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Alterações no gene *mgrB* associadas a resistência às polimixinas em isolados de *Klebsiella pneumoniae* recuperadas de pacientes internados em um hospital de Porto Alegre.

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“Todas as vitórias ocultam uma abdicação”.

(Simone de Beauvoir)

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Alterações no gene *mgrB* associadas a resistência às polimixinas em isolados de *Klebsiella pneumoniae* recuperadas de pacientes internados em um hospital de Porto Alegre.

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Resumo

O aumento na incidência de enterobactérias multirresistentes acarretou o retorno no uso de polimixinas para o tratamento de infecções graves. Em *Klebsiella pneumoniae*, as polimixinas atuam no lipídeo da membrana externa, alterando sua carga, promovendo a lise celular. Genes envolvidos no equilíbrio da membrana externa podem estar associados a resistência as polimixinas. O *mgrB* é um regulador dos sistemas de dois componentes PhoP/PhoQ e PmrA/PmrB, e sua inativação pode estar relacionada a resistência às polimixinas. Neste contexto, buscamos investigar alterações no gene *mgrB* em isolados de *Klebsiella pneumoniae* resistentes a polimixina B. Foram analisados 37 isolados, oriundos de um hospital de Porto Alegre. Todos os isolados foram selecionados através da Concentração Inibitória Mínima e posteriormente submetidos a Reação em Cadeia da Polimerase, sendo alguns enviados para sequenciamento, os quais apresentaram diferentes alterações no *mgrB*: mutações pontuais, inserção de sequência e deleção do gene. Em relação a outros estudos, a proporção encontrada foi inversa, apresentando maior incidência de mutações pontuais, podendo estar relacionado a disseminação clonal. Acredita-se que a resistência as polimixinas não ocorra por um mecanismo único, mas por diversos fatores que levem a inativação do sistema, os quais ainda devam ser melhor elucidados.

Palavras chave: resistência, polimixinas, enterobactérias, gene *mgrB, Klebsiella pneumoniae*

INTRODUÇÃO

As polimixinas são antibióticos de última linha para o tratamento de infecções graves causadas por enterobactérias multirresistentes [1,2,4,5]. O retorno do interesse nessa classe de antibióticos como opção terapêutica, após anos praticamente abandonada, deve-se, essencialmente, ao aumento crescente da resistência aos carbapenêmicos, observada em diferentes regiões do mundo, incluindo o Brasil [1-3]. A maior frequência de utilização das polimixinas aumentou a pressão seletiva sobre as bactérias, favorecendo a emergência e a disseminação da resistência a essas drogas [1,3].

Tal resistência vem sendo descrita em todo mundo, com uma tendência de aumento a cada ano [2]. Na Europa, há relatos de múltiplos surtos de isolados de *Klebsiella pneumoniae* resistentes às polimixinas, especialmente atribuídos ao clone epidêmico internacional ST258. Na América do Norte, a resistência às polimixinas e aos carbapenêmicos é baixa; mesmo assim, surtos de *K. pneumoniae* resistente às polimixinas pertencentes ao mesmo clone são descritos [4]. No Brasil, o primeiro relato de resistência à polimixina B em enterobactérias foi em 2006 [9]. Estudos dessa época demonstraram um percentual de 1,8% de resistência às polimixinas em *K. pneumoniae*, evoluindo, em 2013, para 15% em populações de *K. pneumoniae* produtoras de KPC [1, 20].

As polimixinas são polipeptídios catiônicos que têm um mecanismo de ação complexo. Inicialmente, atuam ligando-se ao lipopolissacarídeo (LPS) bacteriano, mais especificamente no lipídeo A, da membrana externa dos bacilos gram-negativos, o qual apresenta carga negativa. Ao se ligar no LPS, elas deslocam cátions divalentes, alterando a polarização da membrana, culminando na lise celular.

As bactérias são capazes de desenvolver resistência às polimixinas através da diminuição da carga negativa do LPS, reduzindo ou evitando a ligação da droga [2,6]. Existem duas principais modificações no LPS relacionadas à resistência às polimixinas: a adição de fosfoetanolamina (pETN) ou a adição de 4-amino-4-desoxi-L-arabinose (L-Ara4N). A adição de L-Ara4N confere níveis mais altos de resistência devido a redução das cargas negativas do LPS a próximo de zero. Estudos mostram que em *Salmonella enterica* resistentes à polimixina B, a presença de L-Ara4N é 5 vezes maior do que em isolados susceptíveis [10]. A adição desses grupos está associada a alterações em genes cromossômicos ou, conforme descrito mais recentemente, a expressão de genes presentes em plasmídeos (*mcr*) [16].

Cromossomicamente, as modificações do LPS podem ser resultado de alterações nos sistemas de regulação de dois componentes, PhoP/PhoQ e PmrA/PmrB, mas também estar associadas a alterações no gene *mgrB.* [5,6,11]. PhoQ é uma proteína transmembrana que capta diferentes sinais ambientais, dentre eles a presença de peptídeos catiônicos, pH ácido e baixa concentração de íons Mg^{2+} [5,11,12]. A captação desse sinal pela PhoQ leva a fosforilação de PhoP, e o sinal ativa *operons* que controlam as modificações da membrana externa, através do sistema PmrA/PmrB. Isso induz a produção da proteína MgrB, que irá reprimir a fosforilação de PhoP, através da inibição da atividade de PhoQ quinase, reduzindo a transferência de pETN e L-Ara4N para o lipídeo A. Isso evita a diminuição das cargas negativas no LPS, exercendo, portanto, um *feedback* negativo sobre o sistema de regulação PhoP/PhoQ [11].

Tem sido demonstrado que a inativação do gene *mgrB* por sequências de inserção, mutações *missense*, deleções de pequenas sequências ou até do *locus* completo desse gene exercem um *feedback* positivo no sistema PhoQ/PhoP, favorecendo a transferência dos grupos pETN e L-Ara4N para o LPS, sendo esse o principal mecanismo de resistência às polimixinas em *K. pneumoniae* [1,3,4]. Assim, o objetivo desse estudo foi avaliar

possíveis alterações genéticas presentes no gene *mgrB* de isolados clínicos de *K. pneumoniae* resistentes à polimixina B.

METODOLOGIA

Foram avaliados 37 isolados de *K. pneumoniae* resistentes à polimixina B, recuperados de pacientes internados em um hospital de Porto Alegre, Rio Grande do Sul, Brasil. Os isolados foram obtidos de diferentes espécimes clínicos, coletados entre julho de 2018 e agosto de 2019. Um isolado por paciente foi considerado. O estudo foi aprovado pelos Comitês de Ética da Universidade Federal do Rio Grande do Sul e do hospital do qual os isolados foram recuperados. No laboratório de Bacteriologia da Faculdade de Farmácia, os isolados bacterianos foram mantidos a -20°C.

A susceptibilidade à polimixina B foi avaliada determinando-se a Concentração Inibitória Mínima (CIM), através da técnica de microdiluição em caldo, conforme recomendado pelo EUCAST e CLSI [18]. A técnica foi realizada em placas de poliestireno de 96 poços, utilizando-se caldo Mueller Hinton 2 cátion-ajustado (Sigma-Aldrich, Missouri, EUA), onde uma diluição seriada de polimixina B (Sigma-Aldrich, Missouri, EUA) foi realizada, obtendo-se concentrações de 64 μg/mL a 0,125 μg/mL. Um poço de controle de esterilidade (sem adição do inóculo) e controle de crescimento (sem adição do antimicrobiano) foi considerado para cada isolado. Foi preparada uma suspensão bacteriana em solução salina estéril com turvação correspondente ao tubo 0,5 da escala de McFarland, a qual foi diluída e adicionada a cada poço de maneira a obter uma concentração bacteriana final de aproximadamente 5 x 10^5 UFC/mL em cada poço. Após incubação a 35-37ºC por 18 a 20 horas, a CIM foi definida e interpretada de acordo com o EUCAST: isolados com CIM > 2 μg/mL foram considerados resistentes à polimixina B [19].

Para a amplificação do gene *mgrB*, a Reação da Polimerase em Cadeia (PCR) foi realizada de acordo com Cannatelli e colaboradores (2014). Os isolados foram repicados em placas de Agar Triptona de Soja (Oxoid, Basingstone, Reino Unido) e incubados a 35-37ºC *overnight*. A extração de DNA foi realizada por lise térmica: colônias isoladas foram suspendidas em 500μL de tampão TE (Tris-EDTA, pH 7,8), homogeneizadas em vórtex por 10 segundos e incubadas primeiramente a 80ºC em banho seco por 20 minutos e, posteriormente, em banho de gelo (aproximadamente 0°C) por 20 minutos; após, as amostras foram centrifugadas a 15.000 rpm por 3 minutos e o sobrenadante contendo o DNA extraído foi utilizado para as reações de PCR.

A amplificação dos genes foi realizada em uma reação com volume final de 25 μL, contendo tampão para PCR (Invitrogen, Mariland. EUA), $MgCl_2$ (Invitrogen, Mariland, EUA), 1U Platinum Taq DNA Polimerase (Invitrogen, Mariland, EUA), 2,5 mM de dNTP's (Invitrogen, Mariland, EUA), além das sequências iniciadoras (5'-AAGGCGTTCATTCTACCACC-3' e 5'-TTAAGAAGGCCGTGCTATCC-3') a 10 mM (QuatroG, Porto Alegre, Brasil). As reações foram realizadas em termociclador Veriti (Applied Biosystems, Perkin Elmer, EUA) iniciando a desnaturação a 94ºC por 3 minutos, seguindo de ciclos de 45 segundos a 94ºC por 30 segundos, a 51ºC por 2 minutos, e a 72ºC por 20 segundos, repetidos 30 vezes, finalizando por um ciclo de extensão final a 72ºC por 5 minutos. O produto do PCR foi analisado após eletroforese em gel de agarose a 1,5%, por 20 minutos a 100V. A visualização foi realizada utilizando-se Gelred (QuatroG, Porto Alegre, Brasil) sob luz ultravioleta. Um padrão de peso molecular de 100pb (Ludwig, Alvorada, Brasil) foi adicionado à corrida para controle do tamanho dos amplicons. Todos os isolados que apresentaram amplificação foram considerados positivos para o gene mgrB.

Um subgrupo de isolados resistentes à polimixina B apresentando o gene *mgrB* supostamente intacto (com amplicon de tamanho esperado) e com aumento no tamanho do amplicon, recuperados de hemoculturas de pacientes internados em unidades hospitalares distintas foram selecionados para serem submetidos ao sequenciamento do gene, com o objetivo de analisar possíveis alterações de sequência, associadas ao fenótipo observado.

Para tanto, foi realizada purificação dos produtos de PCR destes isolados utilizando 2 μL de ExoSAP-IT (Applied Biosystems, Perkin Elmer, EUA,) para cada 5 μL de produto de reação. A solução foi submetida a termociclador Veriti (Applied Biosystems, Perkin Elmer, EUA) por 30 minutos a 37ºC seguido de 15 minutos a 80ºC. À amostra purificada adicionou-se 1,4μL da sequência iniciadora *forward* e/ou *reverse* (5 mM), sendo, então, encaminhadas para sequenciamento (Sanger), realizado no aparato ABI 3500 Genetic Analyzer (Applied Biosystems, Perkin Elmer, EUA). As sequências obtidas foram analisadas utilizando-se a ferramenta *BioEdit Sequence Alignment Editor*, para alinhamento. As sequências foram comparadas com outras sequências do gene *mgrB* depositadas no *GenBank*, do *National Center of Biotechnology Information* (NCBI) e, também, no *DNA Data Bank of Japan* (DDBJ), usando o programa *Basic Local Alignment Search Tool* (BLAST).

RESULTADOS

Nesse estudo, foram analisados 37 isolados de *K. pneumoniae* recuperados de pacientes internados em um hospital da cidade de Porto Alegre, entre julho de 2018 e agosto de 2019. A Tabela 1 apresenta sítio de isolamento, data de coleta e local de internação, bem como a MIC de polimixina B dos isolados avaliados e os resultados de PCR. Para 1 (um) paciente, dados referentes à internação e data de coleta não estavam disponíveis.

Os isolados foram obtidos principalmente de culturas de urina (n=18; 48,6%) e hemoculturas (n = 10; 27,0%). Outros sítios clínicos de isolamento incluíram aspirado traqueal, escarro e fáscia. Um isolado (2,7%) foi proveniente de cultura de vigilância (swab retal). Em relação ao local de internação, os isolados foram recuperados especialmente de pacientes internados em unidades clínicas de internação (n=15; 40,5%), sendo que as do 5° (n=6; 16,2%) e 10° (n=5; 13,5%) andares do hospital foram as mais representadas. Nove isolados (24,3%) foram recuperados de pacientes internados em Unidade de Tratamento Intensivo (UTI), especialmente a UTI do segundo andar (n = 6; 16,2%) do hospital.

As CIM para a polimixina B variaram de 8 μg/mL a > 64 μg/mL sendo em geral bastante elevadas. De fato, 29 isolados (78,4%) apresentaram CIM iguais ou superiores a 32 μg/mL (Tabela 1). Dentre os 37 isolados, 30 (81%) apresentaram como produto de PCR um amplicon de 144pb, tamanho esperado para o gene *mgr*B, utilizando as sequências iniciadoras descritas acima. Seis isolados (16,2%) amplificaram um produto com mais de 1000pb, conforme pode ser visualizado na Figura 1. Interessantemente, a exceção de um (CIM = 16 μg/mL), todos esses isolados apresentaram CIM igual ou superior a 64 μg/mL. Por fim, para um isolado não foi possível realizar a amplificação do gene *mgrB*, indicando a deleção do gene. Esse isolado foi recuperado do aspirado traqueal de um paciente proveniente da unidade de internação do 6º andar do hospital, apresentando CIM de 32 μg/mL para a polimixina B.

A partir desses resultados, 10 isolados carreando *mgrB* intacto e um apresentando amplicon de *mgrB* de aproximadaente 1400pb, recuperados de hemoculturas e de aspirado traqueal foram selecionados para o sequenciamento, sendo como critério utilizado o sítio de coleta mais estéril.

Os 10 isolados com *mgrB* intacto demonstraram sequencias do gene muito semelhantes entre si. Ao compará-los, eles apresentaram divergência de apenas um nucleotídeo na posição 91: seis deles tinham guanina nessa posição, enquanto os outros quatro, uma citosina, promovendo alteração do códon. A partir do BLAST, foi possível verificar que eles alinharam, com identidade superior a 99%, com as sequências MH368669.1 e KJ129597.1 [6] referentes a um gene *mgrB* não funcional, conforme descrito na Tabela 2.

Por outro lado, o isolado contendo o amplicon de elevado peso molecular demonstrou 98,02% de identidade com a sequência HG008893.1, compatível com um gene *mgrB* interrompido por sequência de inserção semelhante à IS5.

DISCUSSÃO

O retorno da utilização das polimixinas como opção terapêutica para o no tratamento de infecções por *K. pneumoniae* e outras enterobactérias resistentes aos carbapenêmicos trouxe uma nova realidade: a emergência e disseminação da resistência às polimixinas. Estudos demonstram que essa resistência pode estar associada à presença de genes plasmidiais (*mcr-1*) ou à múltiplas alterações em genes cromossômicos. Modificações no gene *mgrB* parecem ser o principal mecanismo cromossomal de resistência à polimixina B especificamente em *K. pneumoniae*.

Da população avaliada nesse estudo, 16,2% carreavam um gene *mgrB* com tamanho (em pb) elevado, compatível com a presença de sequências de inserção (IS), alteração já bem descrita por outros autores [5-6,8,13,15]. Várias IS foram observadas, porém IS5 e IS5-*like* parecem ser as mais frequentemente descritas na literatura interrompendo *mgrB* [6,8]. Cannatelli e colaboradores (2014) relatam ter encontrado três variações de IS, sendo uma delas a IS5-*like*. Poirel e colaboradores (2015) também relatam a presença de variações de IS, algumas pertencentes ao grupo IS5.

O isolado 439 do nosso estudo demonstrou alta similaridade com o gene descrito por Cannatelli e colaboradores (2013), apresentando IS5 inserida no *mgrB*. Conforme observado por Macesic e colaboradores (2019), a presença de IS parece ser exclusiva de *mgrB*, não sendo verificada em outros genes envolvidos nos mecanismos cromossômicos de resistência à polimixina B em *K. pneumoniae*. Embora não estejam bem estabelecidas as causas dessa ocorrência, os autores sugerem que tais IS podem ter origem plasmidial sendo, a partir daí, transferidas para o cromossomo bacteriano. Esse pode ser um dos motivos da diversidade de sequências que vêm sendo observadas em diversos estudos, premissa que merece maior esclarecimento.

Demonstrada a importância da proteína MgrB na regulação da homeostasia celular, a deleção do gene, completa ou parcial, acarretando perda de função, pode ter diversas implicações para a célula. A deleção parcial do gene já foi descrita por alguns autores [14,17]. Cannatelli e colaboradores (2013) relatam que a deleção total do gene *mgrB* conferiu resistência a colistina em um isolado mutante. Em outro momento, os mesmos autores relatam deleções parcial ou total do gene *mgrB* [6]. Giordano e colaboradores (2018) observaram a ocorrência de deleção parcial de 11pb, uma alteração significante para o gene, em isolados de um hospital da Itália, ocorrência já relatada anteriormente por Giani e colaboradores (2015) em outro local da Itália. Em nosso estudo, foi observada uma *K. pneumoniae* com deleção completa do gene, com MIC de 32 μg/mL para a polimixina B. A avaliação de outros genes envolvidos no mecanismo de resistência às polimixinas, como os genes responsáveis pela codificação dos sistemas de dois componentes PhoP/PhoQ e PmrA/PmrB se faz necessária para definir se essa deleção é a alteração molecular exclusivamente responsável pela resistência observada.

Conforme ressaltado por Macesic e colaboradores (2019), embora a adição de IS no gene *mgrB* seja o mecanismo central de resistência à polimixina B em *K. pneumoniae*, uma proporção considerável (40% para os autores citados e 81% em nosso estudo) de isolados resistentes apresentam *mgrB* com amplicon intacto (de tamanho esperado), reforçando a participação de outras vias moleculares nesse mecanismo de resistência.

Ao analisar as sequências do gene *mgrB* de isolados com tamanho de amplicon esperado em nossa população, foi observada uma identidade de 99 a 100% com um gene *mgrB* não funcional descrito por Cannatelli e colaboradores (2013). Poirel e colaboradores (2015) relatam que cepas selvagens possuem 47 aminoácidos na proteína MgrB. Já, Olaitan e colaboradores (2014) descrevem sequências com *stop* códon prematuro, codificando apenas 29 aminoácidos. Nossos isolados também apresentaram similaridade elevada às sequencias descritas por Olaitan e colaboradores (2014), levando a crer que também transcrevam um *stop* códon prematuro. Isso acarretaria a não funcionalidade ou funcionalidade parcial da proteína, o que pode ser um mecanismo auxiliar na resistência às polimixinas quando encontradas outras alterações associadas a genes dos sistemas de dois componentes.

Ao comparar entre si as sequências do gene *mgrB* que demonstraram o tamanho esperado de amplicon no PCR, foi observada a presença de uma mutação *missense* na posição 91. Essa mutação (presença de citosina ou guanina) dá origem a códons distintos e, consequentemente, diferentes aminoácidos nesta localização, o que pode gerar alteração conformacional na proteína MgrB, tornando-a menos específica ou reduzindo sua atividade. Conforme ressaltado por Macesic e colaboradores (2019), a resistência às polimixinas em *K. pneumoniae* é multifatorial, estando relacionada a um conjunto de complexos mecanismos genéticos. Proteínas com especificidade reduzida ou com menor atividade podem corroborar para a ocorrência final do fenótipo de resistência.

Além dos isolados avaliados nesse estudo serem provenientes de uma mesma instituição de saúde, os genes *mgrB* sequenciados apresentaram uma similaridade elevada entre si conforme discutido anteriormente. Assim, não se pode descartar a possibilidade de uma expansão clonal. De fato, a ocorrência e disseminação de clones resistentes a colistina já foi descrita e deve ser considerada [14]. Por outro lado, a pressão seletiva do uso de polimixinas, induzindo o desenvolvimento de resistência associada a mecanismos genéticos diversos, sem relação clonal entre os isolados também já foi reconhecida [15]. A avaliação de outros genes associados aos mecanismos de resistência às polimixinas, bem como a análise da diversidade genética dos nossos isolados devem ser realizadas para elucidar a epidemiologia molecular dessa resistência.

Apesar do número limitado de isolados, nosso estudo demonstra que, mesmo que a inserção de sequências no *mgrB* seja uma alteração relevante, a maioria de nossos isolados apresentou outras alterações moleculares na sequência do gene, reiterando o caráter multifatorial da resistência às polimixinas em *K. pneumoniae.*

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Tabela 1: Resultados da Concentração Inibitória Mínima (CIM) para Polimixina B em comparativo ao sítio de coleta da amostra, local de internação do paciente e data de coleta.

ISOLADO	LOCAL DE INTERNAÇÃO	SÍTIO DE COLETA	DATA DE COLETA	CIM POLIMIXINA (μg/mL)	GENE *mgrB*
148	UI 5° andar	Urina	16/07/18	32	Intacto
163	UCE	Urina	23/07/18	32	Intacto
169	Emergência	Swab retal	29/07/18	16	IS
212	UTI 2° andar	Hemocultura	20/11/18	8	Intacto
371	Emergência	Urina	03/06/19	32	Intacto
377	-	Urina	-	>64	IS
379	Emergência	Urina	14/06/19	>64	Intacto
381	Emergência	Urina	12/06/19	32	Intacto
382	UI 5° andar	Escarro	05/07/19	16	Intacto
389	UI 9° andar	Urina	18/06/19	>64	IS
397	UI 9° andar	Hemocultura	22/06/19	16	Intacto
398	Emergência	Urina	28/06/19	>64	Intacto
405	Emergência	Urina	03/07/19	32	Intacto
412	UI 5° andar	Urina	10/07/19	64	Intacto
416	UI 10° andar	Urina	10/07/19	64	Intacto
420	UTI 2° andar	Aspirado traqueal	12/07/19	16	Intacto
422	UI 5° andar	Aspirado traqueal	05/07/19	16	Intacto
423	UI 10° andar	Urina	11/07/19	64	IS
424	UTI 2° andar	Aspirado traqueal	19/07/19	32	Intacto
425	Emergência	Aspirado traqueal	15/07/19	64	Intacto
427	UI 5° andar	Urina	16/07/19	32	Intacto
428	UI 6° andar	Aspirado traqueal	16/07/19	32	Deleção do gene
429	UI 3° andar	Fáscia	15/07/19	64	Intacto
435	UI 5° andar	Hemocultura	22/07/19	64	Intacto
439	UTI 8° andar	Hemocultura	29/07/19	64	IS
442	Emergência	Escarro	01/08/19	16	Intacto
446	Emergência	Urina	02/08/19	32	Intacto
449	UTI 8° andar	Urina	25/07/19	32	Intacto
450	UTI 2°andar	Urina	07/08/19	32	Intacto
457	UI 10° andar	Hemocultura	10/08/19	32	Intacto
458	UTI 8° andar	Hemocultura	11/08/19	64	Intacto
461	Emergência	Urina	08/08/19	64	IS
468	UCE	Hemocultura	12/08/19	32	Intacto
469	UTI 2° andar	Hemocultura	11/08/19	32	Intacto
470	UI 10° andar	Urina	12/0819	64	Intacto
473	UTI 2° andar	Hemocultura	18/08/19	16	Intacto
474	UI 10° andar	Hemocultura	18/08/19	32	Intacto

*UI: Unidade de internação; UCE: Unidade de Cuidados Especiais; UCI: Unidade de Cuidados Intensivos; UTI: Unidade de Tratamento Intensivo; IS: *insertion sequence*

Tabela 2: Identidade com as sequencias MH368669.1 (*Klebsiella pneumoniae* strain Kpn-4644Lar nonfunctional MgrB (*mgrB*) gene, complete sequence) e KJ129597.1(*Klebsiella pneumoniae* subsp. pneumoniae isolate KP08C16 mutated MgrB (*mgrB*) gene, complete sequence) encontrada através do BLAST nas plataformas do *National Center of Biotechnology* (NCBI) e *DNA Data Bank of Japan* (DDBJ) em comparação aos isolados com *mgrB* de 144pb.

ISOLADO	BLAST NCBI (%)	BLAST DDBJ (%)
212	99,31	99
397	99,31	99
420	100	100
435	100	100
457	99,31	99
458	99,31	99
468	99,31	99
469	100	100
473	99,31	99
474	100	100

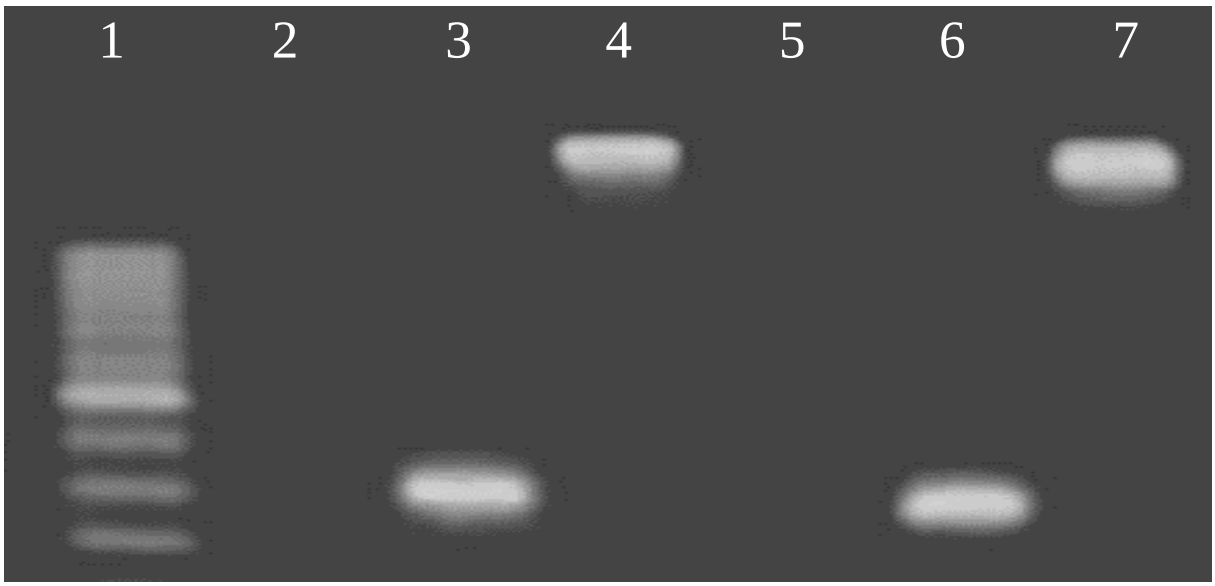

Fig1 Gel de agarose com Gelred. Da esquerda para a direita: (1) marcador de 100pb, (2) controle negativo de reação, (3) controle positivo do amplicon para *mgrB*, (4) controle do amplicon com inserção, (5) amostra sem amplificação, (6) amostra com *mgrB* de tamanho normal e (7) amostra com IS

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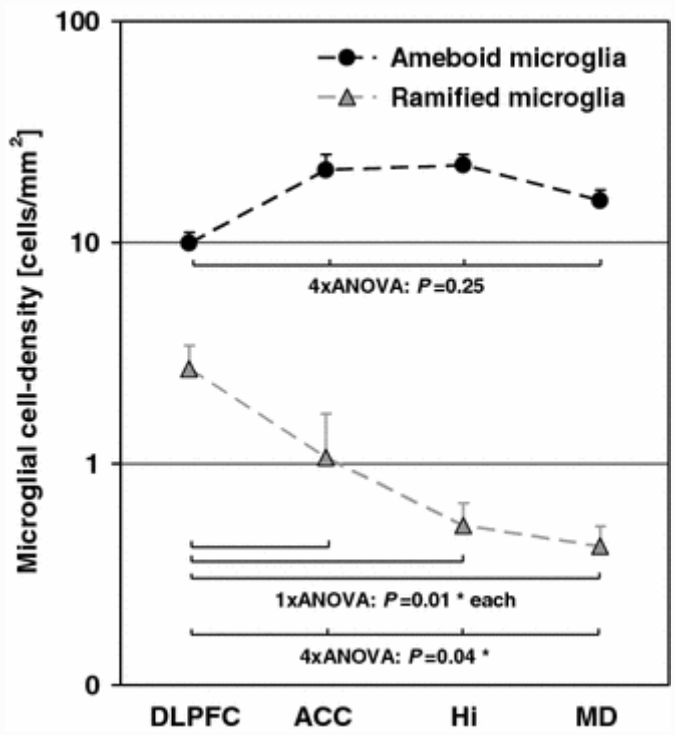

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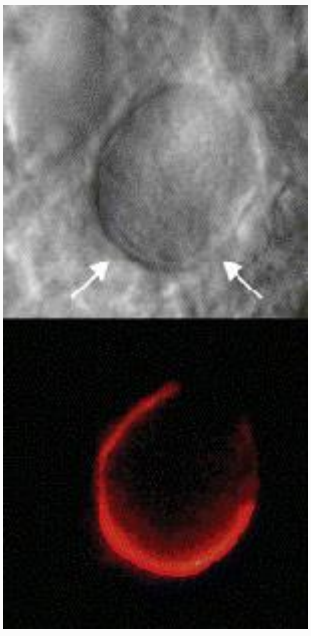

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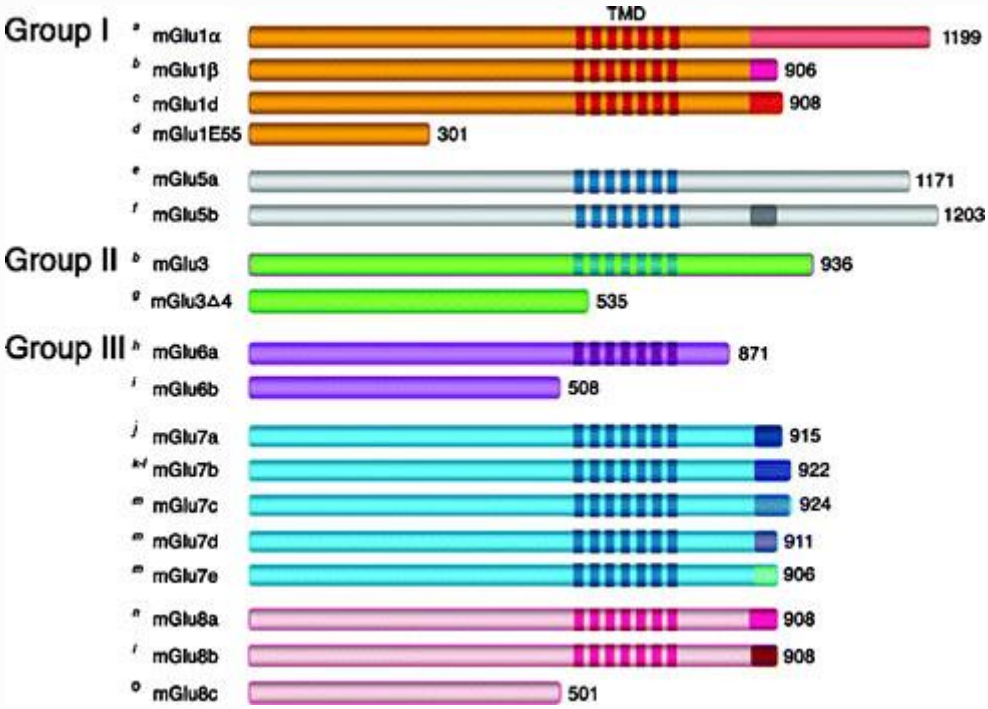

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