenclature is being used.

Bacterial nomenclature

When genus and species are given together use a capital letter for the genus and a lowercase letter for the species and italicize both e.g. *Staphylococcus aureus*. After the initial use in the text of the full name of an organism the generic name should then be abbreviated to the initial letter, e.g. *E. coli.*

When the genus is used as a noun or adjective use lowercase roman unless the genus is specifically referred to e.g. 'staphylococci and streptococci' but 'organisms of the genera *Staphylococcus* and *Streptococcus*'.

The name of an order has an initial capital but is not italicized, e.g. Enterobacteriaceae. For genera in the plural, use lowercase roman, e.g. salmonellae.

When the species is used alone use lowercase e.g. viridans streptococci. For trivial names, use lowercase roman e.g. meningococcus.

Authors should use bacterial names present in the *Approved List of Bacterial Names, Amended Edition* (1989), Skermanm, V.B.D., McGowan, V. &Sneath, P.H.A., Eds, ASM Press, Washington, DC, USA (ISBN 1-55581-014-4), with subsequent alterations validly published by announcement in Validation Lists of the *International Journal of Systematic and Environmental Microbiology* (formally the *International Journal of Systematic Bacteriology*). A full list of validly published bacterial names is given at http://www.bacterio.cict.fr/allnames.html

Genetic and amino acid nomenclature

Bacterial genetics. Genotype designations are indicated with italic lowercase three-letter locus codes (e.g. *par*, *his*, *ara*). If several loci are involved in a related function the individual loci are designated by the addition of an uppercase italic letter to the locus code (*parC*, *ompF*).

Phenotype designations (for example the protein product of a bacterial gene) are given in roman type with an initial capital letter (OmpF, LacZ).

Erythromycin gene nomenclature should follow that described in: Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J &Seppala H. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* 1999; **43**: 2823-30.

Yeast genetics. Wild-type alleles are all uppercase and italicized (*LEU2*), mutant alleles are all lowercase and italicized (*leu2*), and gene products are capitalized on the first letter and are not italicized (Leu2).

General. Authors should ensure that they confine discussion of changes in amino acid sequence to the context of the protein (e.g. OmpF) and nucleotide changes to the context of the gene (e.g. *ompF*). Please also be aware of the difference between a mutant (a strain with one or more mutations) and a mutation (a change in the sequence of the genetic material).

Amino acids. The full residue names or three-letter abbreviations are preferred in the text (e.g. a methionine residue at position 184 should be symbolized Met-184). The single letter codes may be used in figures. Amino acid changes should be designated Met-184→Val or M184V.

When comparing nucleotide or amino acid sequences authors should exercise care in the use of the term homology. Homology should only be used when a common evolutionary origin is being implied; it is incorrect to give a percentage homology between two sequences. The wing of a bird and the human arm are homologous structures (they are believed to have a common evolutionary origin), homology cannot be quantified. For sequence comparison authors should use the terms identity and similarity. Sometimes 'equivalent' or 'counterpart' is more appropriate than 'homologue'.

Beta-lactamase nomenclature

Authors submitting articles reporting the identification of new beta-lactamases must provide evidence that they have contacted the relevant clearinghouse (http://www.lahey.org/Studies/) to deposit the new sequence data and receive a unique designation for the new enzyme.

Macrolide-lincosamide-streptogramin resistance determinant nomenclature

Nomenclature for macrolide-lincosamide-streptogramin resistance determinants should follow the structure suggested by: Roberts MC, Sutcliffe J, Courvalin P *et al*. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B antibiotic resistance determinants. *Antimicrob Agents Chemother* 1999; **43**: 2823-30. A new gene must have ≤79%

amino acid identity with all previously characterized MLS genes before receiving a new unique name. Adding subscripts or superscripts to established genes is not acceptable. See: http://faculty.washington.edu/marilynr/. Before submitting a sequence to GenBank or submitting a manuscript for publication, please contact Professor Marilyn Roberts (marilynr@u.washington.edu). Once a new name has been assigned you must indicate in your article that you have received approval by the nomenclature centre for the new gene name.

Tetracycline resistance determinant nomenclature

Nomenclature for tetracycline resistance determinants should follow that suggested by: Levy SB, McMurry LM, Barbosa TM *et al*. Nomenclature for new tetracycline resistance determinants. *Antimicrob Agents Chemother*1999; **43**: 1523-4. A new gene must have ≤79% amino acid identity with all previously characterized *tet*genes before receiving a new unique name. Adding subscripts or superscripts to established genes is not acceptable. See: http://faculty.washington.edu/marilynr/. The Levy Group is responsible for coordinating the naming of new *tet* genes and before submitting a sequence to GenBank or submitting a manuscript for publication, please contact Laura McMurry (laura.mcmurry@tufts.edu). Once a new name has been assigned you must indicate in your article that you have received approval by the nomenclature centre for the new gene name.

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FICI data

Fractional inhibitory concentration index (FICI) experiments are performed in order to study drug interactions and they must be interpreted in the following way:

FICI<=0.5 = synergy

FICI>4.0 = antagonism

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For further information please see the following Editorial:

Odds FC. Synergy, antagonism, and what the checkerboard puts between them. *J Antimicrob Chemother*2003; **52**: 1.

Microarray data

Authors of articles containing microarray data must ensure that the full datasets are lodged with an appropriate publicly available online database (the data must not be supplied for publication as Supplementary data alongside the article). The data should be supplied with the submitted article if they are not already publicly available. The name of the database and the accession numbers should be provided in the article. Authors must ensure that their data are available for public scrutiny from the online publication date of their article at the latest.

Chemistry

General nomenclature. The IUPAC recommendations on chemical nomenclature should be followed (*IUPAC Compendium of Chemical Terminology* (1987, ISBN 0 632 01767 8, Blackwell Scientific Publications, Oxford). All chemical names are run together except those of acids, acetals, esters, ethers, glycosides, ketones and salts, which are printed as separate words; hyphens are used to separate numbers, Greek letters and some configurational prefixes, e.g. *p*-nitrophenol. Italics are used for certain prefixes, e.g. *cis*-, *trans*- and *N*. Small capitals are used for dextro- and laevo- prefixes, e.g. L-glutamine.

Drugs. Spelling of drug names should conform with that given in the latest edition of the British National Formulary. Chemical or generic names of drugs should be used; trade names may be referred to once only upon first use of the generic or chemical name. The content of proprietary formulations should be given if relevant. Generic names should not be abbreviated in the text; abbreviations may be used in Tables if there is limited space. If compounds are referred to by code name or company number either the structure or a reference to a paper illustrating the structure must be given, any previous code names or designations should be given on first use.

Supplier locations are required for all smaller/local suppliers.

References

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detail, and for discussing the results and any relevant issues raised by them. Authors are responsible for ensuring that references are quoted accurately and not taken out of context. References must not be cited in the synopsis.

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All references should be listed numerically at the end of the text. Each reference should be preceded by a number (not superscript) followed by a full stop. Please see the following examples. Failure to conform to Journal style will result in the manuscript being returned to authors.

Examples

Journal reference (<= three authors)

Sanschagrin F, Levesque RC. A specific peptide inhibitor of the class B metallo-B-lactamase L-1 from *Stenotrophomonasmaltophilia* identified using phage display. *J AntimicrobChemother* 2005; **55**: 252-5.

Journal reference (> three authors)

Williams I, Gabriel G, Cohen H *et al*. Zidovudine-the first year of experience. *J Infect* 1989; **18** Suppl 1: 23-31.

Journal reference (online journal)

Bell A, Lewandowski K, Myers R *et al*. Genome sequence analysis of Ebola virus in clinical samples from three British healthcare workers, August 2014 to March 2015. *Euro Surveill* 2015; **20**: pii=21131.

Whole book

Long HC, Blatt MA, Higgins MC *et al*. *Medical Decision Making*. Boston: Butterworth-Heinemann, 1997.

Book chapter

Manners T, Jones R, Riley M. Relationship of overweight to haitus hernia and reflux oesophagitis. In: Newman W, ed. *The Obesity Conundrum*. Amsterdam: Elsevier Science, 1997; 352-74.

NCCLS/CLSI methods

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Panel on Antiretroviral Guidelines for Adults and Adolescents. *Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents*. Department of Health and Human Services. http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf.

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