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INFLUÊNCIA DA CÁPSULA POLISSACARÍDICA NA AÇÃO BACTERICIDA DA INDOLICIDINA SOBRE *Streptococcus pneumoniae*

Bragança Paulista

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EPÍGRAFE

"Por vezes sentimos que aquilo que fazemos

não é senão uma gota de água no mar.

Mas o mar seria menor se lhe faltasse

uma gota."

Madre Teresa de Calcutá

RESUMO

Streptococcus pneumoniae é responsável por um alto índice de morbidade e mortalidade em todo o mundo. A cápsula polissacarídica confere proteção contra a fagocitose e influencia muitos aspectos da patogênese do pneumococo. Os polissacarídeos capsulares (CPS) são altamente imunogênicos e apresentam grande variabilidade estrutural, com mais de 100 sorotipos descritos até o momento. Peptídeos antimicrobianos (AMPs) são um importante mecanismo da defesa inata contra muitos patógenos. Um exemplo de AMP é a indolicidina, um peptídeo catiônico produzido por neutrófilos bovinos, com efeitos bactericidas. Foi demonstrado que a CPS interfere na capacidade lítica dos AMPs frente aos pneumococos, mas os efeitos da variabilidade da cápsula na suscetibilidade à indolicidina não foram explorados. O presente trabalho teve como objetivo determinar os efeitos da cápsula na resistência à indolicidina *in vitro*. Um ensaio bactericida foi desenvolvido para determinar a suscetibilidade de pneumococos à morte por indolicidina, comparando cepas de tipo selvagem x mutantes sem cápsula. Diferentes sorotipos de pneumococo exibiram resistência variável à indolicidina (variação de 20 a 80% na viabilidade bacteriana após tratamento com 7,5 µg/ml de indolicidina). A resistência à ação do AMP se correlacionou de forma positiva com a carga superficial da cápsula: as bactérias com maior eletronegatividade apresentaram maior resistência à indolicidina, com exceção do sorotipo 3. Curiosamente, o efeito da expressão da cápsula na resistência à indolicidina foi dependente do sorotipo; pneumococo sorotipo 2, com baixo potencial zeta, foi mais resistente à indolicidina quando a cápsula estava presente (40% *vs.* 80%), enquanto a bactéria de sorotipo 4, com carga superficial menos negativa, foi mais resistente na ausência da cápsula (80% *vs.* 10%). A adição de CPS livre reverteu parcialmente os efeitos líticos da indolicidina sobre os pneumococos, enquanto a adição de anticorpos anticapsulares acentuou a ação bactericida do peptídeo, sugerindo um possível novo mecanismo protetor induzido por vacinas pneumocócicas baseadas em polissacarídeos.

Palavras-chave: Peptídeo. Polissacarídeo. *Streptococcus pneumoniae*.

ABSTRACT

Streptococcus pneumoniae is responsible for high morbidity and mortality worldwide. The polysaccharide capsule confers protection against phagocytosis and influences many aspects of pneumococcal pathogenesis. The capsular polysaccharides (CPS) are highly immunogenic and exhibit great structural variability, with more than 100 serotypes described so far. Antimicrobial peptides (AMPs) are an important innate defense mechanism against many pathogens. An example of AMP is indolicidin, a cationic peptide produced by bovine neutrophils, with bactericidal effects against bacteria. CPS has been shown to interfere with the ability of AMPs to kill pneumococci, but the effects of capsule variability in susceptibility to indolicidin have not been explored. The present work aimed to determine the effects of capsule on resistance to indolicidin *in vitro*. A bactericidal plate assay was designed to determine the susceptibility of pneumococci to killing by indolicidin, comparing wild type x mutant strains lacking capsule. Different pneumococcus serotypes exhibited variable resistance to indolicidin (20 to 80% range in bacterial viability after treatment with 7.5 µg/ml indolicidin). Resistance to the action of AMP was positively correlated with the surface charge of the capsule: bacteria with the highest electronegativity had high resistance to indolicidin, with the exception of serotype 3. Interestingly, the effect of capsule expression on resistance to indolicidin was serotype-dependent; pneumococcus serotype 2, with low zeta potential, was more resistant to indolicidin when the capsule was present (40% *vs.* 80%), while serotype 4 strain, with a less negative surface charge, was more resistant in the absence of capsule (80% *vs.* 10%). The addition of free CPS partially reversed indolicidin lytic effects on pneumococci, while the addition of anticapsular antibodies accentuated the bactericidal action of the peptide, suggesting a possible new protective mechanism induced by pneumococcal vaccines affected by polysaccharides.

Keywords: *Peptide*. *Polysaccharide*. *Streptococcus pneumoniae*.

LISTA DE SÍMBOLOS E ABREVIAÇÕES

AMPs	Peptídeos Antimicrobianos
CPS	Cápsula Polissacarídica
PS	Polissacarídeos Capsulares

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1. INTRODUÇÃO

1.1 *Streptococcus pneumoniae*

As infecções por *Streptococcus pneumoniae* (pneumococo) são um problema de saúde pública mundial; todos os anos, cerca de um milhão de pessoas – na maior parte crianças e idosos de países em desenvolvimento – morrem de doenças provocadas por essa bactéria (34). Trata-se de um microrganismo Gram-positivo encapsulado, que coloniza o trato respiratório humano de forma assintomática e pode se tornar invasivo em situações como coinfecção pelo vírus influenza ou deficiência nos mecanismos de defesa (1); ao invadir sítios estéreis como os pulmões, meninges e sangue, o pneumococo provoca intensa resposta inflamatória, que pode ser fatal (2).

O envoltório celular do pneumococo é composto de três estruturas (Figura 1): a membrana plasmática mais interna, formada por uma bicamada lipídica; a parede celular constituída de peptideoglicanos e ácido teicóico, que ancora várias proteínas de superfície (como a proteína PspA); e a cápsula polissacarídica na porção mais externa, bastante variável em espessura e composição química (3).

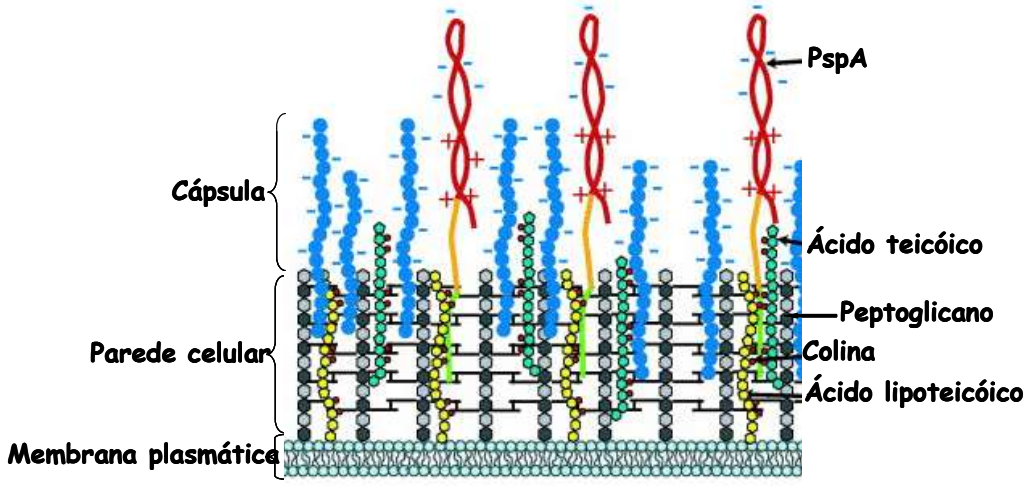

FIGURA 1: Envoltório celular de *Streptococcus pneumoniae*. São mostradas as estruturas presentes no envoltório celular do pneumococo: i) membrana plasmática, sendo a porção mais interna, formada por uma bicamada lipídica; ii) parede celular, constituída por peptideoglicanos e ácidos tecóicos, a PspA (proteína de superfície de pneumococo A) está ancorada na parede celular; iii) e a porção mais externa, a cápsula

polissacarídica, que é variável em espessura e composição química, essa variabilidade química, é usada para classificar o pneumococo em 100 sorotipos diferentes. Fonte: Figura adaptada de Darrieux et al (2007).

A cápsula polissacarídica (CPS) é um fator de virulência importante, envolvido na evasão da deposição de proteínas do Sistema Complemento e consequente fagocitose durante a invasão do hospedeiro humano. Por sua variabilidade estrutural, a composição da cápsula é utilizada como critério de classificação da bactéria em 100 sorotipos (4).

As variações estruturais nos polissacarídeos capsulares resultam em diferenças na carga elétrica da cápsula. O potencial zeta mede a magnitude das interações eletrostáticas entre partículas. Medidas de potencial zeta de diferentes sorotipos de pneumococo indicam ampla variação na eletronegatividade da cápsula, que se reflete em variações no potencial de colonização e na resistência à fagocitose (20).

Os polissacarídeos capsulares (CPS) são imunogênicos e constituem a base das vacinas pneumocócicas atualmente em uso. Há dois tipos de formulações baseadas em polissacarídeos: aquelas contendo os CPS livres, e as conjugadas – onde os CPS são quimicamente ligados a proteínas carreadoras não pneumocócicas, como o toxóide diftérico. As vacinas conjugadas, por incluírem o componente proteico, são capazes de induzir anticorpos protetores em crianças menores de 5 anos (5, 6). Estas vacinas são eficazes no controle das doenças invasivas e também foram capazes de reduzir a colonização da nasofaringe – a primeira etapa da infecção, comum a todas as doenças causadas pelo pneumococo – por mecanismos ainda não esclarecidos (7).

1.2 PEPTÍDEOS ANTIMICROBIANOS

Peptídeos antimicrobianos (AMPs) são proteínas de baixa massa molecular, capazes de inibir o crescimento de bactérias, vírus e fungos. Fazem parte do sistema imune inato de diversas classes de seres vivos (8) e constituem parte da defesa inata dos organismos. A maioria dos AMPs são catiônicos e anfipáticos e agem desestabilizando as membranas dos microrganismos (9).

Até o momento, 116 AMPs diferentes foram identificados em humanos, podendo ser encontrados em diferentes tecidos e superfícies epiteliais como pele, olhos, cavidade oral, auricular, intestino, sistema nervoso e urinário (10). A classe de AMPs mais investigada, são os AMPs que

possuem atividade antibacteriana, sendo em sua maioria catiônicos e anfipáticos (11, 12). Alguns AMPs aniônicos também possuem atividade antibacteriana, como o maximin-H5, proveniente da pele de sapos (13).

A carga positiva dos peptídeos antimicrobianos é crucial para sua atuação contra bactérias. Ao contrário das membranas plasmáticas de células eucarióticas – que são compostas por lipídeos neutros, as membranas citoplasmáticas das bactérias Gram-positivas e Gram-negativas são ricas em lipídios altamente eletronegativos, como fosfatidilserina (PS), cardiolipina (CL) ou fosfatidilglicerol (PG). Essas estruturas conferem à membrana bacteriana uma carga negativa, que atrai peptídeos positivamente carregados (14).

Além da sua carga positiva, os peptídeos antimicrobianos apresentam elevado teor de resíduos hidrofóbicos, como o triptofano; isso permite que os AMPs se insiram nas regiões interfaciais das bicamadas lipídicas, facilitando a interação dos peptídeos antimicrobianos com a membrana celular das bactérias (15). O ancoramento dos AMPs à membrana bacteriana leva a formação de poros, e consequentemente à ruptura da célula microbiana (14).

A desestabilização da membrana plasmática dos microrganismos na ação dos AMPs pode ocorrer através de alguns mecanismos (Figura 2), como: i) modelo de barril (*barrel-stave*) os AMPs se inserem perpendicularmente a bicamada lipídica formando uma estrutura semelhante a um barril, onde posteriormente, haverá formação de poros; ii) modelo carpete ou detergente: a região anfipática do AMP entra em contato com a membrana plasmática, revestindo uma porção da membrana, como um tapete, com isso o AMP consegue penetrar a bicamada lipídica, levando também a formação de poros; iii) modelo de poro toroidal: os AMPs se inserem perpendicularmente a bicamada lipídica, com suas regiões hidrofóbicas ligadas a ela e a porção hidrofílica voltadas para o poro formado (9, 13).

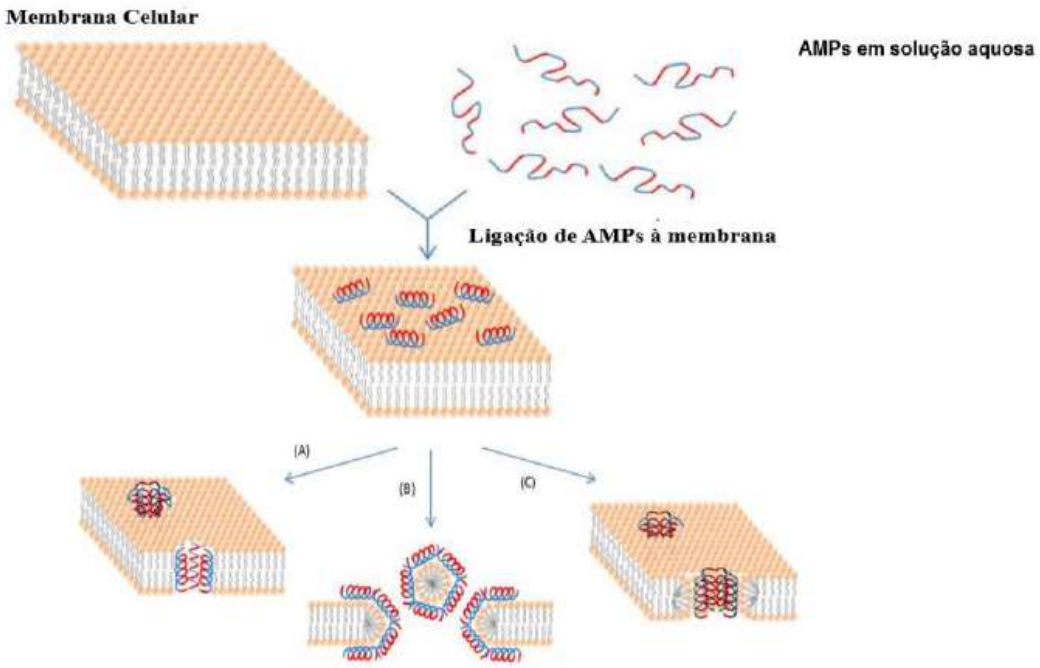

FIGURA 2: Representação de alguns mecanismos de ação dos AMPs na membrana plasmática. São mostrados três modelos de mecanismos de ação dos AMPs sobre a membrana plasmática. A) Modelo de *Barrel-Stave*: inserção dos AMPs perpendicularmente a bicamada lipídica formando uma estrutura semelhante a um barril. B) Modelo *Carpet-like* ou *Detergent-like*: a região anfipática do AMP entra em contato com a membrana plasmática e reveste uma porção da membrana, como um tapete, com isso o AMP consegue penetrar a bicamada lipídica. C) Modelo *Toroidal Pore*: inserção do AMP perpendicularmente à bicamada lipídica, onde as regiões hidrofóbicas do AMP estão ligadas a bicamada lipídica e as suas regiões hidrofílicas estão voltadas para o poro formado. Adaptado de BAHAR; REN (2013) (16).

Além dos mecanismos descritos na Figura 2, os AMPs também podem apresentar outras ações na membrana, como o afinamento (*Membrane thinning*), onde os AMPs irão se inserir em apenas um lado da bicamada lipídica e levar a formação de uma lacuna entre as moléculas lipídicas, levando as moléculas lipídicas vizinhas a preencher essa lacuna, afinando dessa forma a bicamada lipídica. E, por último, há o mecanismo de agregação (*Aggregate*), onde os AMPs se fixam à membrana paralelamente à superfície, e em se inserem na membrana verticalmente para formar

estruturas semelhantes a esferas e consequentemente a ruptura celular (13). Além do efeito bactericida direto, os AMPs podem apresentar outros efeitos, como pro-apoptóticos, anticarcinogênicos, neutralizadores de toxinas patogênicas ou atuando como imunomoduladores (17, 18).

Foi demonstrado que bactérias gram-positivas desenvolveram diversos mecanismos de resistência frente aos AMPs, dentre esses mecanismos, destacam-se: bombas de efluxo e sistema de transporte, expressão e repressão gênica induzida pelos peptídeos, sequestro e inativação de AMP (19). Outro mecanismo existente é a modificação da cápsula polissacarídica. Sorotipos que apresentam a estrutura capsular mais eletronegativa, são capazes de evitar a ação lítica dos AMPs frente ao pneumococo (20).

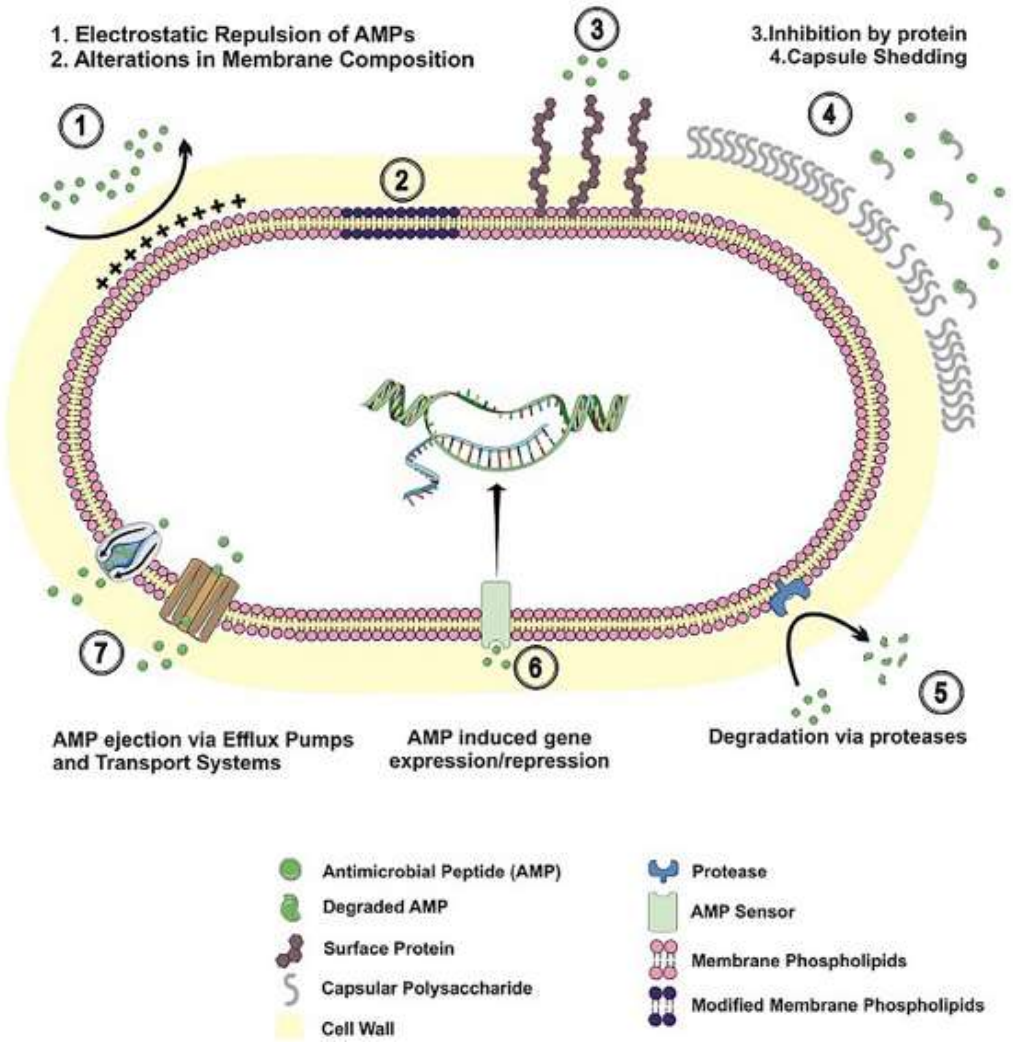

FIGURA 3: Diferentes mecanismos de resistência utilizados por bactérias Gram-positivas para evitar a ação dos AMPs. São mostrados diversos mecanismos de resistência que as bactérias desenvolveram frente aos AMPs. 1) Repulsão eletrostática dos AMPs: modificações no ácido teicóico da parede celular por meio da D-alanina (*dlt operon*) que reduz a carga negativa da molécula. 2) Alterações na composição da membrana: esse mecanismo tem o objetivo de impedir que o AMP se insira na membrana plasmática, uma forma de impedir é através da inserção de D-alanina nos ácidos lipoteicóicos (D-alanilação), esse processo reduz a carga negativa da membrana, inibindo a interação com os AMPs. 3) Inibição por proteína: esse mecanismo leva a inativação e/ou degradação do AMP. 4) Desintegração da cápsula: pode ser desencadeado pela atividade da autolisina (LytA). 5) Degradação via protease: ocorre através da clivagem do AMP pelas proteases. 6) Expressão e repressão gênica induzida pelos AMPs: pode ocorrer através de diversos modos, como fatores Sigma, sensores celulares, reguladores, entre outros. 7) Bombas de efluxo e sistema de transporte: expulsão de AMPs utilizando bombas de efluxo ou transportadores ABC. Fonte: Figura de Assoni et al (2021) (19).

1.3 INDOLICIDINA

A indolicidina é um peptídeo antimicrobiano produzido por neutrófilos durante a sua ativação. Pertence à família das catelicidinas (21). A indolicidina apresenta atividade inibitória contra bactérias, fungos, vírus e células cancerígenas, além de uma possível atividade quimiotática para células de defesa como neutrófilos, monócitos e linfócitos T (22).

A indolicidina é composta por 13 resíduos de aminoácidos – *(ILPWKWPWWPWRR-Am).* A região C-terminal da molécula é a responsável pela ação antimicrobiana; esta região sofre um processo denominado amidação, com perda do grupamento –OH da porção terminal carboxila (-COOH), que é substituído por um grupamento amina (-NH) (23, 24).

A ação da indolicidina se dá através do rompimento de membranas bacterianas (25) e da inibição da síntese de DNA (26). Foi evidenciado que o mecanismo de ação da indolicidina sobre a membrana plasmática se dá através do modelo *Carpet-like* – descrito na Figura 2 (24, 27, 28). Em bactérias Gram-negativas, foi demonstrado que a indolicidina é capaz de permear rapidamente a parede celular, atingindo o alvo de ação, a membrana plasmática, onde forma canais que levam à sua ruptura (29, 30). Um efeito semelhante foi observado em *Streptococcus pneumoniae* e *Staphylococcus aureus* (bactérias Gram-positivas), sugerindo que a indolicidina é capaz de atravessar a espessa barreira da parede celular e promover a desestabilização da membrana

plasmática. A desestabilização da membrana celular bacteriana resulta da interação entre a membrana carregada negativamente e a indolicidina com carga positiva, conteúdo hidrofóbico e alta concentração de triptofano (2, 31). Também foi demonstrado que a indolicidina tem a capacidade se ligar à dupla hélice do DNA, impedindo a replicação e a transcrição e amplificando sua ação antimicrobiana (2, 32), além de possuir atividade anti-HIV, através da enzima HIV-integrase (16, 33).

Estudos do nosso grupo demonstraram que o pneumococo é parcialmente resistente à ação lítica da indolicidina, graças à presença da proteína de superfície de pneumococo A (PspA), que impede a ligação do peptídeo à membrana bacteriana (Milani et al, manuscrito em preparação). No entanto, o papel de outros componentes da bactéria na ação da indolicidina não foi investigado.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a influência da cápsula polissacarídica na ação bactericida da indolicidina sobre *Streptococcus pneumoniae*.

2.2 OBJETIVOS ESPECÍFICOS

- ✓ Determinar a sobrevivência de pneumococos de diferentes sorotipos capsulares após tratamento com indolicidina.

- ✓ Comparar a ação da indolicidina em pneumococos selvagem e mutantes que não expressam cápsula.

- ✓ Avaliar os afeitos da adição de polissacarídeos purificados na ação da indolicidina sobre pneumococos.

- ✓ Investigar o papel de anticorpos anti-capsulares na morte de pneumococos na presença da indolicidina.

3. ARTIGO EM PREPARAÇÃO

3.1 CAPÍTULO I

- O manuscrito descreve os efeitos da cápsula polissacarídica sobre a ação da indolocidina no pneumococo. Foram comparadas bactérias selvagens e mutantes sem cápsula, bem como isolados que expressam diferentes sorotipos capsulares. O papel dos CPS livres e dos anticorpos anticapsulares também foi avaliado. Os resultados deste trabalho demonstram que a contribuição da cápsula polssicarídica para a proteção contra ação do AMP é influenciada por sua carga elétrica e que anticorpos anticapsulares são capazes de ampliar a ação bactericida do AMP. A manuscrito está em fase de finalização para submissão à revista Frontiers in Microbiology.

INFLUENCE OF THE POLYSACCHARIDE CAPSULE ON THE BACTERICIDAL ACTIVITY OF INDOLICIDIN ON S*treptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae is responsible for high morbidity and mortality worldwide. The polysaccharide capsule confers protection against phagocytosis and influences many aspects of pneumococcal pathogenesis. The capsular polysaccharides (CPS) are highly immunogenic and exhibit great structural variability, with more than 100 serotypes described so far. Antimicrobial peptides (AMPs) are an important innate defense mechanism against many pathogens. An example of AMP is indolicidin, a cationic peptide produced by bovine neutrophils, with bactericidal effects against several bacteria. CPS has been shown to interfere with the ability of AMPs to kill pneumococci, but the effects of capsule variability in susceptibility to indolicidin have not been explored. The present work aimed to determine the effects of capsule on resistance to indolicidin *in vitro*. A bactericidal plate assay was designed to determine the susceptibility of pneumococci to killing by indolicidin, comparing wild type x mutant strains lacking capsule. Different pneumococcal serotypes exhibited variable resistance to indolicidin, which correlated with the capsule net charge. Interestingly, the effect of capsule expression on resistance to indolicidin was

dependent on the serotype; bacteria with lower zeta potential were more resistant to indolicidin when capsule was present, while those with less negative surface charge were more resistant in the absence of capsule. The addition of free CPS partially reversed the lytic effects of indolicidin over pneumococci, while the addition of anticapsular antibodies accentuated the peptide bactericidal action, suggesting a possible new protective mechanism induced by polysaccharide-based pneumococcal vaccines.

Keywords: *Capsular polysaccharide*. *Indolicidin*. *Streptococcus pneumoniae*.

1. INTRODUCTION

Streptococcus pneumoniae (pneumococcus) infections are a worldwide public health problem. Every year, about one million people, mostly children and the elderly in developing countries, die of diseases caused by this organism (42). The pneumococcus is an encapsulated Gram-positive microorganism that colonizes the human upper respiratory tract asymptomatically. In situations, such as during concomitant infection by respiratory viruses, including influenza virus, or deficiency in host defense mechanisms (1). *S. pneumoniae* can invade disseminate to other sites such as the lungs, meninges, and blood, pneumococci cause an intense inflammatory response that can be fatal (2).

The pneumococcal cell envelope is composed of three main structures: the innermost plasma membrane, formed by a lipid bilayer; the cell wall consisting of peptidoglycans and teichoic and lipoteichoic acid, which anchors several surface proteins; and the polysaccharide capsule in the outermost portion, which is quite variable in thickness and chemical composition (3).

The polysaccharide capsule (PSC) is an important virulence factor, involved in the evasion of Complement System protein deposition and consequent phagocytosis during human host invasion (4). Due to its structural variability, the composition of the capsule is used as a classification criterion for the bacterium and currently contains more than 100 individual serotypes (5).

Capsular polysaccharides (CPS) are immunogenic and form the basis of pneumococcal vaccines currently in use. Conjugated vaccines are effective in the control of invasive diseases and were also able to reduce nasopharynx colonization - the first stage of infection, common to all diseases caused by pneumococcus - by mechanisms not yet clarified (6).

Antimicrobial peptides (AMP) are low molecular mass proteins capable of inhibiting the growth of bacteria, viruses, and fungi. They are part of the innate immune system of several classes of living organisms (7). Most AMPs are cationic and amphipathic and act by destabilizing the membranes of microorganisms (8). To date, 116 different AMP have been identified in humans and can be found in different tissues and epithelial surfaces such as skin, eyes, oral cavity, auricular, intestine, nervous system and urinary tract (9).

The positive charge of antimicrobial peptides is crucial for their action against bacteria. Unlike eukaryotic plasma cell membranes, which are composed of neutral lipids, the cytoplasmic membranes of Gram-positive and Gram-negative bacteria are rich in highly electronegative lipids, such as phosphatidylserine (PS), cardiolipin (CL), or phosphatidylglycerol (PG). These structures give the bacterial membrane a negative charge, which attracts positively charged peptides. The anchoring of AMPs to the bacterial membrane in most cases leads to the formation of pores and subsequent rupture of the microbial cell or effects on metabolism and translation (10-12).

In addition to their positive charge, antimicrobial peptides have a high content of hydrophobic residues, such as tryptophan; this allows the AMP to penetrate the interfacial regions of lipid bilayers, facilitating the interaction of antimicrobial peptides with the underlying bacterial cell membrane (13).

Besides a direct bactericidal effect, AMPs can exert activities in other ways, such as being pro-apoptotic, anticarcinogenic, pathogenic toxin neutralizers, or acting as immunomodulators (14, 15).

To counteract AMPs' bactericidal activities, bacteria have evolved an arsenal of resistance mechanisms, including efflux pumps and transport systems, AMP sequestration and inactivation, competition, and envelope modifications that promote AMP repulsion or inhibit their ability to bind to the cell membrane (16). These latter modifications usually affect surface charge, limiting the interaction between the bacterial membranes and the positively charged AMPs. One defense mechanism used by pneumococci is its polysaccharide capsule, which shows great variability in

chemical composition, resulting in structures that range from highly negative to those closer to neutrality. Serotypes with the most negative capsular structures have been associated with increased resistance to in vitro phagocytosis by neutrophils and an increased ability to colonize the host (17).

Indolicidin is an antimicrobial peptide belonging to the cathelicidin family, secreted by neutrophils during their activation (18). It displays inhibitory activity against bacteria, fungi, viruses, and cancer cells, as well as a possible chemotactic activity for defense cells such as neutrophils, monocytes, and T lymphocytes (19).

Indolicidin is a short linear peptide, composed of 13 amino acid residues - (ILPWKWPWWPWRR-Am). The C-terminal region of the molecule is responsible for the antimicrobial action; this region undergoes a process called amidation, with loss of the -OH group of the carboxyl-terminal portion (-COOH), which is replaced by an amine group (20, 21).

The action of indolicidin occurs through the rupture of bacterial membranes (22). In Gram-negative bacteria, indolicidin has been shown to rapidly permeate the cell wall to reach its target, the plasma membrane, where it forms channels that lead to bacterial rupture (23, 24). A similar effect was observed in *Streptococcus pneumoniae* and *Staphylococcus aureus* (Gram-positive bacteria), suggesting that indolicidin is able to cross the thick barrier of the cell wall and promote plasma membrane destabilization. The positive charge of the AMP, combined with its high hydrophobic content and high concentration of tryptophan (2, 25) contribute to the bactericidal activity. It has also been demonstrated that indolicidin enters bacterial cells, binds to the DNA double helix, thereby preventing replication and transcription and amplifying its antimicrobial action (2, 26).

Previous data from our group show that pneumococci are partially resistant to the lytic action of indolicidin, thanks to the presence of pneumococcal surface protein A (PspA (Milani et al, manuscript in preparation)), a surface-exposed protein able to bind to lactoferrin and prevent its lytic bactericidal effects (27, 28). However, the contribution of the polysaccharide capsule, an exposed structure known to affect bacterial resistance to phagocytosis and AMPs such as defensins, to indolicidin has not yet been investigated. In this study we evaluated the influence of the polysaccharide capsule in protecting the bacteria against the action of indolicidin and whether the protective effect of the capsule varies according to its composition. Using wild type and mutant

pneumococcal strains, as well as adding purified polysaccharides and anti-capsular antibodies, we investigated the role of capsule for resistance to killing by indolicidin.

2. MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE. The bacterial strains used in the study are presented in Table 1. Bacterial strains were grown in Todd-Hewitt medium containing 0.5% yeast extract (THY; purchased from Kasvi) and stocks were kept frozen at -80ºC. The night before each experiment, 20 µl of the frozen stock of the pneumococcus strain were plated on blood agar and incubated overnight at 37 ºC in microaerobic conditions.

TABLE 01: *Streptococcus pneumoniae* isolates used.

Strain	Serotype	Source	Reference
St 245/00	14	IAL[1]	(29)
A66.1	3	UAB[2]	(30, 31)
D39	2	UAB[2]	(30)
TIGR 4	4	UAB[2]	(32)
AM 1000	_	LU[3]	(33)
HR 1001	_	LU[3]	(34)
St 0603	6B	BCH[4]	(35)
P1079	1	UFG[5]	(29, 36)
P1031	23F	UFG[5]	(29, 36)
P1153	9V	UFG[5]	(36)
P69	10A	IAL[1]	(29, 36)

[1]IAL – Instituto Adolfo Lutz

[2]UAB – University of Alabama at Birmingham, USA

[3]LU – Lund University, Malmo, Sweden

[4]BCH – Boston Children's Hospital, USA

[5]UFG – Universidade Federal de Goiás, Brazil

The next morning, bacterial colonies were transferred to 7 ml of THY media and incubated at 37 ºC and the optical density ($O.D._{600nm}$) was monitored until reaching an $O.D._{600nm}$ between 0.3 - 0.4.

BACTERICIDAL ASSAY. Bacterial cultures (5 ml) at the desired $O.D._{600nm}$ were transferred to another tube and the bacteria were pelleted by centrifugation and washed with 5 ml of sterile PBS solution and resuspended in 2 ml of sterile PBS. Next, the bacterial suspensions were incubated in the presence of increasing concentrations of indolicidin (ANASPEC, code AS-60999), ranging from 7.5 to 120 µg/ml, diluted in phosphate buffered saline (PBS) to a final volume of 100 µl. The untreated control samples were incubated with PBS alone.

The samples were incubated at 37°C for 1 h, serially diluted and plated on blood agar. The number of bacteria surviving treatment was calculated as the colony forming units per ml (CFU/ml) for each group compared with the untreated control.

EFFECTS OF THE ADDITION OF CAPSULAR POLYSACCHARIDES ON THE ACTION OF INDOLICIDIN. To assess the contribution of the polysaccharide capsule to the lytic action of indolicidin on pneumococci, wild type and isogenic non-capsular mutant strains were subjected to treatment with indolicidin as described above.

The effect of free polysaccharides on indolicidin activity was investigated by adding 10 or 20 µg of free CPS of serotypes 1, 14 and 6B (ATCC) to the bacterial suspension 15 min prior to incubation with the peptide.

MOUSE IMMUNIZATION. The animal experiments were approved by the São Francisco University Animal Ethics Committee (protocol 003.04.2021). Female BALB/c mice (Obtained

from CEMIB – UNICAMP, Brazil) were immunized i.p. with 3 doses of 10 µg of CPS 1 and 6B (ATCC) using 100 µg of Al(OH)3 as an adjuvant, in a final volume of 0.5 ml, at 12 days intervals. Blood was collected through retroorbital bleeding and serum from coagulated blood was stored at -20 ºC.

EFFECT OF ANTI-CAPSULAR ANTIBODIES ON THE BACTERICIDAL ACTION OF INDOLICIDIN. To determine the ability of anti-capsular antibodies to block the potential protective action of the capsule against the effects of indolicidin, wild type pneumococci P1079 (serotype 1) and St 0603 (6B) were incubated in the presence of serum from mice immunized with purified CPS 1 and 6B, respectively, 15 mi prior to treatment with indolicidin. The control group was incubated in presence of serum from mice injected with adjuvant alone in saline. Both groups were submitted to serial dilution and plated. The number of bacteria surviving treatment was determined in each group and compared with the control.

ZETA POTENTIAL MEASUREMENT. The strains were grown on 5% sheep blood agar for 24 hours under anaerobic conditions as described above. A bacterial suspension at 1.5 x 10^8 CFU/ml was prepared immediately after cultivation and washed twice by centrifugation at 3,000 rpm for 5 minutes in a 1 mM NaCl solution. The precipitate was diluted in 2 ml of 1 mM NaCl and the experiment was performed in Zeta Plus Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY), starting with bacterial particle size measurement using Particle Sizing software (Brookhaven Instruments Corporation, Holtsville, NY), followed by measurement of zeta potential using Zeta Plus software (Brookhaven Instruments Corporation, Holtsville, NY). The experiment was carried out in duplicate, in two separate experiments.

STATISTICS. Statistical analyses were performed using the analysis of variance (ANOVA) followed by Dunnett's (treatment versus control) or Tukey's (to compare the differences among different treatments) post-test for multiple comparisons. Pearson's correlation was used to evaluate the relationship between zeta potential and resistance to indolicidin treatment. Student's t-test was used to evaluate the action of anti-capsular antibodies. All experiments were performed in

quadruplicates and repeated twice. Differences were considered statistically significant if P≤0.05. The statistical analyses and all graphs were performed using GraphPad Prism 9.

3. RESULTS

PNEUMOCOCCAL RESISTANCE TO INDOLICIDIN IS INFLUENCED BY THE CAPSULE SEROTYPE

Pneumococci expressing different capsule types (1, 2, 3, 4, 6B, 9V, 10A, 14 and 23F) were subjected to treatment with increasing concentrations of indolicidin (ranging from 7.5 to 120 µg/ml). As shown in Figure 1, the pneumococcal strains greatly varied in their ability to resist lysis and killing by indolicidin. Serotypes 9V, 23F and 2 showed the highest resistance to indolicidin and were only susceptible to concentrations of 15 µg/ml and higher (Supplementary Figure 1). Serotypes 1, 3, 4, 6B, 10A and 14, on the other hand, were susceptible to indolicidin killing at the lowest concentration, 7.5 µg/ml.

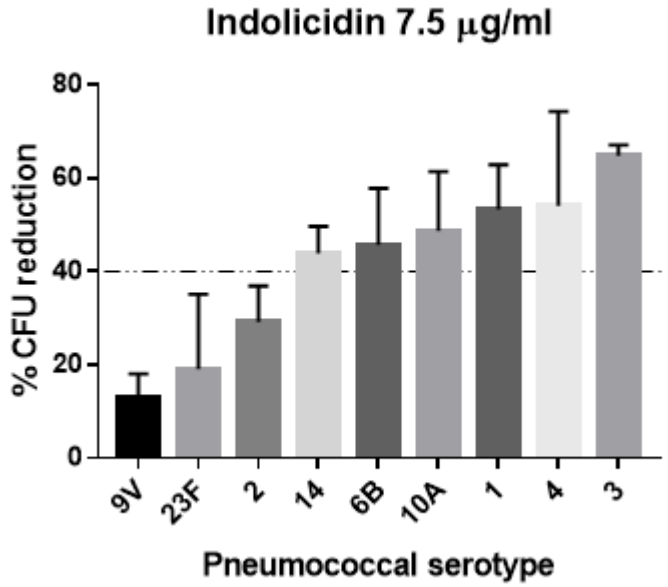


Figure 1. Susceptibility of *Streptococcus pneumoniae* to indolicidin. Bacterial strains of different serotypes were treated with 7.5 µg/ml indolicidin and plated. The percentage of bacterial reduction after treatment is shown for each strain. The dashed line represents the cut-off value above which there was a significant bacterial reduction.

Since polysaccharide capsules present variations in net charge, the zeta potential of each strain was calculated and plotted against the percent reduction in bacterial viability after indolicidin treatment. The analysis indicates a significant positive correlation between electronegativity and resistance to killing by indolicidin (Figure 2). The exception was serotype 3, which showed a very low zeta potencial, and a low resistance to the action of indolicidin. A previous study correlating zeta potential and colonization also found a discrepant result with serotype 3, indicating a characteristic of the serotype (20).

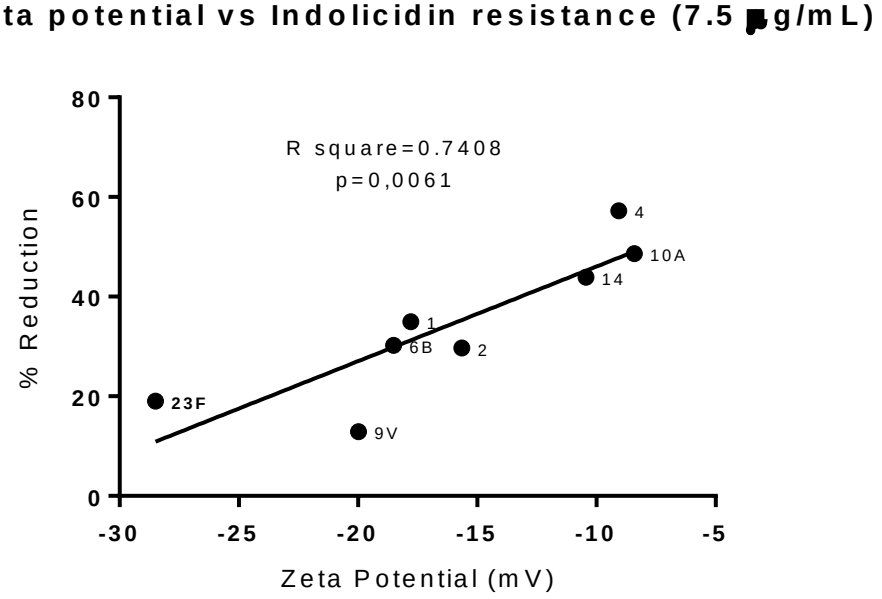


Figure 2. Correlation between of *Streptococcus pneumoniae* surface charge and resistance to killing by indolicidin. Surface charge was determined by calculating the zeta potential of each individual serotype. Resistance is represented by the percent reduction of each bacterial strain after 1 hour treatment with 7.5 µg/ml indolicidin. The data were analyzed using Pearson's correlation.

THE ABSENCE OF CAPSULE DIFFERENTLY IMPACTS RESISTANCE TO INDOLICIDIN

Once the susceptibility of the D39 (serotype 2) and TIGR4 (serotype 4) strains to lysis by indolicidin was established, a comparative analysis was performed using the respective capsule-free isogenic mutants AM1000 and HR1001.

The absence of capsule had opposite impacts on resistance to indolicidin for D39 and TIGR4 (Figure 3). At the lower concentrations of indolicidin (15 µg/ml) no differences in survival were observed between D39 and its capsule negative derivative, AM1000 (Figure 3A). However, at a higher concentration of indolicidin (30 µg/ml) the wild-type strain showed an increased resistance against killing, with 40% reduction in comparison with 80% killing in the capsule-negative strain, suggesting a protective effect of the capsule (Figure 3B). On the other hand, the capsule negative mutant in the TIGR4 background showed an increased resistance to the AMP, indicated by a smaller percentage of bacterial reduction after indolicidin-treatment (Figure 3A). This effect was intensified at the higher concentration of indolicidin (30 µg/ml), which caused an 80% reduction in survival of TIGR4, compared with only 14% reduction in the mutant strain (Figure 3B). Taken together, these results suggest that different capsules affect resistance to indolicidin in different ways.

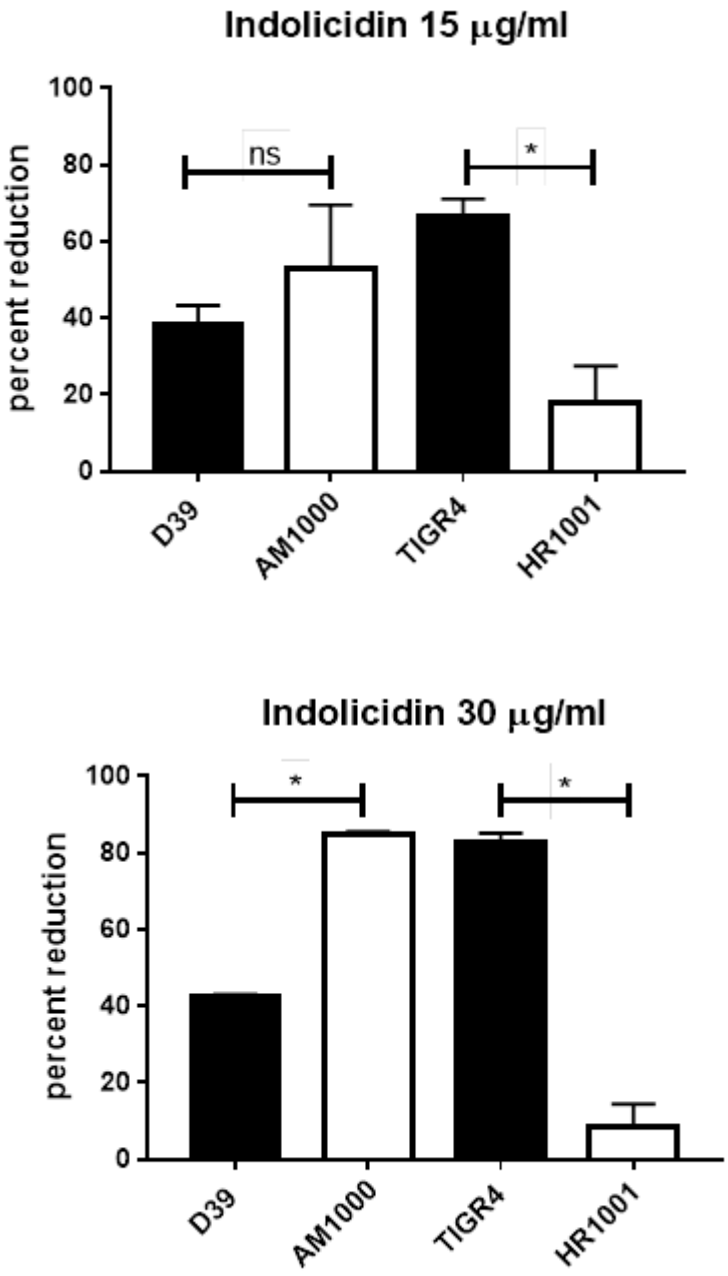
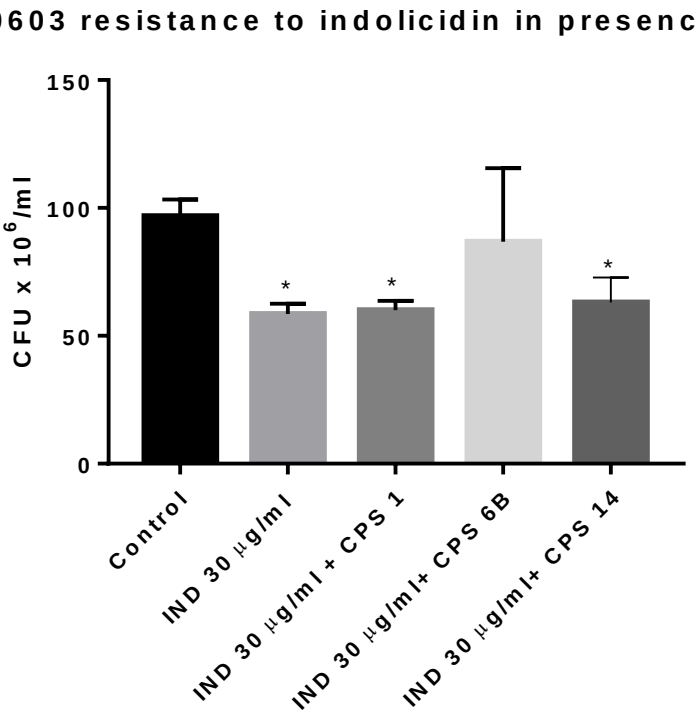


Figure 3. Capsule affects pneumococcal resistance do indolicidin. Wild-type pneumococcal strains D39 (serotype 2) and TIGR4 (serotype 4) and their capsule-negative mutants AM1000 (D39 background) and HR1001 (TIGR background) were treated with indolicidin at 15 μg/ml (A) and 30 μg/ml (B) and plated. The percent reduction in bacterial survival is shown for each group. The statistical analysis performed was ANOVA followed by Tukey. *p<0,05 in comparison with the wild-type strain.; ns: not significant.

EFFECT OF FREE CPS ON INDOLICIDIN LYTIC ACTION.

The experiments were performed using St. 245/00 (serotype 14) and St. 0603 (serotype 6B) incubated with free CPS 1, 6B and 14 prior to indolicidin treatment. Treatment of both strains with indolicidin (7.5 µg/ml for St 245/00 and 30 µg/ml for St 0603) led to a significant reduction in viability (Figure 4). The addition of purified CPS 6B partially reverted this effect, resulting in bacterial counts similar to those in the control group. The protective effect of CPS 6B was shown both against the serotype 6B (Figure 4A) and 14 strains (Figure 4B). CPS 1 and 14, on the other hand, did not affect pneumococcal killing by indolicidin.



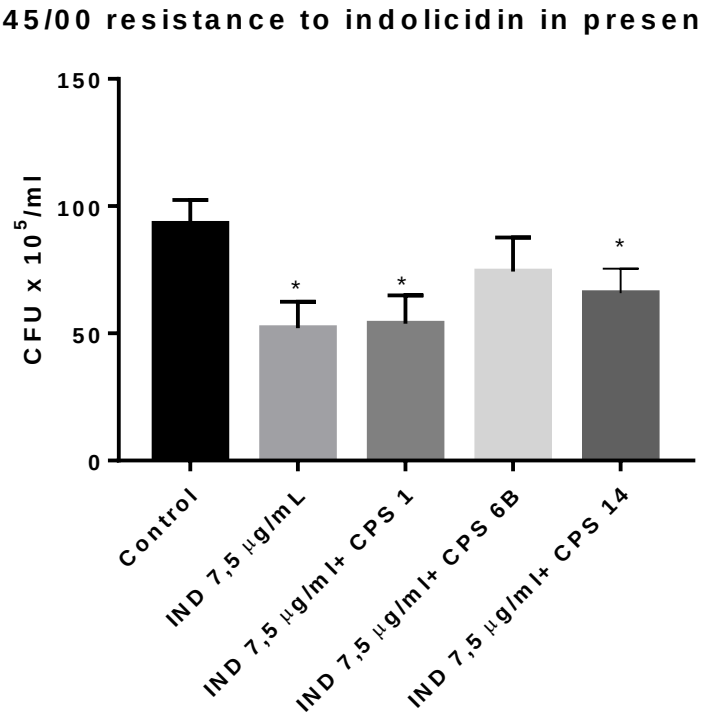


Figure 4. Effect of capsular polysaccharides on the lytic action of indolicidin. Peumococcal strains St 0603, serotype 6B (A) and St 245/00, serotype 14 (B) were incubated with purified CPS 1, 6B and 14 prior to treatment with indolicidin. The control group was incubated with PBS only. The number of bacteria surviving treatment is shown for each group. The statistical analysis performed was ANOVA followed by Dunnett. *p<0,05 in comparison with the control.

EFFECT OF ANTI-CAPSULAR ANTIBODIES ON INDOLICIDIN LYTIC ACTION.

To evaluate the effect of anti-capsular antibodies over indolicidin killing, pneumococcal strains P1079 (serotype 1) and St. 0603 (serotype 6B), were preincubated with sera from mice immunized with the homologous CPS, followed by treatment with indolicidin. The results of this analysis are shown in Figure 5. Incubation with anti-CPS1 promoted an increase in bacterial killing by indolicidin in comparison with control sera (Figure 5A). This effect may be attributed to the antibodies concealing protective epitopes in the capsule, thus blocking further interactions with the peptide. A similar effect was observed with antisera against the serotype 6B strain (St 0603) (Figure 5B).

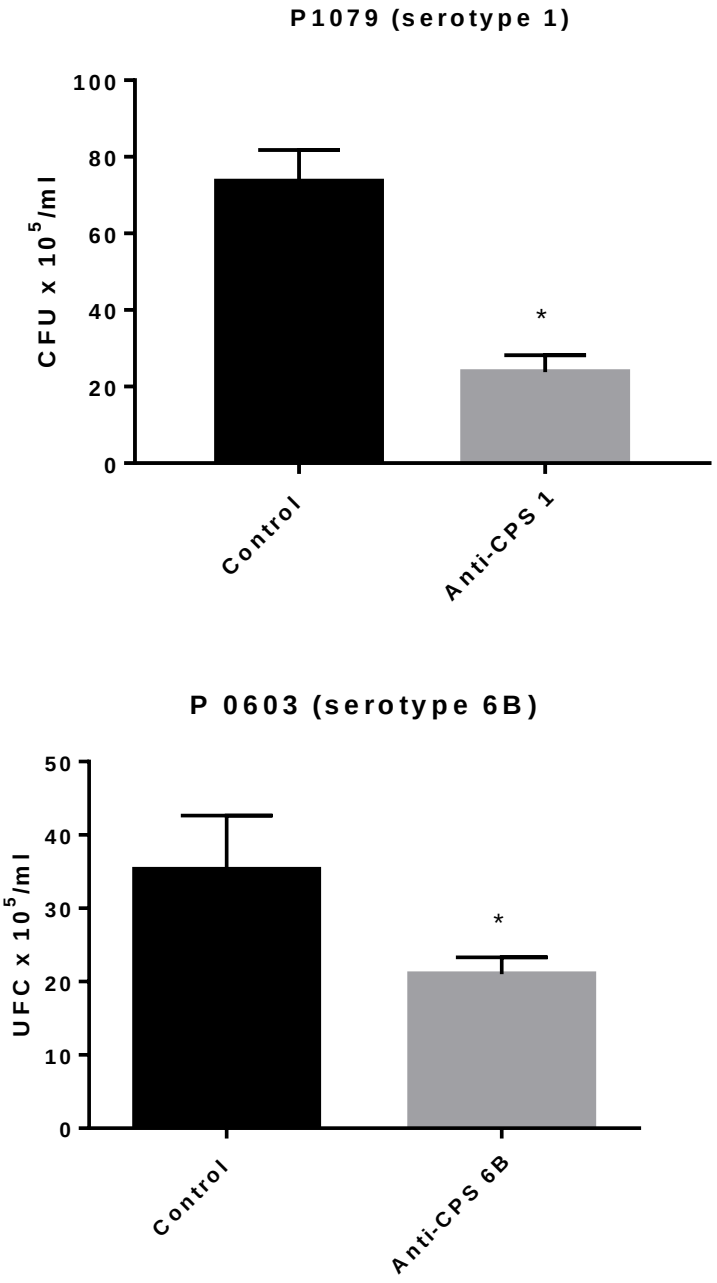


Figure 5. Effect of anti-capsular antibodies on the lytic action of indolicidin. Pneumococcal strains St 1079, serotype 1 (A) and 0603, serotype 6B (B) were incubated with sera from mice immunized with CPS 1 and 6B, respectively, prior to treatment with indolicidin. The control group was incubated with sera from sham immunized mice. The number of bacteria surviving treatment is shown for each group. The statistical analysis performed was Student's t-test. *$p<0.05$ in comparison with the control.

4. DISCUSSION

The polysaccharide capsule plays a central role in pneumococcal pathogenesis, protecting the bacterium against phagocytosis (37). Recent data also demonstrates a role for the capsule in favoring pneumococcal translocation through the vascular endothelium and enhancing intracellular survival – key steps involved in transition from carriage to invasive disease (38). The contribution of capsule to pneumococcal virulence is further emphasized by the high protection observed with the current polysaccharide-based vaccines against invasive diseases (6).

However, capsules present high structural and serological diversity; to date, more than 100 pneumococcal serotypes have been identified (5). In addition, the degree of protection that the polysaccharide capsule confers varies according to the serotype (37, 39).

In the present study, we investigated the contribution of the capsule to resistance or susceptibility of *Streptococcus pneumoniae* against the lytic action of indolicidin, an antimicrobial peptide belonging to the cathelicidin family.

Initially, *S. pneumoniae* isolates bearing different capsular types (Table 1) were exposed to a 1 h treatment with increasing concentrations of indolicidin. At the end of the treatment, a range of variations in killing was observed. Serotypes 9V, 23F and 2 were more resistant to indolicidin when compared with serotypes 1, 3, 4, 10A and 14. A dose-dependent effect was observed with all strains tested, with a more pronounced bacterial reduction at higher indolicidin concentrations.

To determine if resistance to indolicidin was affected by the capsule charge, we measured the zeta potential of the pneumococcal strains and plotted the results against the killing assay data. A strong positive correlation was observed between capsule net charge and resistance to the peptide, indicating that capsule charge could predict pneumococcal susceptibility to indolicidin *in vitro*.

A study conducted by Li et al. (2013) (17) investigating the contribution of surface charge to neutrophil mediated killing, also found that more electronegative capsules were related to increased resistance to phagocytosis and an increased ability to colonize the human nasopharynx. The present results further aid in this hypothesis, suggesting that capsule charge can influence several virulence attributes in this bacterium.

Next, we sought to determine if the ability to produce capsules affected killing by indolicidin, by comparing two pneumococcal strains of different serotypes with their capsule-negative isogenic mutants. The type 2 strain D39 was significantly more resistant to lysis with a high dose of indolicidin in comparison with its mutant, AM1000. The serotype 4 strain TIGR4, on the other hand, revealed an opposite effect, with the mutant displaying increased resistance to killing in relation to the wild-type counterpart. A possible explanation for this apparent discrepancy may be found on the surface charge of these strains; D39 was highly electronegative, while TIGR4 showed a zeta potential closer to neutrality. Therefore, we postulate that the effect of the capsule over the action of indolicidin will depend on the ability of such polysaccharides to prevent the lytic action of the AMP.

Previous work evaluating resistance of *S. pneumoniae* expressing diverse serotypes to human defensins HNP1-3 reported similar results (1). The unencapsulated TIGR4 was much more resistant to HNP1-3 when compared to the encapsulated strain. For D39, no differences in killing were observed between the wild type and mutant strains, in the concentrations of AMP tested. In the present work, the increased resistance of the wild type D39 in relation with the mutant was only apparent when higher concentrations of indolicidin were used, suggesting that other factors beyond the capsule may be involved in resistance to the AMP.

Considering the variable effects of capsules on resistance to indolicidin, we investigated whether purified CPS 1, 6B and 14 could influence killing by the AMP. Of the three CPS tested, only 6B was able to partially reverse killing of strain types 14 and 6B by indolicidin. It is not clear why CPS 1, which had similar zeta potential, did not affect bacterial killing by the AMP. It is possible that other factors like surface proteins and cell wall charge may contribute to the bacterium resistance to the AMP.

A study using free CPS 6B added to sensitive *Klebsiella pneumoniae* cultures showed a protective effect against killing by polymyxin B and HNP-1 (40). Furthermore, this protective effect was observed with negatively charged CPS, but not with cationic or uncharged ones. The authors conclude that the protective effect of CPS against killing by AMPs is dependent on their charge, similarly to our results. They also postulate that in presence of AMPs, bacteria may shed negatively charged CPS which can prevent lysis. This is an interesting suggestion, that would require more experiments in order to be confirmed.

Lastly, we tested the ability of anti-capsular antibodies to interfere with indolicidin activity. Anti-CPS1 and anti-CPS6B promoted an increase in pneumococcal killing by indolicidin, suggesting a possible new protective mechanism induced by polysaccharide-based pneumococcal vaccines. It also indicates that the ability of CPS to reduce killing by indolicidin involves a direct interaction between the polysaccharide and the AMP; the presence of antibodies would limit this interaction, thus abrogating the protective effects of the capsule and allowing the peptide to access the subjacent bacterial membrane, promoting killing.

Habets and coleagues (2012) (41), compared the susceptibility of several pneumococcal isolates to the human cathelicidin LL-37 and the alpha-defensin HNP-1 and found that carriage isolates were more resistant to the AMPs than clinical isolates. This finding suggests that AMPs act as selection force for pneumococci during colonization – the first step in all pneumococcal diseases.

5. CONCLUSION

Different pneumococcal serotypes exhibited variable resistance to indolicidin, which correlated with the capsule net charge; bacteria with lower zeta potential were more resistant to indolicidin when capsule was present, while those with less negative surface charge were more resistant in the absence of capsule.

Our results are in accordance with previous work using other antimicrobial peptides, indicating that the capsule has broad (however diverse) effects on the lytic activity of CAMPs. Free CPS 6B was able to partially reverse the bactericidal action of indolicidin over pneumococci, while anti-capsular antibodies favor the AMP activity. We postulate that electronegative CPS protect pneumococci from indolicidin by hijacking the AMP, thus preventing it from reaching the subjacent cell membrane.

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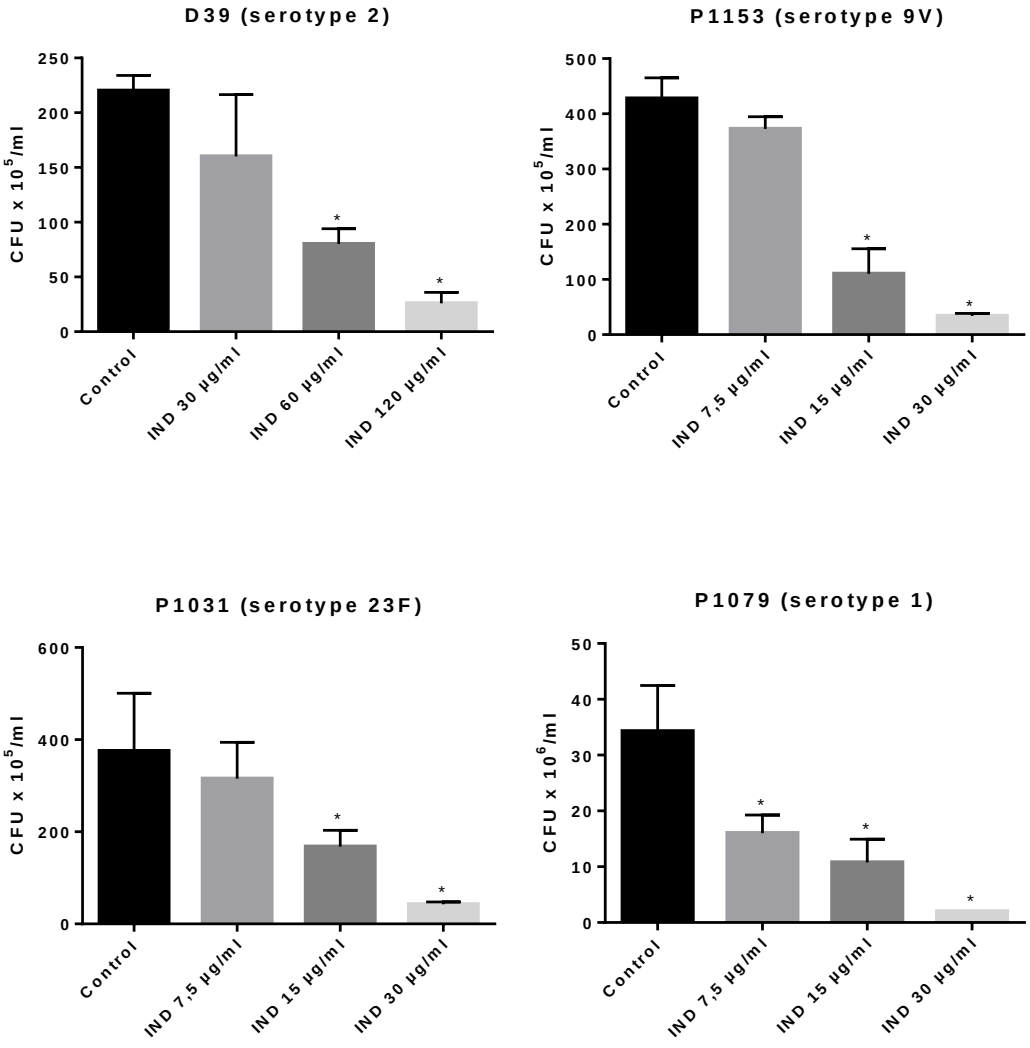
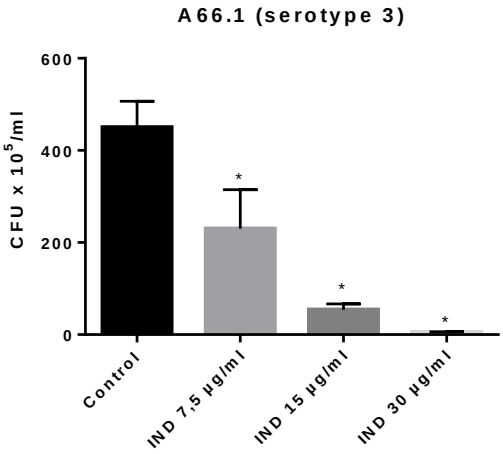
Suplementar Figure I

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400
200
0
*
*
*
Control
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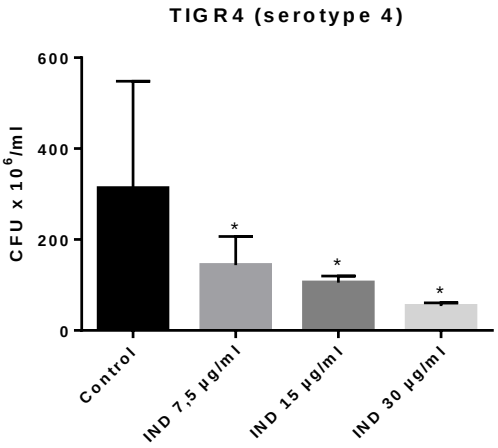
IND 15 µg/ml
IND 30 µg/ml


TIGR4 (serotype 4)
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400
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0
*
*
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Control
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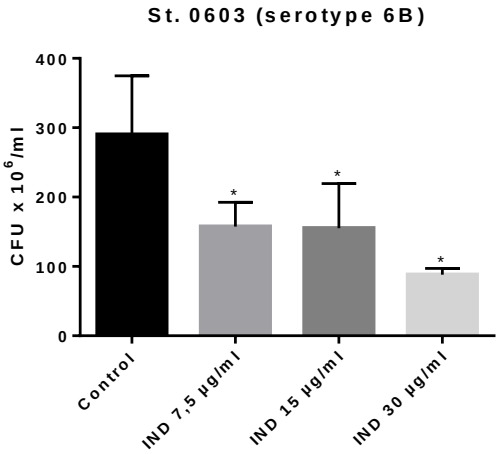
St. 0603 (serotype 6B)
CFU x 10^6/ml
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300
200
100
0
*
*
*
Control
IND 7,5 µg/ml
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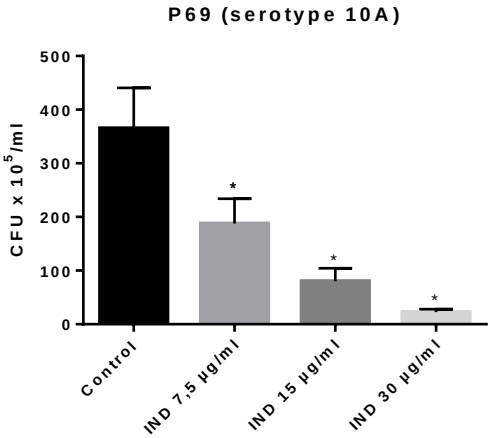
IND 30 µg/ml


P69 (serotype 10A)
CFU x 10^5/ml
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400
300
200
100
0
*
*
*
Control
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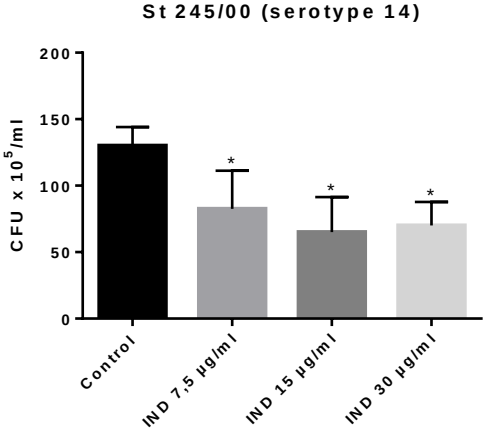
IND 30 µg/ml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CFU x 10^5/ml
200
150
100
50
0
*
*
*
Control
IND 7,5 µg/ml
IND 15 µg/ml
IND 30 µg/ml

Supplementary figure 1. Susceptibility of different *Streptococcus pneumoniae* serotypes to indolicidin. Bacterial strains were treated with increasing concentrations of indolicidin and plated. The control group was incubated with PBS only. The numbers of bacteria surviving treatment is shown for each strain. *$p<0.05$ in comparison with control.

4. CONCLUSÃO

Diferentes sorotipos de pneumococo exibiram resistência variável à indolicidina; que se correlacionou com a carga superficial da cápsula; bactérias com baixo potencial zeta foram mais resistentes à indolicidina quando a cápsula estava presente, enquanto aquelas com carga superficial menos negativa foram mais resistentes na ausência da cápsula. Nossos resultados corroboram trabalhos anteriores usando outros tipos de peptídeos antimicrobianos (como as defensinas), indicando que a cápsula tem amplos (porém diversos) efeitos sobre a atividade lítica de AMPs. A adição de CPS livre do sorotipo 6B foi capaz de reverter parcialmente os efeitos bactericidas da indolicidina sobre o pneumococo, enquanto os anticorpos anti-capsulares favoreceram a atividade do AMP. Postulamos que a CPS eletronegativa protege os pneumococos da indolicidina sequestrando o AMP, evitando assim que ele alcance a membrana celular subjacente e cause a lise da bactéria.

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Anexo I: Parecer do comitê de ética no uso de animais (CEUA) referente à obtenção de anticorpos capsulares em camundongos**.**

CEUA
Comitê de Ética na Utilização de Animais

USF
UNIVERSIDADE SÃO FRANCISCO

Bragança Paulista, 15 de Março de 2021

Projeto de Pesquisa: "Papel da cápsula polissacarídica na ação de peptídeos antimicrobianos catiônicos sobre o pneumococo."

Área de Conhecimento: Imunologia
Autor: Profa. Michelle Darrieux Sampaio Bertoncini
Colaboradores: Prof. Thiago Converso; Aluna Natalha Waz
Instituição: Universidade São Francisco
Protocolo: 003.04.2021
IP. Ciuca: 200.225.122.34
CIAEP/CONCEA Nº 01.226.2014
Vigência do Projeto: 01/05/2021 – 20/12/2022
Número: 30 animais
Espécie: Camundongo isogênico
Linhagem: BALB/c
Peso: 20 gr/5-7 semanas
Total de Animais: 30 Camundongos isogênicos
Espécie: 30 Femeas
Procedência do Animal: Biotério/CEMIB - UNICAMP

Prezado Pesquisador,
O Comitê de Ética em Pesquisa com Uso de Animais de Pesquisa – CEUA, da Universidade São Francisco analisou em reunião no dia 15/04/2021, o projeto de pesquisa, sob a responsabilidade de Vossa Senhoria. Após avaliação dos documentos o relator considerou o projeto como APROVADO.

Parecer: **APROVADO**

Profa. Giovanna Barbarini Longato
Coordenadora do Comitê de Ética com Uso de Animais de Experimentação