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Mestrado

AÇÃO DA SERINOPROTEASE PIC PRODUZIDA POR *Escherichia coli* SOBRE PROTEÍNAS DA MATRIZ EXTRACELULAR E DA CASCATA DE COAGULAÇÃO SANGUÍNEA

JANAÍNA VASCONCELOS GOMES

São Luís
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão, como requisito para a obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Afonso Gomes Abreu Junior.

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BANCA EXAMINADORA

Prof. Dr. Afonso Gomes Abreu Junior. (Orientador)
Universidade Federal do Maranhão

Profa. Ludmila Bezerra da Silva
Universidade Federal do Maranhão

Profa. Hivana Patricia Melo Barbosa Dall'Agnol
Universidade Federal do Maranhão

Prof. Luis Claudio Nascimento da Silva
Uniceuma-Centro Universitário do Maranhão

“O período de maior ganho em
conhecimento e experiência é o período
mais difícil da vida de alguém”.

Dalai Lama

Dedico

A minha família.

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RESUMO

A serinoprotease Pic (*protein involved in colonization*) é uma proteína autotransportadora identificada em *E. coli* enteroagregativa (EAEC), *Shigella flexneri* e *Citrobacter rodentium*. Dentre os papéis biológicos para Pic já foram descritos hemaglutinação, atividade mucinolítica, degradação do fator V da cascata de coagulação e clivagem de glicoproteínas de superfície de leucócitos, que estão envolvidas no tráfico, migração e inflamação. Nosso grupo de pesquisa já demonstrou a sua atuação no sistema complemento humano o que confere a essas bactérias a capacidade de driblar os mecanismos de defesa do sistema imune inato favorecendo, desta forma, o desenvolvimento e manutenção do quadro de sepse. Devido a essas habilidades, levantou-se a hipótese de que Pic teria ação sobre outras moléculas importantes do hospedeiro como as proteínas envolvidas com a cascata de coagulação sanguínea e moléculas da matriz extracelular. Desta forma, o objetivo deste estudo foi investigar a ação da serinoprotease Pic sobre componentes da matriz extracelular e da cascata de coagulação sanguínea. Para isso, frações concentradas do sobrenadantes de cultura de *E. coli* produtora de Pic (HB101/Pic) e não produtora de Pic (HB101), bem como BSA foram incubados em diferentes tempos com diversas moléculas da cascata de coagulação sanguínea (plasminogênio, fibrinogênio e fibrina) e da matriz extracelular (colágeno tipo I, colágeno tipo IV, decorina, laminina e fibronectina plasmática) para avaliar tanto uma possível ligação como a degradação destes componentes. Além disto, foi realizado também um ensaio de ativação do plasminogênio em plasmina, uma vez que esta é uma molécula chave na formação de coágulos e na ativação de outras vias do sistema imune. Desta forma, foi possível observar que as frações concentradas do sobrenadantes de cultura de HB101/Pic foi capaz de se ligar a diversas moléculas da matriz extracelular, de maneira significativa, tais como colágenos tipo I e IV, laminina e fibronectina. Ao incubar a proteína Pic com moléculas da coagulação sanguínea foi possível observar ligação com, o plasminogênio, este por sua vez, foi convertido em sua forma ativa, plasmina, na presença do ativador exógeno do tipo uroquinase (uPA). Os demais componentes tanto da cascata como da matriz extracelular não foram clivados por Pic. Acreditamos que a bactéria *E. coli* produtora de serinoprotease Pic liga-se à matriz extracelular por meio de diversos componentes, facilitando assim o processo infeccioso no hospedeiro. A plasmina gerada na presença de Pic também deve contribuir para um efeito sinérgico na degradação de moléculas do sistema complemento, bem como para ativação desregulada e aumentada da cascata de coagulação sanguínea.

Palavras-chave: Pic; matriz extracelular, cascata de coagulação, plasmina, plasminogênio.

ABSTRACT

Serine protease Pic (protein involved in colonization) is an autotransporter protein identified in enteroaggregative E. coli (EAEC), Shigella flexneri and Citrobacter rodentium. Among biological roles for Pic have been described hemagglutination, mucinolytic activity, factor V degradation of the coagulation cascade and cleavage of leukocyte surface glycoproteins, which are involved in trafficking, migration and inflammation. Our research group has already demonstrated its performance in the human complement system, which gives these bacteria the ability to circumvent the defense mechanisms of the innate immune system, thus favoring the development and maintenance of sepsis. Because of these abilities, it was hypothesized that Pic would have action on other important host molecules such as the proteins involved with the blood clotting cascade and extracellular matrix molecules. Thus, the objective of this study was to investigate the action of Pic serinoprotease on components of the extracellular matrix and blood coagulation cascade. For this, concentrated fractions of Pic (HB101 / Pic) and non-Pic (HB101) -producing E. coli culture supernatants as well as BSA were incubated at different times with several molecules of the blood coagulation cascade (plasminogen, fibrinogen and fibrin) and extracellular matrix (collagen type I, collagen type IV, decorin, laminin and plasma fibronectin) to evaluate both a possible binding and the degradation of these components. In addition, a plasmin activation assay was performed in plasmin, as this is a key molecule in the formation of clots and in the activation of other pathways of the immune system. In this way, it was possible to observe that the concentrated fractions of HB101 / Pic culture supernatants were able to bind to several extracellular matrix molecules in a significant manner, such as collagens type I and IV, laminin and fibronectin. By incubating the Pic protein with blood coagulation molecules, it was possible to observe binding with plasminogen, which in turn was converted into its active form, plasmin, in the presence of the exogenous urokinase activator (uPA). The other components of both cascade and extracellular matrix were not cleaved by Pic. We believe that the E. coli bacterium producing serinoprotease Pic binds to the extracellular matrix through several components, thus facilitating the infectious process in the host. Plasmin generated in the presence of Pic should also contribute to a synergistic effect on the degradation of complement system molecules, as well as to deregulated and increased activation of the blood coagulation cascade.

Keywords: Pic; extracellular matrix, coagulation cascade, plasmin, plasminogen.

LISTA DE SIGLAS E ABREVIATURAS

AIEC	*E. coli* aderente e invasiva
ATs	Autotransportadores
BFP	*Bundle forming pilus*
BfpA	*Binding forming pili*
DAEC	*E. coli* de adesão difusa
DEC	*E. coli* diarreiogênica
E.Coli	Escherichia coli
eae	*Attaching and effacing*
EAEC	*E. coli* enteroagregativa
EAF	*E. coli* enteropatogenic adherence fator
EHEC	*E. coli* enteroemorrágica
EIEC	*E. coli* enteroinvasiva
EPEC	*E. coli* enteropatogênica
ETEC	*E. coli* enterotoxigênica
EXPEC	*E. coli* extra-intestinais
FN	Fibronectina
FNc	Fibronectina celular
FNp	Fibronectina plasmática
FT	Fator tecidual
FvW	Fator de Von Willebrand
CID	Coagulação intravascular disseminada
GAGs	Glicosaminoglicanos
LEE	*Locus of enterocyte effacement*
LT	Toxina termo lábel

MEC	Matriz extracelular
MMPs	Metaloproteinases de matriz
PAI	Inibidor do plasminogênio
PDF	Produto de degradação de fibrina
Pic	*Protein involved in colonization*
SPATEs	*Serine protease autotransporter enterobacteriaceae*
ST	Toxina termo estável
STEC	*E. coli* enteroagregativa produtora de shiga toxina
Stx	Toxina Shiga
t-PA	Ativador tecidual do plasminogênio
t-PA	Plasminogenio do tipo tecidual
u-PA	Plasminogenio do tipo uroquinase
UPEC	*E. coli* uropatogênicas

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1. INTRODUÇÃO

A diarreia é considerada uma das maiores causas de mortalidade infantil em crianças com menos de cinco anos de idade, sendo responsável pela morte de cerca de 760.000 a cada ano. A diarreia infecciosa pode ser causada por bactérias, vírus ou parasitas, sendo o rotavírus e a *Escherichia. Coli* (*E. coli*) os agentes etiológicos mais comuns em países em desenvolvimento (World Health Organization, 2014).

Embora faça parte da microbiota intestinal do homem e de outros animais, existem linhagens de *E. coli* que apresentam fatores de virulência específicos, causando infecções intestinais ou extra-intestinais em seu hospedeiro. As cepas patogênicas de *E. coli* geralmente estão associadas à infecção intestinal, sendo chamadas de *E. coli* diarreiogênicas (DEC). Quando colonizam outros sítios anatômicos são denominadas *E. coli* extra-intestinais (ExPEC) e podem levar a infecções do trato urinário, sepse, meningite, dentre outras doenças (CROXEN, 2013). Algumas *E. coli* são produtoras de proteínas denominadas SPATEs (*Serine protease autotransporter Enterobacteriaceae*), que constituem uma superfamília de proteínas cujos membros se parecem com aqueles que pertencem à superfamília tipo tripsina de serino proteases (YEN, et.al, 2008; RUIZ-PEREZ E NATARO, 2014).

A secreção de serinoproteases por microrganismos pode causar danos ao hospedeiro, a exemplo da clivagem de moléculas-chave do sistema complemento, da cascata de coagulação sanguínea e diversas proteínas da matriz extracelular, levando a uma resposta ineficiente do hospedeiro frente à ação de microrganismos produtores dessas proteases. A proteína Pic (*Protein involved in colonization*) é um exemplo dessas SPATEs. É produzida e secretada por *E. coli*, *Shigella flexneri* 2a e *Citrobacter rodentium* (RAJAKUMAR et al., 1998; HENDERSON et al., 1999; BHULLAR et al., 2015) e desempenha vários papéis biológicos, incluindo hemaglutinação, atividade mucinolítica, degradação do fator V da cascata de coagulação e clivagem de glicoproteínas de superfície de leucócitos, que estão envolvidas no tráfico, migração e inflamação (HENDERSON et al., 1999; DUTTA et al., 2002; PARHAM et al., 2004; GUTIERREZ-JIMENEZ et al., 2008;RUIZ-PEREZ et al., 2011; ABREU et al., 2016).

Devido à sua atividade mucinolítica, Pic também promove a colonização intestinal de ratos e coelhos pela clivagem do muco presente na luz intestinal, favorecendo assim a adesão da bactéria aos enterócitos (HARRINGTON et al., 2009; MUNERA et al., 2014;

ABREU et al., 2016). Após estabelecimento da bactéria no sítio de infecção, Pic exerce um papel antagônico estimulando as células caliciformes a hiperproduzirem muco (NAVARROGARCIA et al., 2010), o que contribui para uma colonização persistente, uma vez que fornece uma fonte de nutrientes para o crescimento bacteriano.

Recentemente nosso grupo de pesquisa mostrou que Pic também reduz a atividade do sistema complemento pela inativação das três vias: via clássica, alternativa e das lectinas (ABREU et al., 2015). Além disso, diversos patógenos expressam uma série de proteínas de membrana com capacidade de interagir com moléculas da matriz extracelular, da cascata de coagulação e com células do hospedeiro.

A matriz extracelular (MEC) é uma rede macromolecular tridimensional não celular que fornecem auxílio estrutural e bioquímico para as células circundantes. Elas atuam como receptores para vários patógenos e vários trabalhos indicam que as proteínas da MEC podem estar presentes na superfície das células intestinais participando como receptores bacterianos (WESTERLUND E KORHONEN, 1993; WALIA et al., 2004; KONKEL et al., 2005), a exemplo da fibronectina que foi a primeira proteína descrita a atuar como um receptor celular para patógenos bacterianos (YÁÑEZ, 2016). Além destas, moléculas geradas na cascata de coagulação sanguínea podem interagir com as proteínas da MEC.

A cascata de coagulação sanguínea é ativada pela ação proteolítica sequencial de pró-enzimas por proteases do plasma, resultando na formação de trombina que, então, quebra a molécula de fibrinogênio em monômeros de fibrina (FRANCO, 2001). Neste processo designado de fibrinólise participam o plasminogênio e o ativador tecidual do plasminogênio, protease que transforma o plasminogênio em plasmina. Proteínas bacterianas são também capazes de interagir com plasminogênio e ativá-lo em sua superfície. Assim, a plasmina gerada pode degradar componentes da matriz extracelular, fibrina, fibrinogênio, além de fatores e co-fatores de coagulação (fator V e fator VIII) (BAJZAR, 2000; RODRIGUES et al., 2012).

Assim, devido a essa habilidade da proteína Pic de degradar moléculas do tecido conjuntivo, contribuindo para os processos de invasão e destruição tecidual observados durante a infecção por essas bactérias, levanta-se a hipótese de que Pic teria ação sobre proteínas envolvidas com a com a cascata de coagulação sanguínea e moléculas da matriz extracelular. Este estudo torna-se relevante devido a alta prevalência de DEC nos países em desenvolvimento que potencialmente aumentam o risco de doença, incluindo a sepse.

De acordo com García et al. (2012), na última década houve um aumento acentuado nas infecções causadas por *E. coli* resistente a antibióticos, o que poderia afetar o prognóstico de pacientes com sepse. Portanto, entender como a proteína Pic se comporta na corrente sanguínea se torna fundamental para melhor esclarecimento das causas da sepse, além de caracterizar os fatores de virulência que contribuem para a permanência da bactéria na corrente circulatória, etapa crucial para o desenvolvimento de vacinas e possíveis inibidores proteicos

2. REFERENCIAL TEÓRICO

2.1. *Escherichia coli*

E. coli foi descrita em 1885 pelo pediatra alemão Theodore Escherich, primeiramente sob a denominação de *Bacillus coli* comune. Porém, após uma revisão foi renomeada para *E. coli* (CHEN; FRANKEL, 2005). Faz parte da família Enterobacteriaceae, que possui importantes patógenos humanos, especialmente em ambientes hospitalares, onde causam os mais variados tipos de infecção, tais como infecções do trato urinário, pneumonias, meningites, abscessos, feridas cirúrgicas, sepse e, principalmente, infecções intestinais causando diarreia (SADER et al., 2001; PATERSON, 2006).

E. coli é uma bactéria comum do trato gastrointestinal de humanos e animais, porém algumas cepas possuem fatores de virulência que conferem a este patógeno a habilidade de causar infecções, sendo estes classificados como bactérias patogênicas intestinais (SILVA et al., 2014). Trata-se de um bacilo Gram negativo, oxidase-negativo, capaz de crescer tanto em ambientes aeróbicos quanto anaeróbicos, preferencialmente a 37 °C, podendo ainda ser móvel ou não. É facilmente isolada de amostras fecais através do cultivo em meios seletivos e a mudança de pH do meio. A fermentação da lactose pode ser usada para diferenciar fermentadoras de não-fermentadoras de lactose. (CROXEN et al., 2013).

Os diferentes tipos de *E. coli* podem ser identificados por antissoros específicos que podem variar de acordo com os antígenos existentes na superfície da membrana bacteriana ou em outras estruturas como flagelos. Esses antígenos podem ser flagelares (H), capsulares (K) ou somáticos (O) (NATARO E KAPER,1998). Destes, dois tipos principais de antígenos de superfície constituem a base para a classificação

sorológica: o antígeno ‘O’ ou somático que define o sorogrupo e o antígeno ‘H’ ou flagelar (FIALHO, 2008).

Existem clones de *E. coli* altamente adaptados que têm adquirido atributos específicos de virulência e que, desta forma, conferem a estas bactérias uma maior habilidade para adaptarem-se a novos nichos, permitindo causar um amplo espectro de doenças. Esses atributos de virulência são frequentemente codificados por elementos genéticos que podem ser mobilizados em diferentes cepas para criar novas combinações de fatores de virulência (KAPER; NATARO; MOBLEY, 2004).

Uma vez adquiridos fatores de virulência, as *E. coli* podem causar doenças que vão desde as infecções intestinais até aquelas que afetam órgãos extra-intestinais, causando infecções do trato urinário, sepses e meningites. São exemplos as *E. coli* uropatogênicas (UPEC) e as *E. coli* causadoras de meningite em neonatos (NATARO; KAPER, 1998; KAPER; NATARO; MOBLEY, 2004).

O potencial das cepas em causar infecções pode variar de acordo com os fatores de virulência específicos para cada patógeno entérico. O grande espectro de mecanismos fisiopatológicos e o tipo de diarreia exigiram a classificação das DECs em patótipos, separados em seis categorias, considerando seus mecanismos de virulência específicos, as síndromes clínicas que causam, os sorotipos O:H, os aspectos epidemiológicos e/ou os tipos de interação com linhagens celulares *in vitro*. Esses patótipos de *E. coli* diarreiogênicas são: *E. coli* enteropatogência (EPEC); *E. coli* enterotoxigênica (ETEC); *E. coli* produtora de toxina de Shiga (STEC); *E. coli* enteroinvasiva (EIEC); *E. coli* enteroagregativa (EAEC) e *E. coli* difusamente aderente (DAEC) e E. coli invasiva aderente (AIEC) (CROXEN et al., 2013)

As EPEC são capazes de causar a lesão A/E (*attaching and effecing*), que é caracterizada pela perda das microvilosidades das células intestinais e formação de um pedestal com adesão íntima da bactéria aos enterócitos, além do acúmulo de actina polimerizada e rearranjo de outros componentes do citoesqueleto (MOON et al., 1983; KAPER; NATARO; MOBLEY, 2004; HUANG et al., 2002). Os genes responsáveis por essa lesão são codificados em uma ilha de patogenicidade denominada região LEE (*locus of enterocyte effacement*) (KAPER; NATARO; MOBLEY, 2004). A região LEE contém 41 genes e é complexamente regulada. Codifica um sistema de secreção tipo III (SSTIII) e o plasmídeo EAF, que contém sequências que codifica a proteína BfpA (*Binding Forming Pili*) (NATARO E KAPER, 1998). Esse Sistema SSTIII, transloca proteínas

efetoras da bactéria para o citoplasma da célula hospedeira (DENG et al., 2004; HACKER; KAPER, 2000). Além desses efetores, algumas cepas de EPEC também codificam proteínas associadas à virulência, a exemplo de EspC, uma serinoprotease que atua como uma enterotoxina, causando efeitos citopáticos em células de cultura de tecidos (NAVARRO-GARCIA et al., 2004) e segmentos intestinais de ratos (MELLIES et al., 2001), além de promover a clivagem de importantes substratos biológicos, como o fator V da cascata de coagulação, pepsina e espectrina (DUTTA et al., 2002).

As EPEC são divididas em duas categorias: típicas e atípicas. EPEC típica são identificadas pela presença do gene *eae* (EPEC *attaching and effacing*) e plasmídeo EAF (EPEC *adherence factor*). O plasmídeo EAF contém os genes envolvidos com a biogênese de uma adesina fimbrial do tipo IV denominada *bundle forming pilus* (BFP), que é responsável pela interação entre bacterias e adesão localizada das EPEC à célula hospedeira, levando à formação de micro colônias (TRABULSI; KELLER; GOMES, 2002). As EPEC atípica estão associadas à diarreia endêmica, surtos de diarreia e casos de diarreia persistente (HEDBERG et al., 1997; YATSUYANAGI et al., 2003; NGUYEN et al., 2006). Os estudos realizados no Brasil também têm demonstrado associação com diarreia endêmica em crianças (BUERIS et al., 2007; MORENO et al., 2008; SCALETSKY et al., 2010).

As ETEC aderem à mucosa do intestino delgado causando diarreia por enterotoxinas ST (toxina termo estável) e LT (toxina termo lábel). Essas toxinas ocasionam lesão no momento da colonização na superfície da mucosa intestinal (TRABULSI; ALTERTHUM, 2005). No organismo de seus hospedeiros colonizam a superfície da mucosa intestinal e pela ação de suas toxinas, causam diarreia aquosa, principalmente em recém nascidos (NATARO; KARPER, 1998).

Nas EIEC ocorre invasão epitelial, seguido de fagocitose e lise dos vacúolos endocíticos, multiplicação intracelular, movimento direcional pelo citoplasma e invasão de células epiteliais adjacentes que são indistinguíveis dos sintomas da disenteria causada pelas espécies de *Shigella*, que invadem e proliferam em células epiteliais. Todas essas características são coordenadas pelo plasmídeo que codifica os genes de invasão (TRABULSI; ALTERTHUM, 2005). Alguns estudos têm mostrado que *E. coli* e *Shigella* são taxonomicamente indistinguíveis a nível de espécie (PUPO; LAN; REEVES, 2000; WEI et al., 2003), sendo ambos os patógenos intracelulares facultativos e causadores da disenteria bacilar (CROXEN et al., 2013).

As EHEC passaram a constituir uma subcategoria STEC e caracterizam-se por apresentar genes que codificam a produção das toxinas Shiga. Dois grupos de toxina de Shiga (Stx1 e Stx2) podem ser produzidas por STEC. Estas citotoxinas recebem este nome por serem iguais a toxina produzida por *Shigella dysenteriae* I e seus receptores são encontrados em células renais e intestinais (STEC) (KAPER; NATARO; MOBLEY, 2004), cujo principal sorotipo é o O157:H7 (NATARO E KAPER, 1998) e assim como EPEC, alguns sorotipos de STEC são capazes de causar lesões do tipo A/E.

Em humanos, STEC é responsável por um amplo espectro de doenças que varia desde diarreias brandas até severas e sanguinolentas, evoluindo para complicações graves como colite hemorrágica, síndrome urêmica hemolítica e púrpura trombótica trombocitopênica (NATARO E KAPER, 1998).

EAEC é responsável por diarreia persistente, com duração de mais de 14 dias, em crianças e adultos, tendo prevalência em países em desenvolvimento. A persistência da diarreia em crianças é similar à causada por ETEC, causando danos leves, porém significantes, na mucosa (NATARO E KAPER, 1998; BHUNIA, 2008).

DAEC provocam um alongamento das microvilosidades na mucosa *in vitro*, são caracterizadas pela formação de agregados sobre as células do cólon epitelial. Secretam toxinas, porém não têm efeito necrosante no tecido (NATARO E KAPER,1998; NATARO E KAPER, 1998; BHUNIA, 2008).

AIEC tem sido implicada como um dos agentes causadores da doença de crohn, afetando principalmente o intestino delgado. Não há um único agente causador da doença de crohn identificado, e a hipótese atual é que a doença é causada por uma combinação de fatores, incluindo a genética, a microbiota intestinal, fatores ambientais e patógenos entéricos (BARRETT et al., 2008).

Uma característica comum entre esses diferentes grupos de *E. coli* é a secreção de proteases, a exemplo das SPATEs (*Serino Protease Autotransporters of Enterobacteriaceae*).

2.2. *Serino Protease Autotransporters of Enterobacteriaceae (SPATEs)*

SPATEs, também conhecidas como autotransportadoras (AT), são uma família de proteases extracelulares produzidas por Enterobacteriaceae, secretadas pelo sistema de secreção do tipo V de bactérias Gram-negativas (HENDERSON; NATARO, 2001).

As AT são transportadas através da membrana interna por meio do sistema Sec. Uma vez no periplasma, o domínio C-terminal se dobra como um barril na membrana externa, formando um poro. O domínio do passageiro é então translocado através da membrana externa, clivado do β-barril e liberado no meio extracelular, onde ele cumprirá sua função, geralmente relacionada à virulência da linhagem produtora (DAUTIN,2010) (Figura 1).

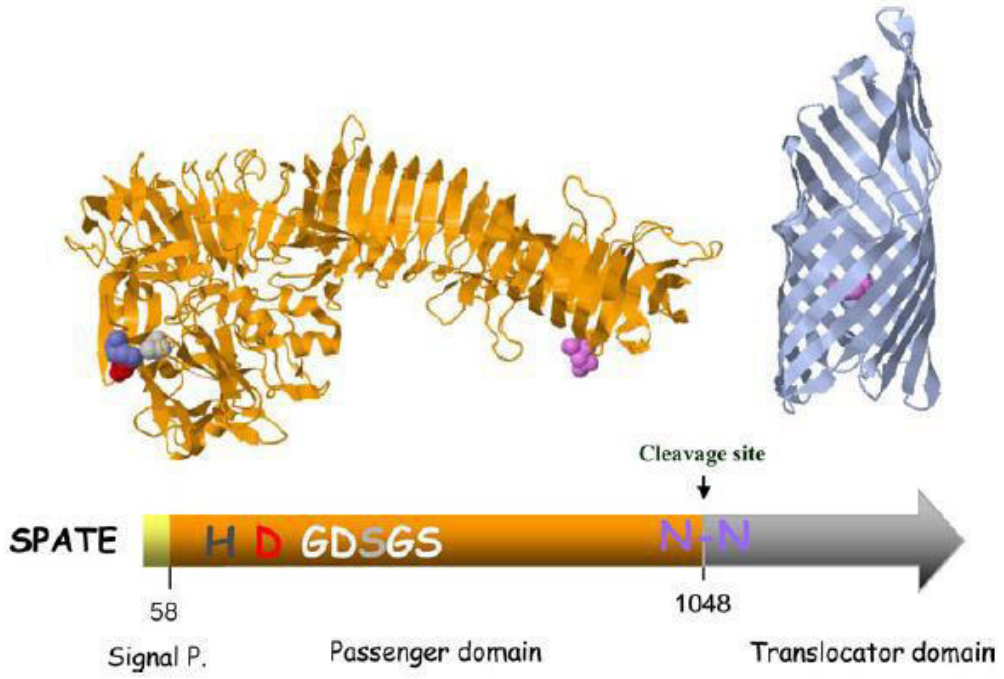

FIGURA 1: Estrutura geral das SPATEs. **FONTE:** RUIZ-PEREZ E NATARO (2014).

Com base nas relações filogenéticas e na similaridade funcional, as SPATEs foram divididas em dois grupos (DUTTA et al, 2002) (Figura 2). Os membros do primeiro grupo, a exemplo de Pet, EspC, EspP, Sat e SigA, apresentam efeitos citopáticos nas células epiteliais do hospedeiro através da internalização e clivagem das proteínas do hospedeiro intracelularmente (RUIZ-PEREZ; NATARO, 2014). Por exemplo, Pet e EspC têm como alvo a proteína α-fodrina (espectrina) de ligação a actina, que afeta o citoesqueleto (CANIZALEZ-ROMAN; NAVARRO-GARCIA, 2003). Em contraste, os membros do segundo grupo clivam principalmente proteínas extracelulares, a exemplo de Vat, SepA, EatA, EspI e Pic (RUIZ-PEREZ; NATARO, 2014).

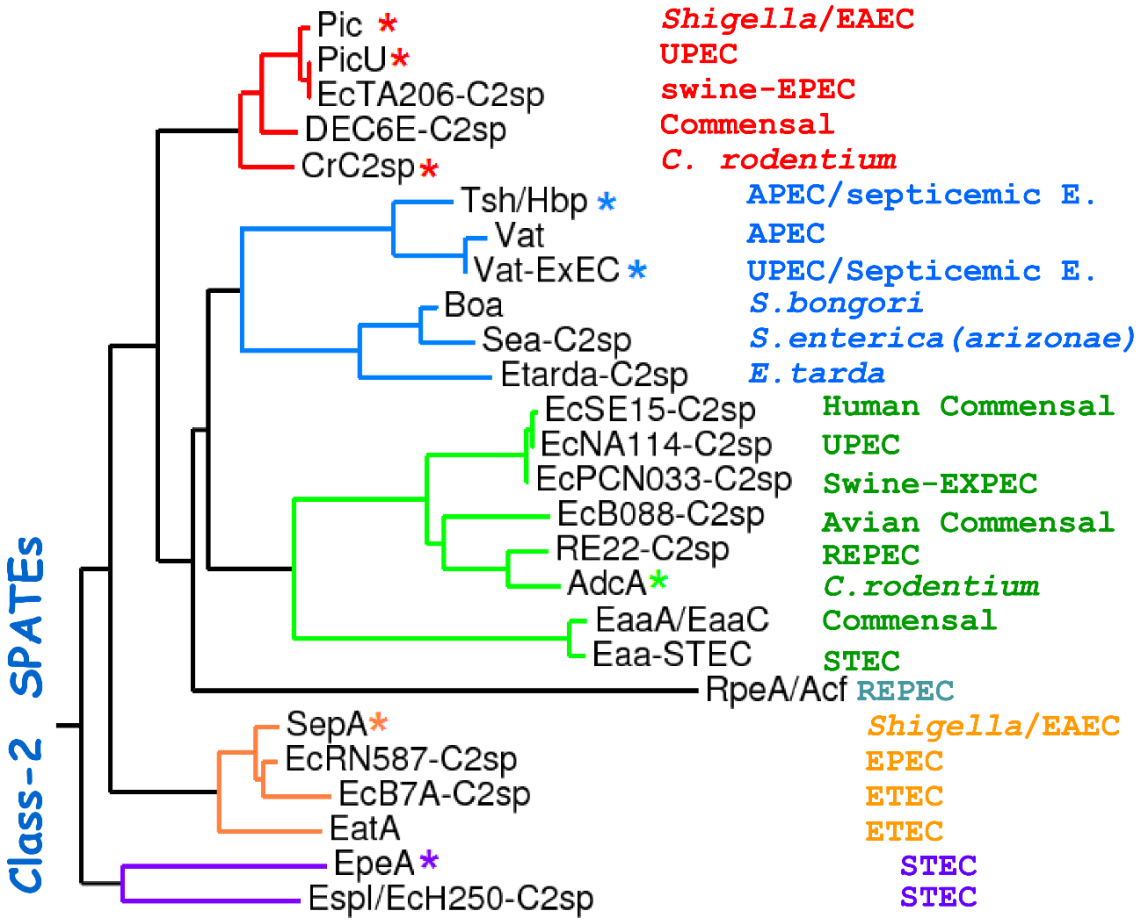

FIGURA 2: Relações de sequência de proteínas SPATE de classe 2. **FONTE:** RUIZ-PEREZ, 2013 modificado por Ayala-Lujan et. al.,2014.

2.3. *Protein Involved in Colonization (Pic)*

Pic é uma serinoprotease produzida por diversos patógenos, dentre eles: EAEC (HENDERSON et al., 1999), *Shigella. flexneri* 2a (RAJAKUMAR; SASKAWA; ADLER, 1997) EPEC (ABREU et. al., 2013; 2016) e UPEC (PARHAM et al., 2004), *E. coli* híbrida EAEC / produtora de toxina Shiga (sorotipo O104: H4) (MUNERA et. al., 2014; RASKO et. al, 2011), bem como um homólogo, PicC, foi recentemente descrito em *Citrobacter rodentium* (CELIK et.al., 2012).

Dentre as diversas atividades biológicas descritas, Pic contribui para a resistência da bactéria ao soro por um mecanismo dependente de sua atividade proteolítica (HENDERSON et al, 1999), bem como degradação do fator V da cascata de coagulação, hemaglutinação (DUTTA et al., 2002) e clivagem de glicoproteínas de

superfície de leucócitos, envolvidas no tráfico, migração e inflamação (RUIZ-PEREZ et al., 2011). Além disso, o sistema complemento, braço crucial da resposta imune inata contra patógenos bacterianos invasores, possui como principal função reconhecer e destruir as células-alvo. Entretanto, algumas bactérias desenvolveram mecanismos de evasão ao sistema complemento, com a produção de serinoproteases (ABREU et al., 2017).

Abreu et al. (2015) demonstraram que Pic produzida por EAEC medeia a evasão imune pela clivagem direta de moléculas do complemento, reduzindo significativamente a ativação do complemento por todas as 3 vias. Pic é capaz de clivar C3, uma molécula central da cascata do complemento, assim como C3b e proteínas das vias clássica e das lectinas, como C4 e C2. A degradação dos componentes do complemento foi observada com a proteína purificada e também com o sobrenadante de culturas de *E. coli* HB101, que expressam Pic. Ensaios proteolíticos adicionais utilizando soro humano como fonte de proteínas do complemento indicaram que Pic também é ativa em um meio mais fisiológico, sendo capaz de clivar C3, C4 e C2.

Até agora, várias funções *in vitro* ou *ex vivo* foram atribuídas a Pic, incluindo a atividade mucinolítica, considerada importante para os patógenos entéricos colonizarem os intestinos de seus hospedeiros, ajudando-os a penetrar na camada de muco que reveste o epitélio intestinal (GUTIÉRREZ-JIMÉNEZ et. al.,2008; HARRINGTON et al., 2009) favorecendo assim a adesão da bactéria aos enterócitos (HARRINGTON et al., 2009; MUNERA et al., 2014). Após estabelecimento da bactéria no sítio de infecção, Pic exerce um papel antagônico ao estimular células caliciformes a hiperproduzirem muco (NAVARRO-GARCIA et al., 2010). Por conseguinte, foi proposto que Pic poderia ter um papel nutricional na colonização. Especificamente, Pic pode permitir que bactérias usem muco ou mucina como fonte de nutrientes, quando outras fontes não estão disponíveis (HARRINGTON et al, 2009).

SPATEs de classe 2, como Pic, também possuem propriedades imunomoduladoras pela clivagem das moléculas O-glicosiladas encontradas na superfície das células imunes, quimiocinas e proteínas do complemento (RUIZ-PEREZ et.al.,2011; AYALA-LUJAN et. al., 2014). A atividade de Pic sobre glicoproteínas baseia-se na natureza sacarídica dos substratos. A protease apresenta reduzida atividade proteolítica sobre substratos desglicosilados, e reduzida capacidade de ligação à mucina na presença de monossacarídeos (GUTIERREZ-JIMENEZ et.al.,2008; RUIZ-PEREZ et. al., 2011).

Além disso, estudos realizados com leptospira têm mostrado que proteases produzidas por esta bactéria tem ação direta sobre moléculas da matriz extracelular (FRAGA et al., 2016; SILVA et al., 2018).

2.4. *Matriz Extracelular*

As células residem em um microambiente dinâmico dentro dos tecidos conjuntivos denominado matriz extracelular (MEC), composta por cerca de 300 proteínas, conhecidas como matrizes centrais, e inclui proteínas como colágeno, proteoglicanos (PGs) e glicoproteínas (HYNES E NABA; 2011)

A matriz extracelular não serve apenas como uma rede para estabilização da estrutura física dos tecidos, mas também possui função importante nas interações célula-célula e célula-matriz, participando deste modo da integridade dos tecidos (ROBERT-LOBAT, 2012).

Sabe-se que a MEC tem impacto na migração, diferenciação, proliferação, apoptose e adesão celular (FRANTZ et al., 2010). As células são capazes de se ligar à MEC através de receptores e proteínas específicos da superfície celular, integrinas, caderinas e selectinas. Macromoléculas na MEC são capazes de reter fatores de crescimento e outras citocinas, tornando a MEC um reservatório crucial em estágios de desenvolvimento e fisiológicos, onde podem ser liberadas no microambiente por degradação de matriz por metaloproteinases de matriz (MMPs), controlando sua acessibilidade ao microambiente (KIRKPATRICK; SELLECK, 2007; ROZARIO; DESIMONE, 2010). Sabe-se que esta função de reservatório da matriz é de grande importância em relação à influência do fenótipo celular. Células dentro da matriz secretam as moléculas de MEC, construindo assim sua própria rede de suporte externa e podem reformulá-la degradando e substituindo a matriz em torno delas (HANSEN et al, 2015). A interação com patógenos bacterianos entéricos pode ocorrer durante a inflamação ou a abertura de junções estreitas (FUJIMOTO; NAKAJIMA; YAOITA, 2007). Portanto, a ligação a proteínas da MEC pode facilitar a colonização, invasão e / ou sinalização por patógenos intestinais.

A nível celular, a MEC também fornece uma “linguagem bioquímica e mecânica” que comanda processos fundamentais como vias de sinalização celular (BOTTARO et al., 2002), forma e função celular (ALFORD; RANNELS, 2001), mudanças na organização e diferenciação do citoesqueleto (CLARK et al., 1986),

formação de aderências focais e fibras de estresse (CLARK, 1993), alterações de proliferação e migração (VOROTNIKOVA et al., 2010), estimulação da polaridade e expressão gênica (WANG et al., 2004), indução de atividade metastática (MURRAY et al., 2000), resposta a fatores de crescimento (CLARK et al., 1995) e informações que regulam a localização apropriada de contingências celulares específicas dentro da matriz (BROWN; BADYLAK, 2014). Além disso, as interações célula-matriz desempenham papéis críticos ao longo da vida: durante o desenvolvimento embrionário e a organogênese (DEQUACH et al., 2010), angiogênese (DVORAK et al., 1987), cicatrização de feridas (SERINI et al., 1998), doença e metástase (OTRANTO et al., 2012).

Dentre as principais proteínas da MEC, pode-se destacar o colágeno, a fibronectina, a elastina, a laminina, os glicosaminoglicanas e proteoglicanas.

2.4.1 *Colágeno*

Os colágenos são proteínas fibrosas sintetizadas pelos fibroblastos e células afins. Atualmente são conhecidos 27 tipos diferentes de colágenos codificados por 41 genes dispersos em pelo menos 14 cromossomos. Os tipos I, II, III, V e XI são colágenos intersticiais ou fibrilares e os mais abundantes. O tipo IV é não fibrilar (forma lâminas ao invés de fibrilas), e é o principal componente da lâmina basal, junto à laminina. O colágeno de tipo I é o mais abundante e é o predominante nas áreas de tecido fibroso denso, como no derma cutâneo, tendões, córnea e ossos. O colágeno de tipo III é predominante no tecido conjuntivo frouxo. O colágeno de tipo IV é não-fibrilar e se encontra principalmente fazendo parte das membranas basais (ANDRADE, 2003; KUMAR; ABBAS; FAUSTO, 2005).

2.4.2 *Fibronectina (FN)*

A fibronectina desempenha papel fundamental na estruturação tridimensional da MEC, fornecendo conexão para as células através de receptores, como as integrinas. Além da adesão, influencia diretamente o comportamento celular na distribuição, migração e morfologia, organização do citoesqueleto, expressão gênica e transformação oncogênica (ROZARIO; DESIMONE, 2010; SCHWARZBAUER; DESIMONE, 2011; LABAT-ROBERT, 2012; LENSELINK, 2013). É uma glicoproteína de alto peso molecular encontrada em todos os tipos de tecidos e que interage com as células em todos

os estágios do reparo de feridas, já estando presente em grande quantidade na fase de hemostasia no coágulo de fibrina-fibronectina. A fibronectina se liga à heparina, fibrina, colágeno, células e citocinas e à própria fibronectina (LENSELINK, 2013).

A fibronectina encontrada no plasma (FNp) é largamente produzida por hepatócitos e apresenta-se na forma solúvel. Outras formas de fibronectinas são produzidas por numerosas células incluindo fibroblastos, células epiteliais e macrófagos, são referidas como fibronectina celular (FNc). Uma vez secretadas, ambas FNp e FNc podem ser incorporadas à matriz extracelular. As formas de fibronectina do plasma e celular desempenham funções distintas durante a progressão da cicatrização de feridas. FNp circula no plasma sanguíneo na forma inativa e é armazenada nos grânulos de plaquetas até ser ativada em resposta a ferida e estimulação da cascata da coagulação (ROMBERG,1997; MIDWOOD; TO, 2011).

2.4.3 *Glicosaminoglicanos (GAGS)*

São polissacarídeos lineares longos, não flexíveis e com cadeias não ramificadas presentes na matriz extracelular e superfície celular da maioria dos animais, tanto em forma livre quanto ligados a proteínas. São constituídos de combinações repetidas de dissacarídeos, estes formados por unidades de açúcar aminado ligadas a ácido idurônico ou glicurônico. Já foram descritos vários tipos de glicosaminoglicanos, como a heparina, o sulfato de heparan, o ácido hialurônico, sulfato de queratan e sulfato de condroitina (VALCARCEL, et al 2017; SUGAHARA AND KITAGAWA, et al. 2002).

Os GAGs desempenham várias funções, tais como a hidratação dos espaços ao redor das células, formando géis que variam quanto ao tamanho dos poros e a densidade da carga que atua como filtro regulador da passagem de moléculas pelo meio extracelular, ligando fatores de crescimento e outras proteínas que servem como sinais para as células, com a função de bloquear, ativar ou guiar a migração celular através da matriz (KRESSE, SCHÖNHER; 2001).

2.4.4 *Proteoglicanos*

Os proteoglicanos são moléculas formadas por um eixo proteico, ao qual se ligam covalentemente cadeias laterais de glicosaminoglicanos e encontram-se presentes em grânulos citoplasmáticos, na membrana celular ou na matriz extracelular. São conhecidos por apresentarem grande afinidade com uma variedade de ligantes, incluindo

fatores de crescimento, moléculas de adesão, componentes da matriz, enzimas e inibidores de enzima (WU et. al; 2005).

Na matriz extracelular, os proteoglicanos interagem com as proteínas fibrosas. A interação com o colágeno, por exemplo, possibilita a organização das fibras de colágeno na matriz extracelular. Os GAGs também estão envolvidos na ligação de cátions (como sódio, potássio e cálcio) e na retenção de água nos tecidos, possuindo papel fundamental na hidratação da matriz extracelular e na regulação de movimentos moleculares por meio desta. Alguns proteoglicanos, presentes na membrana celular, podem estabelecer ligações com fatores de crescimento e hormônios, apresentando papel fundamental no controle do crescimento celular e servindo como sinal para as células sofrerem divisão, podendo controlar, deste modo, processos importantes como o desenvolvimento de tumores e a embriogênese (NELSON; COX, 2008).

Além da função estrutural, os proteoglicanos podem mediar funções biológicas em processos fisiológicos normais ou em doença, a exemplo de regulação da atividade de moléculas sinalizadoras (ALMEIDA et al., 2000), controle do tráfego de células e moléculas (IRVING-RODGERS et al., 2010), correceptores (MYTHREYE , BLOBE; 2009), proliferação celular (MATAVELI et al., 2009), seletividade da membrana basal (HONARDOUST et al., 2008), adesão e migração celular (RUOSLAHTI; 1989), resposta imune (SETTEMBRE et al., 2008) e interação com proteínas fibrosas da matriz, formação de complexos macromoleculares com ácido hialurônico na matriz extracelular (MICHELACCI et al., 1979).

2.4.5 *Metaloproteinases de matriz (MMPs)*

São as principais enzimas envolvidas na degradação da MEC. Sua atividade é baixa em condições normais, mas aumenta durante os processos de reparo ou remodelação e em tecido doente ou inflamado. As MMPs são produzidas como proteinases solúveis ou ancoradas à membrana celular e clivam componentes da MEC com ampla especificidade de substrato (BONNANS; CHOU AND WERB, 2014)

A ativação de MMP ocorre primariamente via clivagem proteolítica ou por modificação do grupo tiol por oxidação. Coletivamente, as MMPs podem degradar todas as proteínas da MEC e suas ações proteolíticas na MEC têm papéis cruciais na organogênese (LU, TAKAI, WEAVER, WERB; 2011). Além disso, as MMPs são capazes de processar e degradar diversos componentes da cascata de coagulação.

2.5. Cascata de coagulação sanguínea

O fluxo sanguíneo adequado resulta da interação equilibrada entre a homeostase e a fibrinólise. A homeostase depende da interação entre a parede do vaso (vasoconstrição), agregação plaquetária e coagulação, e baseia-se numa cascata de reações proteolíticas sequenciais realizadas por proteases de serina, cuja atividade enzimática é fortemente regulada. A fibrinólise envolve a degradação proteolítica da fibrina por uma serino protease, a plasmina, para prevenir a formação excessiva de coágulos e a subsequente trombose (BORISSOFF et al., 2011; RAU et al., 2007).

Atualmente, o novo conceito da coagulação é descrito como um processo complexo baseado no modelo de superfícies celulares na hemostasia e permite um melhor entendimento dos problemas clínicos observados em alguns distúrbios da coagulação, por enfatizar o papel central de superfícies celulares especificas no controle e direcionamento dos processos hemostáticos (SILVA & MELO, 2016), dividindo-se em três fases distintas mas interligadas entre si: iniciação, amplificação e propagação (RODRIGUES et al., 2012).

A fase de iniciação, classicamente denominada via extrínseca, ocorre nas células que expressam fator tecidual (FT) na sua superfície. Estas células revestem os vasos sanguíneos e após lesão vascular ligam-se ao fator VII presente no plasma dando início ao processo de coagulação. O complexo FT/FVIIa promove a ativação dos fatores IX e X, tornando-os Xa e fator IXa. O fator Xa localizado na superfície celular liga-se ao fator Va e forma o complexo protrombinase FXa/FVa que converte pequenas quantidades de protrombina em trombina. Além disso, o fator IXa gerado pode-se deslocar para outra célula ou para a superfície das plaquetas tornando-as ativas e assim iniciar a fase de amplificação da coagulação (Figura 3).

Na etapa de amplificação a trombina formada na fase de iniciação proporciona a ativação de mais plaquetas, o que contribui para fomentar a adesão plaquetária. Outra função da trombina é a liberação e a ativação do fator VIII que, na sua forma inativa, se encontra ligado ao fator de Von Willebrand (FvW) (tem um papel importante na adesão plaquetária), e a ativação dos fatores V e XI na superfície plaquetária. Desta forma, após a ativação dos fatores da superfície das plaquetas segue-se a fase da propagação.

A fase de propagação classicamente denominada via intrínseca, ocorre nas plaquetas ativadas que são recrutadas para o sítio da lesão. O fator IXa ativado durante a

fase de iniciação associa-se ao fator VIIIa na superfície das plaquetas dando origem ao complexo tenase que por sua vez é capaz de ativar maior quantidade de fator Xa. O fator Xa une-se ao cofator Va ligado à superfície das plaquetas na fase de amplificação, o que resulta na formação do complexo protrombinase, que converte a protrombina em trombina. A trombina é responsável pela clivagem do fibrinogênio em monômeros de fibrina, que dá estabilidade ao tampão plaquetário inicial (SILVA & MELO, 2016).

Assim, forma-se um coágulo sólido, que, posteriormente é lisado pelo sistema fibrinolítico. Neste processo designado de fibrinólise participam o plasminogênio e o ativador tecidual do plasminogênio (t-PA), protease que transforma o plasminogênio em plasmina. Por ser uma enzima proteolítica de amplo espectro, a plasmina digere fibrina, fibrinogênio, e a maioria dos fatores e co-fatores de coagulação. Deste modo, o local lesado acaba por ser reparado e restabelece-se o fluxo sanguíneo normal (Figura 4) (RODRIGUES et al., 2012).

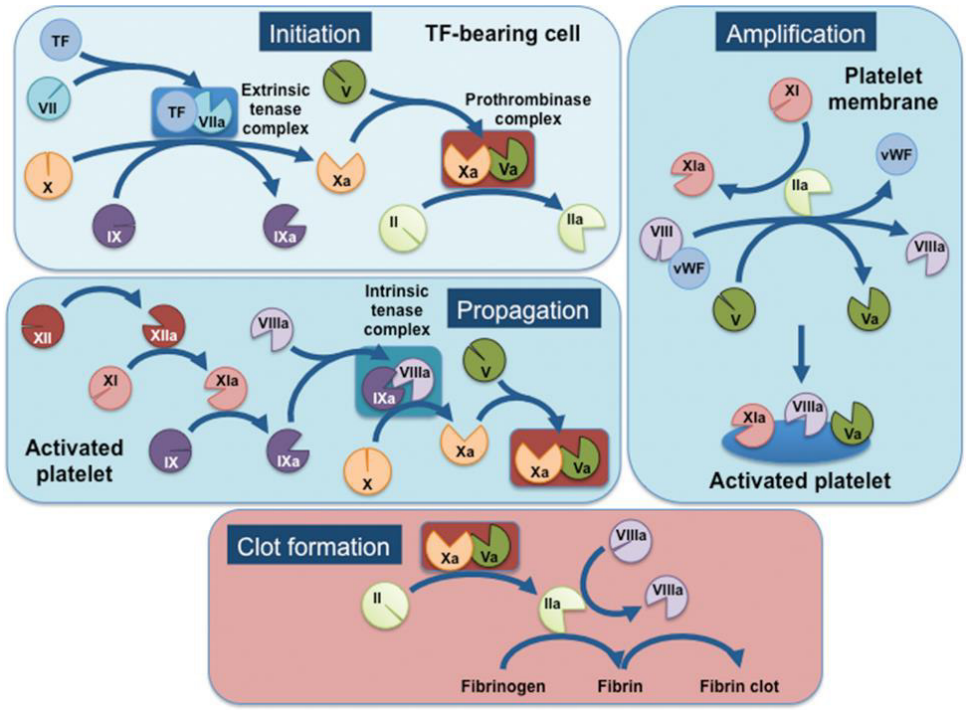

FIGURA 3: Visão geral esquemática da cascata de coagulação do sangue. O modelo é dividido em fases de iniciação, propagação, amplificação e formação de coágulos. Fatores de coagulação do sangue são indicados em algarismos romanos, e as formas ativas são indicadas por um pequeno 'a'. II indica protrombina e trombina IIa. vWF, fator von Willebrand. **FONTE:** SPONK et. al; 2014 (modificado).

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar a ação serinoprotease Pic sobre componentes da matriz extracelular e cascata de coagulação sanguínea.

3.2. Objetivos Específicos

- Avaliar o efeito da proteína Pic sobre proteínas de matriz extracelular;
- Analisar o efeito de Pic sobre moléculas da cascata de coagulação;
- Avaliar se o plasminogênio ligado à Pic é convertido em sua forma ativa, plasmina.

4. CAPÍTULO I

AÇÃO DA SERINOPROTEASE PIC PRODUZIDA POR Escherichia coli SOBRE PROTEÍNAS DA MATRIZ EXTRACELULAR E DA CASCATA DE COAGULAÇÃO SANGUÍNEA

ACTION OF SERINOPROTEASE PIC PRODUCED BY Escherichia coli ON EXTRACELLULAR MATRIX AND BLOOD CELL CASCADE PROTEINS

Janaína Vasconcelos GOMES 1, Afonso Gomes Abreu Júnior 1.

1 Postgraduate Program in Health Sciences, Federal University of Maranhão, São Luís, Maranhão, Brazil.

Correspondence to: Janaína Vasconcelos Gomes, Federal University of Maranhão, Postgraduate Program in Health Sciences, Av. Dos Portugueses, S / N - Bacanga University Campus, CEP: 65085-580, São Luís, Maranhão, Brazil.
Email: janaina.v.gomes@gmail.com

ABSTRACT

Serinoprotease Pic (protein involved in colonization), protein identified in enteroaggregative E. coli, Shigella flexneri and Citrobacter rodentium. Among the biological papers, hemagglutination, mucinolytic activity, degradation of factor V of the coagulation cascade and cleavage of surface glycoproteins of leucocytes involved in trafficking, migration and inflammation were described. We demonstrate their performance in the human complement system conferring to these bacteria the ability to circumvent the defense mechanisms of the innate immune system favoring the development and maintenance of sepsis. The objective of this study was to investigate the action of Pic on components of the extracellular matrix and the blood coagulation cascade. For this, concentrated fractions of Pic (HB101 / Pic), non-Pic (HB101) and BSA-producing E. coli culture supernatants were incubated at different times with several molecules of the coagulation cascade and the extracellular matrix to evaluate possible as degradation of these components. We performed plasminogen activation in plasmin, an important molecule in the formation of clots and in the activation of other pathways of the immune system. We observed that HB101 / Pic bound to several extracellular matrix molecules (collagens type I and IV, laminin and fibronectin). By incubating Pic with blood coagulation molecules, binding and cleavage of plasminogen was found, which was converted to its active form, plasmin, in the presence of the exogenous activator. The other components of both cascade and extracellular matrix were not cleaved by Pic. We believe that Pic-producing E. coli binds to the extracellular matrix through several components, facilitating the infectious process in the host. The plasmin generated in the presence of Pic should generate a synergistic effect on the degradation of molecules in the complement system, as well as for deregulated activation of the blood coagulation cascade.

Keywords: Pic; extracellular matrix, coagulation cascade, plasmin, plasminogen.

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Introduction

Escherichia coli is a commensal bacterium, some pathogenic strains can cause diarrhea or extra-intestinal diseases in healthy and immunocompromised individuals, which generates a serious public health problem since diarrheal diseases are a major cause of morbidity and mortality in children under five years of age[1,2].

The pathogenic strains of E. coli are usually associated with intestinal infection, being called diarrheogenic *E. coli* (DEC). When colonizing other anatomical sites are called extra-intestinal E. coli (ExPEC) and can lead to infections of the urinary tract, central nervous system and bloodstream [1]. In addition, the bacterium is also associated with infections of the bloodstream, which causes high rates of morbidity / mortality and high costs in health care. Some *E. coli* are producers of proteins called SPATEs (Serine protease autotransporter Enterobacteriaceae), which constitute a superfamily of proteins whose members resemble those belonging to the trypsin-like superfamily of serine proteases [3,4]. The secretion of these serine proteases can cause damage to the host, such as the cleavage of key molecules of the complement system, the blood coagulation cascade and various extracellular matrix proteins, leading to an inefficient response of the host to the action of microorganisms producing these proteases.

Protein involved in colonization is an example of such SPATEs. It is produced and secreted by *E. coli, Shigella flexneri 2a and Citrobacter rodentium* [5-7] and performs several biological roles including haemagglutination, mucinolytic activity, degradation of the factor V of the coagulation cascade and cleavage of leukocyte surface glycoproteins, which are involved in trafficking, migration and inflammation [6,8-12]. Due to its mucinolytic activity, Pic also promotes the intestinal colonization of rats and rabbits by the cleavage of mucus present in the intestinal lumen, thus favoring the adhesion of the bacterium to enterocytes [12-14]. After establishing the bacterium at the infection site, Pic plays an antagonistic role by stimulating the goblet cells to hyperproduce mucus [15], which contributes to a persistent colonization, since it provides a source of nutrients for bacterial growth .

Recently our research group has shown that Pic also reduces the activity of the complement system by the inactivation of the three pathways (classical, alternative and lectin pathways) [16]. In addition, several pathogens express a series of membrane proteins capable of interacting with extracellular matrix, coagulation cascade and host cell molecules.

The extracellular matrix (ECM) is a three-dimensional non-cellular macromolecular network that provides structural and biochemical support to the surrounding cells. In the present work, it is shown that ECM proteins can be present on the surface of the intestinal cells as bacterial receptors [17-19]. such as fibronectin which was the first protein described to act as a cellular receptor for bacterial pathogens [20]. In addition, molecules generated in the blood coagulation cascade may interact with MEC proteins.

The blood coagulation cascade is activated by the proteolytic action of proenzymes by plasma proteases, resulting in the formation of thrombin, which then breaks the fibrinogen molecule into fibrin monomers [21]. In this process called fibrinolysis, plasminogen and the tissue activator of plasminogen are involved, a protease that transforms plasminogen into plasmin. Bacterial proteins are also capable of interacting with plasminogen and activating it on its surface. Thus, the plasmin generated can degrade components of the extracellular matrix, fibrin, fibrinogen, and coagulation factors and cofactors (factor V and factor VIII) [22,23].

Thus, due to this ability of the Pic protein to degrade connective tissue molecules, contributing to the processes of invasion and tissue destruction observed during infection by these bacteria. In this way, the objective of this work was to evaluate the role of Pic on molecules of the blood coagulation cascade and the extracellular matrix.

Material and methods

Bacterial samples

In this study, a recombinant Pic-producing strain (HB101 / pPic) HENDERSON [6] and the non-pathogenic strain E. coli HB101 were used. All samples are kept at -80øC in trypticase soy broth (TSB) plus 20% glycerol and grown in Luria-Bertani broth (LB), LB agar or MacConkey, plus appropriate antibiotics when indicated.

Obtaining Protein Pic

The Pic protein was obtained according to a protocol previously described by Navarro-Garcia et al (2010). For this, strain HB101 (pPic) was grown in LB broth with stirring at 250 rpm at 37 ° C for 18h. Thereafter, the bacterial culture was centrifuged at 4,000 rpm for 15 min at 4 ° C. The supernatant obtained was membrane-filtered at 0.22 μm and concentrated using a 100 kDa cut off membrane filter concentrator (Millipore, Bedford, MA, USA). Samples prepared as described above were analyzed by SDS-PAGE under denaturing conditions, according to the protocol described by Laemmli [24]. Protein extracts were mixed to the running buffer (tris-hydroxymethyl) aminomethane 0.25 M hydrochloride; sodium dodecyl sulfate 8%; glycerol 80%; bromophenol blue 0.04%; pH 6.8) plus β-mercaptoethanol (Sigma-Aldrich). Electrophoresis at 20 mA (Power Pac 300 model source, BioRad) was carried out in specific apparatus (Bio-Rad, Berkeley, CA, USA) containing Tris-Glycine buffer (3% tris (hydroxymethyl) aminomethane; sodium dodecyl sulfate 1%). A molecular weight standard was used with bands of 170 to 10 kDa (PageRuler ™ Prestained Protein Ladder) (Fermentas, Burlington, ON, Canada). After the run the gel was stained with Comassie Brilliant Blue (Bio-Rad) dye for about 1 h with gentle shaking. After staining, the gel was incubated with bleach solution (45% methanol, 10% glacial acetic acid) with gentle stirring until the bands corresponding to the proteins could be visualized.

Pic binding to extracellular matrix components and blood coagulation cascade

After obtaining the protein, Pic binding with extracellular matrix macromolecules was analyzed according to a protocol described by Cameron [25], with some modifications. ELISA plates (Nunc-Immuno Plate, MaxiSorp surface) were sensitized with 1 μg of MEC components (laminin, decorin, collagens type I and IV, plasma fibronectin) or components of the coagulation cascade (plasminogen and fibrinogen and fibrin) or BSA in 100 μl of PBS for 2 h at 37 ° C. Then the wells were washed three times with PBS-0.05% Tween-20 (PBS-T) and then blocked with 200 μl of 1% BSA for 1 h at 37 ° C, followed by overnight incubation at 4 ° C. After incubation, 10 μl of concentrated HB101 / Pic culture supernatant fractions in 100 μl PBS were added in each well for a period of 1 h 30 min at 37 ° C to verify a possible interaction with the different substrates. After washing with PBS-T, the bound proteins were incubated for 1 h at 37 ° C with a suitable dilution of the specific antibody. After washing with PBS-T, a further incubation for 1 h at 37øC was performed with rabbit IgG secondary antibody raised in goat (1: 5000) and conjugated with peroxidase. The wells were washed three times and a solution containing orthophenylenediamine (OPD) (0.04%) in citrate / phosphate buffer (pH 5.0) plus H2O2 (0.01%) was added to the reaction. It was incubated for 15 min and then quenched with the addition of 50 μl of 8 M H 2 SO 4. The absorbance at 492 nm was determined on a microplate reader (Multiskan EX; Labsystems Uniscience).

Evaluation of the proteolytic activity of Pic against extracellular matrix components and the blood coagulation cascade

In this assay cultures of Pic or non-producing bacteria were used. Aliquots of the secreted bacteria or the purified protein were incubated with 5 μg of MEC components (laminin, plasma fibronectin and collagen type I and IV) or components of the coagulation cascade (plasminogen and fibrinogen), commercially acquired. Incubations were performed at 37 ° C at different time periods (1, 4 and 18 h) in buffer containing 85 mM NaCl for 2 h. Samples were heated at 100 ° C for 3 min with sample buffer and the cleavage products were analyzed by SDS-PAGE. The gels were stained with bright Coomassie blue. Alternatively, for better visualization of the fragments, the proteins were transferred from the gel to nitrocellulose membranes and then stained with Coomassie.

Evaluation of the plasminogen-linked capacity of Pic to generate plasmin

Concentrated fractions of E. coli HB101 / Pic, HB101 and BSA culture supernatants were immobilized in 96-well plates (1 μg / well) and incubated for 16-20 h at 4 ° C. Between each subsequent step 3 washes were made with PBS-T. Non-specific binding sites were blocked using 3% BSA for 2 h at 37øC. After blocking, the plates were incubated for 1 h with purified plasminogen (2 μg / well). Then, 3U of

Plasminogen Activator Urokinase (uPA) and 250 ng of the plasmin-specific substrate (D-Val-Leu-Lys p-nitroanilide dihydrochloride) were added in a final volume of 100 µL. An incubation with plasminogen + uPA + plasminogen activator inhibitor 1 (PAI-1) was used as a negative control. Absorbance readings were taken at 405 nm after 24 h.

Statistical analysis

All assays were performed in triplicate at least once. They were expressed in mean and standard deviation and were submitted to the T test and ANOVA using the Graph Pad Prism version 6.0 software.

Results

Pic binds to extracellular matrix components

In this study it was possible to observe that the Pic protein was able to bind significantly to several components of the extracellular matrix, such as collagens type I and IV, laminin, different from that observed with the concentrated fractions of the culture supernatant of strain HB101. On the other hand, Pic did not bind to decorin, as can be observed in figure 1.

Pic binds to plasma fibronectin a and to plasminogen, components of the blood coagulation cascade

When analyzing the interaction of Pic with components of the coagulation cascade, it was not possible to observe protein binding with fibrin and fibrinogen. However, it was able to bind plasma fibronectin and plasminogen (Figure 2).

The Pic protein binds but does not promote cleavage of most of the matrix components and the blood clotting cascade

After this analysis, the HB101 / Pic culture supernatant was incubated with the same molecules at different times to verify a possible proteolytic action on these components. For this, Pic was incubated with some components of the extracellular matrix or the coagulation cascade at different times. After analysis of the cleavage products by SDS-PAGE, followed by staining with Comassie Blue, it was possible to observe that Pic was not able to cleave laminin, plasma fibronectin nor fibrinogen. However, it was possible to observe a discrete cleavage of plasminogen in the first hour of incubation, generating a fragment with sizes varying from ~ 39 to 65 kDa (Figure 3).

Pic on binding to plasminogen generates active plasmin

Given that Pic binds to plasminogen and that this molecule is responsible for the generation of plasmin, an assay was performed to see if the plasminogen attached to Pic could be converted to plasmin. For this, plasminogen was added to Pic and subsequently the exogenous activator uPA and a specific substrate for plasmin (D-Val-Leu-Lys-p-nitroanilide dihydrochloride) were added. After 24 h, the generation of plasmin was detected, confirmed by cleavage of the chromogenic substrate, when Pic was used. No cleavage of the substrate was observed in the presence of the negative control BSA (Figure 4).

Discussion

Proteases secreted by pathogens constitute an important class of virulence factors, performing functions related to invasion, immune evasion, nutrition and reproduction of certain parasites and the degradation of components of the extracellular matrix favors the propagation and dissemination of microorganisms in the host [26]. In this study, we analyzed the action of Pic serinoprotease against some components of the extracellular matrix and the blood clotting cascade.

Some microorganisms are capable of degrading ECM components through the production or "sequestering" of host proteases [27]. Plasminogen is an example of host protease that is capable of degrading ECM components and several pathogens have their potential for invasion and increased spread through plasminogen binding [27-30]. Cell adhesion proteins, such as fibronectin and laminin, connect the elements of the matrix to each other and to the cells. These binding proteins act as transmembrane receptors or are stored in the cytoplasm, where they perform specific functions: they promote cell-cell and cell-ECM interaction, participate in the formation and maintenance of neoformed blood vessels, assist in stabilizing the clot and promote the binding of cells to the cytoskeleton, enabling physical forces to be translated into biomolecular responses such as activation or inhibition of growth factors, cell proliferation and differentiation, collagen synthesis and wound contraction [31].

In this study it was possible to show that Pic binds to several components of ECM, such as type I and IV collagens, plasma fibronectin and laminin. Farfan; Inman and Nataro [32] suggest that ECM proteins may be receptors for EAEC, which binds to various proteins present in the intestinal epithelium, such as fibronectin, laminin and type IV collagen.

It has been recognized for some time that fibronectin plays a role in adhesion of EAEC 042 to epithelial cells through interaction with AAF / II fimbriae, the main virulence factor of EAEC known to be involved in both adhesion and inflammation [32-34].

Yanes et. al [20] have demonstrated that fibronectin plays the role of a bridging bond, connecting the bacterium to the surface of the epithelial cell and suggesting that fibronectin-mediated binding is not required for the activation of intracellular signaling cascades involved in the inflammatory response against infection by EAEC.

Interaction with enteric bacterial pathogens may occur during inflammation or opening of narrow junctions [18] Thus, the binding to ECM proteins can facilitate colonization, invasion and / or signaling by intestinal pathogens [17, 19,35] and directly affect blood coagulation.

Coagulation cascade proteins such as fibrinogen and plasminogen were evaluated in our study and it was possible to show that Pic binds to plasminogen and promotes cleavage of it. It is noteworthy that both fibrinogen and plasminogen participate in the process of hemostasis in the procoagulant, anticoagulant functions and fibrinolysis.

The anticoagulant function occurs first, by the presence of the cellular lining itself that prevents contact between the circulating platelets and the subendothelium. These endothelial cells also secrete several other substances that perform anticoagulant activity, among them: heparan sulfate; thrombomodulin, prostacyclin (PGI2), protein C, plasminogen activators, tissue factor inhibitor. They also secrete procoagulant substances, such as: Von Willebrand factor, fibronectin, tissue factor, plasminogen activator inhibitor (PAI) [36].

In fibrinolysis, fibrin degradation is important in that it avoids excessive clot growth, which may lead to vessel occlusion. In the process of fibrin degradation, serine proteases, such as plasmin, are responsible for the cleavage of fibrin. Plasminogen is converted into plasmin by the action of urokinase plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). Plasmin cleaves fibrin by generating the fibrin degradation product (PDF). The inhibitor of plasminogen activator-1 (PAI-1) inhibits t-PA and u-PA, with α2-antiplasmin (α2-AP) being the direct plasmin inhibitor [21, 37-40]. However, plasmin, which results from the cleavage of plasminogen, also acts on other extracellular matrix components such as collagen IV and V, laminin, fibronectin, vitronectin, among others [29].

Thus, in bacterial infections fibrin deposits are formed, and these clots can interfere in the mobility of the bacterium preventing their spread through different tissues. Bacteria with the ability to generate plasmin on their surface can cause fibrinolysis, preventing the formation of fibrin clots and allowing the release of bacteria [41]. Bacteria also utilize the proteolytic abilities of plasmin to promote remodeling of the cell matrix by generating new sites of interaction, combining effector molecules of the host immune system, such as the C3 and C5 proteins that are part of the complement system and activate matrix metalloproteinases help in the destruction of tissue barriers [42]. Like plasmin, Pic is a serine protease capable of cleaving components of the complement system [16]. Thus, Pic in addition to inactivating the three-way complement system, promotes the formation of plasmin. Thus, these findings suggest that Pic may enhance cleavage on complement molecules from the plasmin generated. This has already been

described in works with leptospires, where they use plasmin in its active form through its membrane proteins LigA and LigB [43].

Several pathogens, such as group *A streptococci, Staphylococcus aureus and Yersinia pestis*, generate their own plasminogen activator [44]. In the case of *Y. pestis, Borrelia burgdorferi* and group A streptococci, the interaction with plasminogen has a direct effect on the invasiveness within the host [45-47]. The plasmin generated degrades a series of substrates important for the invasion and evasion of the bacteria [48,49]. It is a proteolytic enzyme that digests fibrin and some other coagulant proteins, such as fibrinogen, factor V, factor VIII, prothrombin, and factor XII, removing minute remnants of the blood clot that could obstruct blood flow [50].

Recently our group showed that Pic, produced by an E. coli F5 isolated from a case of bacteremia, was able to protect the bacteria from the bactericidal action of the immune system in the bloodstream, helping the bacteria to induce a sepsis in animals, leading to them to death in less than 12 hours. Samet et al [51] reports that direct translocation of intestinal E. coli into the blood results in bacteremia. Kuo et al. [52] in their study using EspP, a serine protease produced by EHEC O157: H7, show that EspP alters hemostasis in vitro by decreasing the activities of coagulation factors V, VII, VIII and XII, and prothrombin, reducing the strength of clot and accelerating fibrinolysis.

During sepsis, coagulation activation is an integral part of the host's response to pathogen invasion, as well as the inflammatory response, leading to disseminated intravascular coagulation (DIC) [53-56].

The main pathophysiological mechanisms of CID are activation initiated by inflammatory cytokines dependent on tissue factor coagulation, insufficient control of anticoagulant pathways, and suppression of fibrinolysis mediated by inhibitor 1 of the plasminogen activator inhibitor [57]. Therefore, coagulation, fibrinolysis, calcicrein-kinin system, complement pathways and inflammatory processes are under intense regulation [58,59].

It should be noted that the Pic protein induces the generation of plasmin and that both have direct action in the complement system and in the blood coagulation cascade. We therefore conclude that the Pic protein was able to bind to several extracellular matrix components, such as laminin, plasma fibronectin and type I and IV collagens, and could therefore contribute to the infectious process in the host and that Pic was unable to to bind or even to cleave the fibrinogen, a component of the coagulation cascade, but was able to cleave the plasminogen in turn bound to Pic is converted into its active form, plasmin, in the presence of the exogenous activator uPA. Thus, the generation of plasmin may intensify the cleavage of components of the complement system, in addition to resulting in aggravation of many clinical manifestations of disseminated intravascular coagulation, as well as intensify the process of sepsis mediated by the Pic protein.

Referencias

1- Croxen M A, Law R. J., Scholz R, Keeney K M, Wlodarska M, Finlay B B (2013) Recent advances in understanding enteric pathogenic Escherichia coli. Clin. Microbiol. Rev. 26, 822–880. . http://dx.doi.org/10.1128/cmr.00022-13

2- Gomes T A., Elias W P., Scaletsky I C., Guth B E., Rodrigues J F., Piazza R M., Martinez M B (2016) Diarrheagenic Escherichia coli. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology], 47 Suppl 1(Suppl 1), 3–30. http://dx.doi.org/10.1016/j.bjm.2016.10.015.

3- Yen Y T., Kostakioti M, Henderson I R, Stathopoulos C (2008). Common themes and variations in serine protease autotransporters. *Trends Microbiol.* 16:370-379. http://dx.doi.org/10.1016/j.tim.2008.05.003.

4- Ruiz-Perez F. & Nataro. (2014) Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence .J.P. Cell. Mol. Life Sci. 71: 745. https://doi.org/10.1007/s00018-013-1355-8

5- Rajakumar K, Sasakawa C, Adle B (1997) Use of a novel approach, termed island probing, identifies the Shigella flexneri she pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins. *Infection and immunity*, *65*(11), 4606–4614.

6- Henderson I R, Czeczulin J, Eslava C, Noriega F, & Nataro J P (1999) Characterization of pic, a secreted protease of Shigella flexneri and enteroaggregative Escherichia coli. *Infection and immunity*, *67*(11), 5587–5596.

7- Bhullar K, Zarepour M., Yu H., Yang H., Croxen M., Stahl M, Vallance B A (2015) The Serine Protease Autotransporter Pic Modulates Citrobacter rodentium Pathogenesis and Its Innate Recognition by the Host. *Infection and immunity*, *83*(7), 2636–2650. http://dx.doi.org/10.1128/iai.00025-15

8- Dutta P R, Cappello R, Navarro-García F, Nataro J P (2002) Functional comparison of serine protease autotransporters of enterobacteriaceae. *Infection and immunity*, *70*(12), 7105–7113. http://dx.doi.org/10.1128/iai.70.12.7105-7113.2002.

9- Parham N J, Srinivasan U ,Desvaux M, Foxman B, Marrs C F,Henderson I R (2004) PicU, a second serine protease autotransporter of uropathogenic *Escherichia coli*. FEMS Microbiology Letters, 230: 73-83. http://dx.doi.org/10.1016/s0378-1097(03)00862-0.

10- Gutiérrez-Jiménez J, Arciniega I, Navarro-García F (2008) The serine protease motif of Pic mediates a dose-dependent mucolytic activity after binding to sugar constituents of the mucin substrate. Microb Pathog, 45:115-23; http://dx.doi.org/10.1016/j.micpath.2008.04.006

11- RuizPerez F, Wahid R, Faherty CS, Kolappaswamy K, Rodriguez L, Santiago A, Murphy E, CrossA, Sztein MB, Nataro JP (2011) Serine protease autotransporters from Shigella flexneri and pathogenic Escherichia coli target a broad range of leukocyte glycoproteins. Proc Natl Acad Sci USA, 108:12881-6; http://dx.doi.org/10.1073/pnas.1101006108

12- Abreu A G, Abe C M, Nunes K O, Moraes C T, Chavez-Dueñas L, Navarro-Garcia F, Elias W P (2016) The serine protease Pic as a virulence factor of atypical enteropathogenic Escherichia coli. *Gut microbes*, *7*(2), 115–125. http://dx.doi.org/10.1080/19490976.2015.136775.

13- Harrington S M, Sheikh J, Henderson I R, Ruiz-Perez F, Cohen P S, & Nataro J P (2009) The Pic protease of enteroaggregative Escherichia coli promotes intestinal colonization and growth in the presence of mucin. *Infection and immunity*, *77*(6), 2465–2473. http://dx.doi.org/10.1128/iai.01494-08.

14- Munera D, Ritchie JM, Hatzios SK, Bronson R, Fang G, Schadt EE, Davis BM, Waldor MK.(2014) Autotransporters but not pAA are critical for rabbit colonization by Shiga toxin-producing Escherichia coli O104:H4. Nat Commun; 5:3080; http://dx.doi.org/10.1038/ncomms4080

15- Navarro-Garcia F, Gutierrez-Jimenez J, Garcia-Tovar C, Castro L A, Salazar-Gonzalez H, & Cordova V (2010) Pic, an autotransporter protein secreted by different pathogens in the Enterobacteriaceae family, is a potent mucus secretagogue. *Infection and immunity*, *78*(10), 4101–4109. http://dx.doi.org/10.1128/iai.00523-10.

16- Abreu AG, Fraga TR, Martinez APG, Kondo MY, Juliano MA, Juliano L, Navarro-Garcia F, Isaac L, Barbosa AS, Elias WP (2015)The serine protease Pic from enteroaggregative Escherichia coli mediates immune evasion by the direct cleavage of complement proteins. J Infect Dis; 212:106-15; PMID:25583166; http://dx.doi.org/10.1093/infdis/jiv013
17- Westerlund B, & Korhonen T K (1993) Bacterial proteins binding to the mammalian extracellular-matrix. *Molecular Microbiology*, *9*, 687-694. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01729.x.
18- Walia B, Castaneda F E, Wang L, Kolachala V L, Bajaj R, Roman, J., … Sitaraman, S. V. (2004). Polarized fibronectin secretion induced by adenosine regulates bacterial-epithelial interaction in human intestinal epithelial cells. *The Biochemical journal*, *382*(Pt 2), 589–596. http://dx.doi.org/10.1042/bj20040021
19- Konkel M E. et al. (2005) Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from Campylobacter jejuni. Molecular Microbiology, v. 24, p.953-963. http://dx.doi.org/10.1046/j.1365-2958.1997.4031771.x
20- Yáñez D., Izquierdo M, Ruiz-Perez F, Nataro J P, Girón J A, Vidal R M & Farfan M J (2016) The Role of Fibronectin in the Adherence and Inflammatory Response Induced by Enteroaggregative *Escherichia coli* on Epithelial Cells. *Frontiers in cellular and infection microbiology*, *6*, 166. http://dx.doi.org/10.3389/fcimb.2016.00166.
21- Franco R F (2001). Fisiologia da coagulação, anticoagulação e fibrinólise. Medicina (Ribeirão Preto).34(3/4):229-3.
22- Bajzar L (2000) Thrombin activatable fibrinolysis inhibitor and na antifibrinolytic pathway. Arterioscler Thromb Vasc Biol 20: 2511- 518. http:// dx.doi.org/ 10.1161/01.ATV.20.12.2511.
23- Rodrigues E S, Castilho-Fernandes A, Covas D T, & Fontes A M (2012) Novos conceitos sobre a fisiologia da hemostasia. *Revista da Universidade Vale do Rio Verde*, *10*(1), 218-233. http://dx.doi.org/10.5892/ruvrv.2012.101.218233.
24- Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, v.227, p.680-685
25- Cameron C E (2003) Identification of a Treponema pallidum Laminin-Binding Protein. Infection And Immunity, [s.l.], v. 71, n. 5, p.2525-2533. American Society for Microbiology. http://dx.doi.org/10.1128/iai.71.5.2525-2533.2003.
26- Armstrong P B (2006). Proteases and protease inhibitors: a balance of activities in host–pathogen interaction. Immunobiology, [s.l.], v. 211, n. 4, p.263-281,. Elsevier BV. hpp://dx.doi.org/10.1016/j.imbio.2006.01.002.
27- Singh B et al.(2012) Human pathogens utilize host extracellular matrix proteins laminin and collagen for adhesion and invasion of the host. Fems Microbiology Reviews, [s.l.], v. 36, n. 6, p.1122-1180. Oxford University Press (OUP). http://dx.doi.org/10.1111/j.1574-6976.2012.00340.x.
28- Bergmann S & Hammerschmidt S (2007) Fibrinolysis and host response in bacterial infections.Thrombosis and haemostasis, v. 98, n 3, p 512-20, 2007.
29- Bhattacharya S, Ploplis V A, CASTELLINO F J (2012) Bacterial Plasminogen Receptors Utilize Host Plasminogen System for Effective Invasion and Dissemination. Journal Of Biomedicine And Biotechnology, [s.l.], p.1-19, Hindawi Limited. http://dx.doi.org/10.1155/2012/482096
30- Nomura M (2012) Plasminogen Activation by Lactic Acid Bacteria. Bioscience, Biotechnology, And Biochemistry, [s.l.], v. 76, n. 8, p.1459-1462, Informa UK Limited. http://dx.doi.org/10.1271/bbb.120121.
31- Widgerow A D (2011) Cellular/extracellular matrix cross-talk in scar evolution and control. Wound Repair And Regeneration, [s.l.], v. 19, n. 2, p.117-133. Wiley. http://dx.doi.org/10.1111/j.1524-475x.2010.00662.x.
32- Farfan M J; Inman K G, NATARO J P (2008) The Major Pilin Subunit of the AAF/II Fimbriae from Enteroaggregative Escherichia coli Mediates Binding to Extracellular Matrix Proteins. Infection And Immunity, [s.l.], v. 76, n. 10, p.4378-4384. http://dx.doi.org/10.1128/iai.00439-08.
33- Konar M et al.(2012) Identification of key proteins of cultured human intestinal cells involved in interaction with enteroaggregativeEscherichia coli. Fems Immunology & Medical Microbiology, [s.l.], v. 66, n. 2, p.177-190. Oxford University Press (OUP). http://dx.doi.org/10.1111/j.1574-695x.2012.00998.x.
34- Izquierdo M et al. (2014) Participation of Integrinα5β1 in the Fibronectin-Mediated Adherence of EnteroaggregativeEscherichia colito Intestinal Cells. Biomed Research International, [s.l.]. Hindawi Limited. http://dx.doi.org/10.1155/2014/781246.

35- Dorsey, CW. et al. (2005) Salmonella enterica serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin. Molecular Microbiology, v. 57, p.196-211. http://dx.doi.org/10.1111/j.1365-2958.2005.04666.x
36- Ferreira C N et al. (2010) O novo modelo da cascata de coagulação baseado nas superfícies celulares e suas implicações. Revista Brasileira de Hematologia e Hemoterapia, [s.l.], v. 32, n. 5, p.416-421. Elsevier BV. http://dx.doi.org/10.1590/s1516-84842010000500016.
37- Zhang Y, Cui J, Zhang R, Wang Y, Hong M (2007) A novel fibrinolytic serine protease from the polychaete Nereis (Neanthes) virens (Sars): Purification and characterization. Biochimie, v. 89, p. 93e103.
38- Collen D, Lijnen H R (2004) Tissue-type plasminogen activator: a historical perspective and personal account. Journal of Thrombosis and Haemostasis, v. 2, p. 541-546.
39- Dobrovolsky A B, Titaeva E V (2002) The fibrinolysis system: regulation of activity and physiologic functions of its main components. Biochemistry (Mosc). 99–108
40- Rijken D C & Sakharov D V (2001) Basic principles in thrombolysis: Regulatory role of plasminogen. Thrombosis Research, , [s.l.], v 103, n 1, pS41–S49. https://doi.org/10.1016/S0049-3848(01)00296-1
41- Coleman JL, Benach JL (1999) Use of the plasminogen activation system by microorganisms. Journal of Laboratory and Clinical Medicine. 134(6):567–576.
42- Raymond B B, Djordjevic S (2015). Exploitation of plasmin(ogen) by bacterial pathogens of veterinary significance. Veterinary Microbiology, [s.l.], v. 178, n. 1-2, p.1-13. Elsevier BV. http://dx.doi.org/10.1016/j.vetmic.2015.04.008.
43- Castiblanco-Valencia M M. et al. (2016) Acquisition of negative complement regulators by the saprophyte Leptospira biflexa expressing LigA or LigB confers enhanced survival in human serum. Immunology Letters, [s.l.], v. 173, p.61-68. Elsevier BV. http://dx.doi.org/10.1016/j.imlet.2016.03.005.
44- Lähteenmäki K, Kukkonen M, Korhonen T K (2001) The Pla surface protease/adhesin of Yersinia pestis mediates bacterial invasion into human endothelial cells. Febs Letters, [s.l.], v. 504, n. 1-2, p.69-72. Wiley. http://dx.doi.org/10.1016/s0014-5793(01)02775-2.
45- Gebbia J A. et al. (1999) The plasminogen activation system enhances brain and heart invasion in murine relapsing fever borreliosis. Journal Of Clinical Investigation, [s.l.], v. 103, n. 1, p.81-87. American Society for Clinical Investigation. http://dx.doi.org/10.1172/jci5171.
46- Li Z et al.(1999) Interaction between Group A Streptococci and the Plasmin(ogen) System Promotes Virulence in a Mouse Skin Infection Model. The Journal Of Infectious Diseases, [s.l.], v. 179, n. 4, p.907-914. Oxford University Press (OUP). http://dx.doi.org/10.1086/314654
47- Goguen J D, Bugge T, Degen J L (2000) Role of the Pleiotropic Effects of Plasminogen Deficiency in Infection Experiments with PlasminogenDeficient Mice. Methods, [s.l.], v. 21, n. 2, p.179-183. Elsevier BV. http://dx.doi.org/10.1006/meth.2000.0989.
48- Vieira M L et al.(2009) Plasminogen Acquisition and Activation at the Surface of Leptospira Species Lead to Fibronectin Degradation. Infection And Immunity, [s.l.], v. 77, n. 9, p.4092-4101. American Society for Microbiology. http://dx.doi.org/10.1128/iai.00353-09.
49- Wolff DG, Castiblanco-Valencia MM, Abe CM, Monaris D, Morais ZM, Souza GO, et al. (2013) Interaction of Leptospira Elongation Factor Tu with Plasminogen and Complement Factor H: A Metabolic Leptospiral Protein with Moonlighting Activities. PLoS ONE v 8, n11,p e81818. https://doi.org/10.1371/journal.pone.0081818
50- Guyton A C, Hall JE (2006) Tratado de Fisiologia Médica. 11ª ed. Rio de Janeiro, Elsevier Ed.
51- Samet A et al. (2013)Leukemia and risk of recurrent Escherichia coli bacteremia: genotyping implicates E. coli translocation from the colon to the bloodstream. European Journal Of Clinical Microbiology & Infectious Diseases, [s.l.], v. 32, n. 11, p.1393-1400. Springer Nature. http://dx.doi.org/10.1007/s10096-013-1886-9.
52- Kuo K H M et al. (2016) EspP, an Extracellular Serine Protease from Enterohemorrhagic E. coli, Reduces Coagulation Factor Activities, Reduces Clot Strength, and Promotes Clot Lysis. Plos One, [s.l.], v. 11, n. 3, p.0149830- 0149835. Public Library of Science (PLoS). http://dx.doi.org/10.1371/journal.pone.0149830.
53- Ferasatkish R, Naddafnia H, Alavi SM, Naseri MH. (2007) Diagnosis and treatment of disseminated intravascular coagulation: a case report. ArchIran Med, 10, 404–8. http://dx.doi.org/07103/AIM.0025.
54- Parker R I (2013) Coagulopathies in the PICU. Critical Care Clinics, [s.l.], v. 29, n. 2, p.319-333. Elsevier BV. http://dx.doi.org/10.1016/j.ccc.2012.12.003.

55- Jaeschke R, Webb S A, Annane D, Dellinger RP (2013) The authors reply. Crit Care Med. 41(10):e289–90.http://dx.doi.org/10.1097/CCM.0b013e3182a42e78.
56- Koga T et al.(2011) Successful treatment of sepsis-induced disseminated intravascular coagulation in a patient with idiopathic thrombocytopenic purpura using recombinant human soluble thrombomodulin. Rheumatology International, [s.l.], v. 31, n. 12, p.1657-1659. Springer Nature. http://dx.doi.org/10.1007/s00296-010-1754-1.
57- Gando S, Levi M, Toh CHT (2016) Disseminated intravascular coagulation. Nature Reviews Disease Primers, [s.l.], v. 2, p.16037. Springer Nature. http://dx.doi.org/10.1038/nrdp.2016.37.
58- Rahman S, Cichon M, Hoppensteadt D, Cunanan J, Jeske W, Thethi I et al. (2011) Upregulation of microparticles in DIC and its impact on inflammatory processes. Clin Appl Thromb Hemost, 17:E202–4. http://dx.doi.org/ 10.1177/1076029610397181.
59- Levi M, Poll TVD (2012) Disseminated intravascular coagulation: a review for the internist. Internal And Emergency Medicine, [s.l.], v. 8, n. 1, p.23-32. Springer Nature. http://dx.doi.org/10.1007/s11739-012-0859-9.

Figures and captions

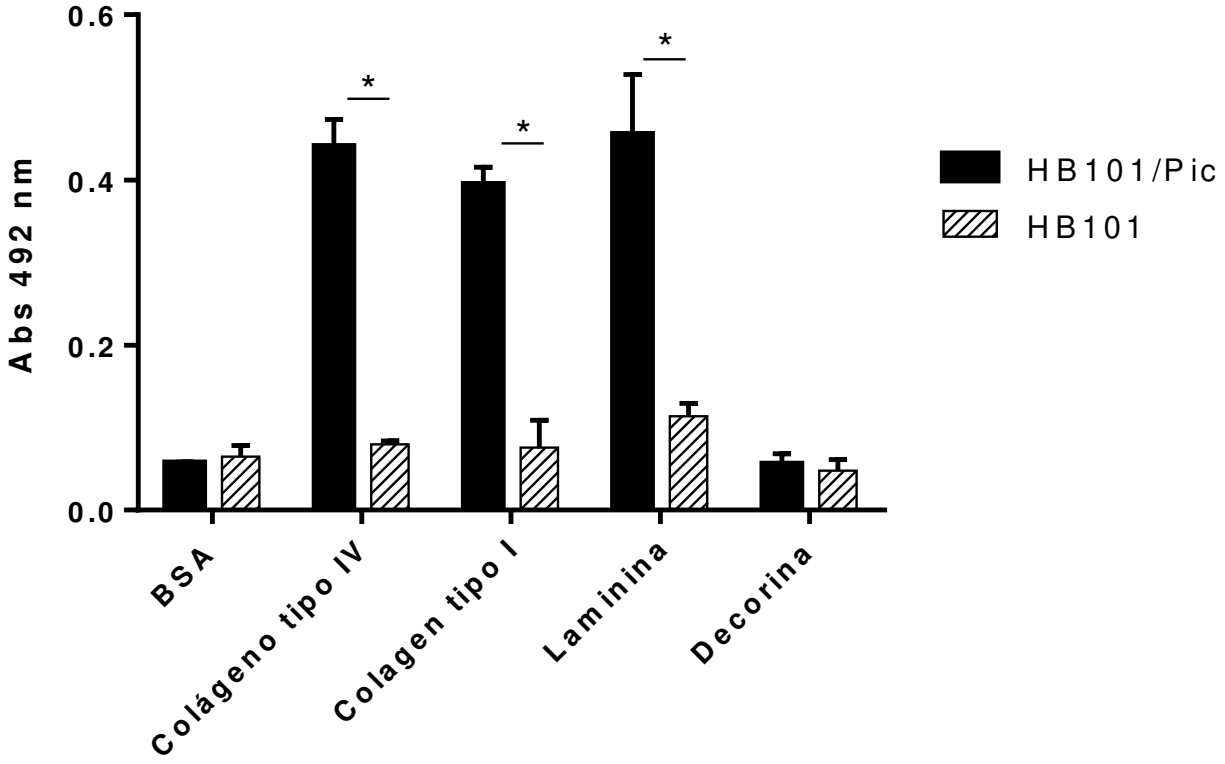

Figure 1: Pic binding assay with extracellular matrix molecules.
Concentrated fractions of HB101 / Pic and HB101 culture supernatants were incubated with collagens type I and IV, laminin, plasma fibronectin and decorin for 1:30 h. The interaction of Pic with the ECM components was evaluated using anti-Pic antibody. BSA (bovine serum albumin).
* $p < 0.05$ when comparing the concentrated fractions of the Pic-containing culture supernatants with the concentrated fractions of the culture supernatants without Pic.

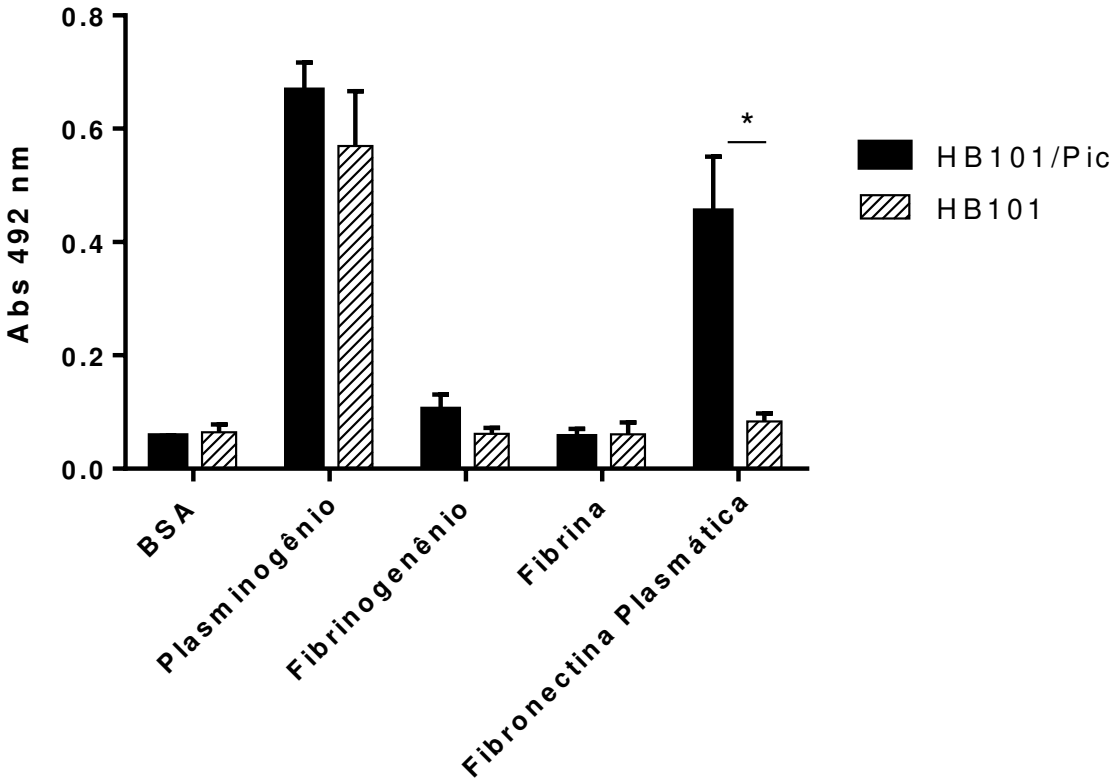

Figure 2: Pic binding assay with coagulation cascade molecules.

Concentrated fractions of the concentrated fractions of HB101 / Pic and HB101 culture supernatants were incubated with plasminogen, fibrinogen and fibrin for 1:30 h. The interaction of Pic with the ECM components was evaluated using anti-Pic antibody. BSA (bovine serum albumin).

* $p < 0.05$ when comparing concentrated fractions of the Pic-containing culture supernatants with the concentrated fractions of the culture supernatants without Pic.

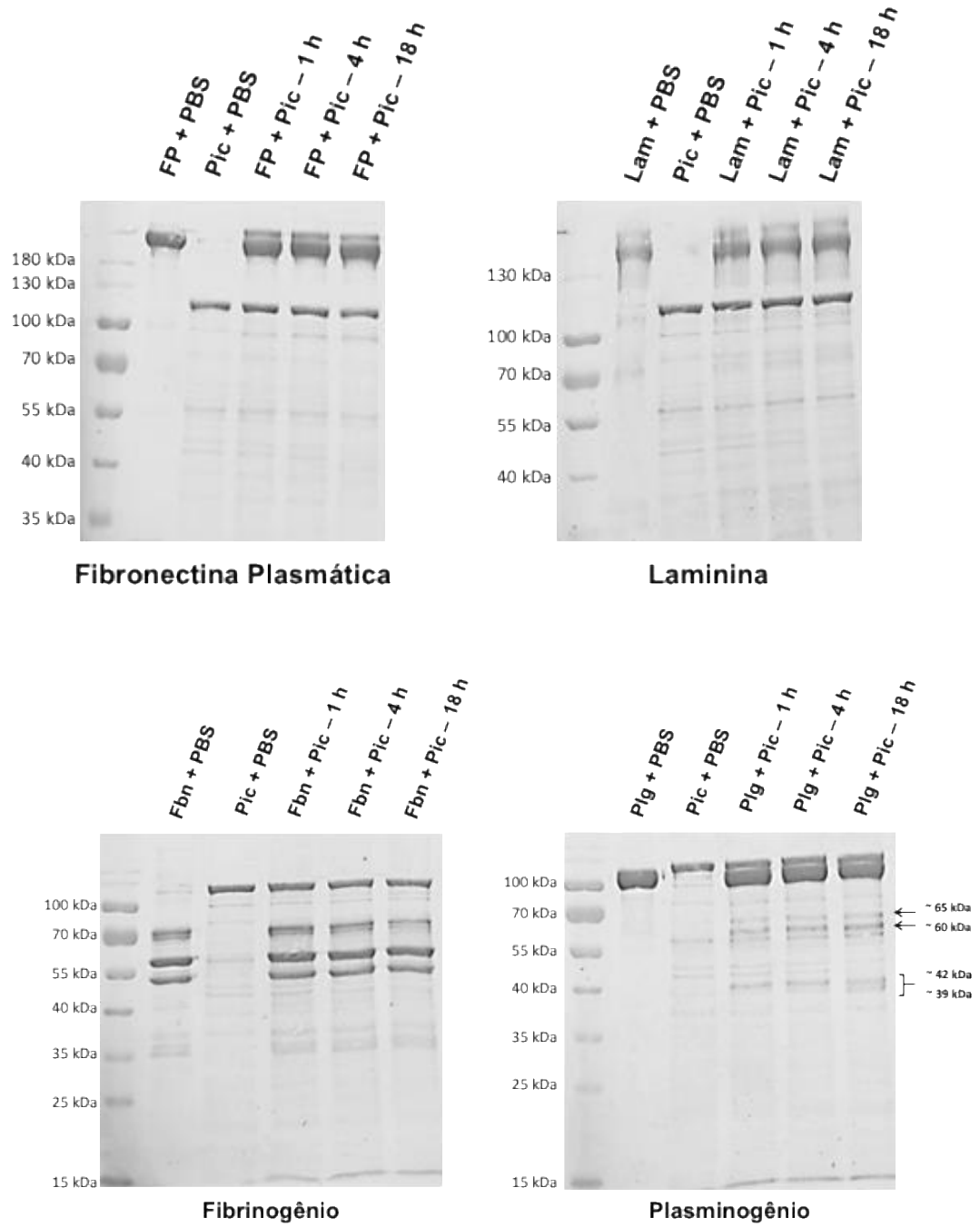

Figure 3: Cleavage test of extracellular matrix components and blood coagulation cascade.
Concentrated fractions of HB101 / Pic culture supernatants were incubated with plasma fibronectin, laminin, fibrinogen and plasminogen for 1, 4, or 18 h at 37 ° C. Cleavage products were visualized after staining with Coomassie-blue. FP (plasma fibronectin), Lam (laminin), Fbn (fibrinogen), Plg (plasminogen), Pic (protein involved in colonization), PBS

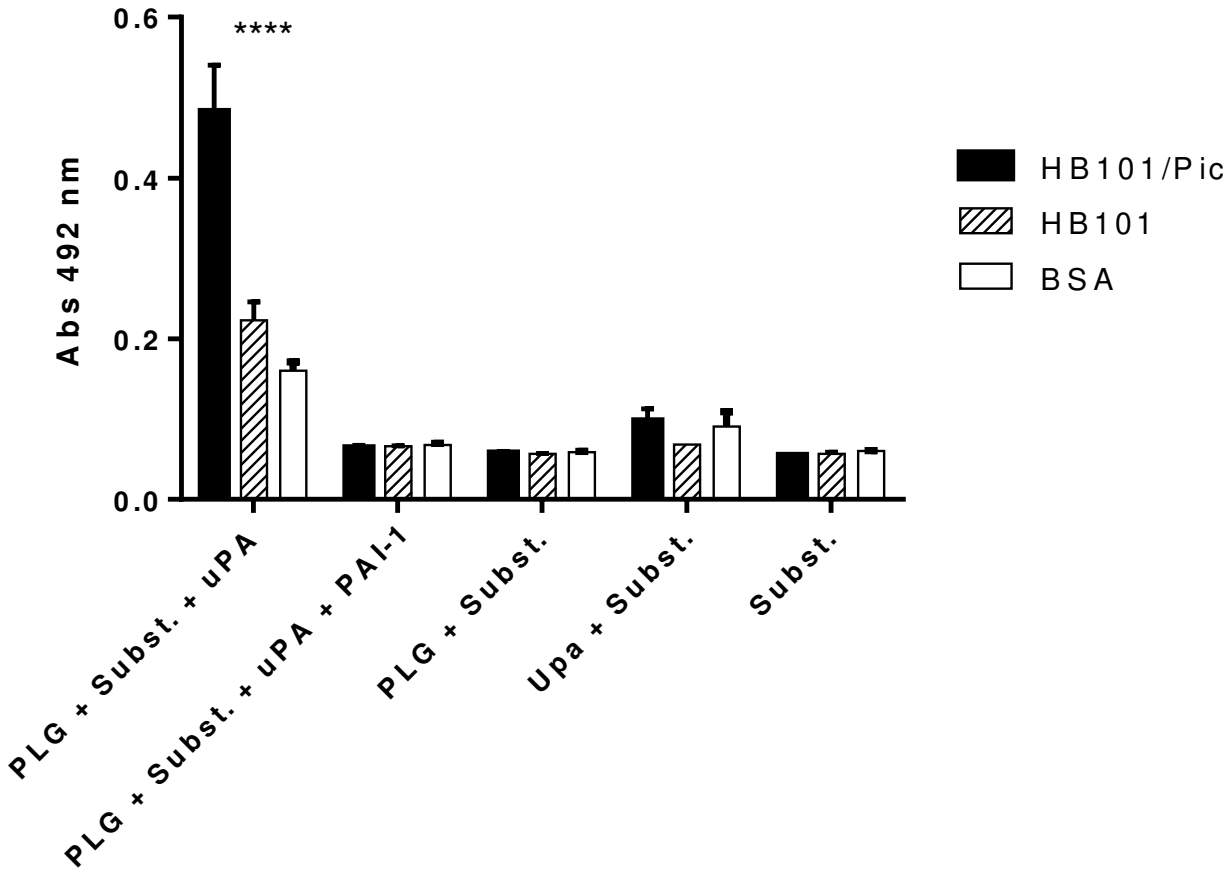

Figure 4: Plasminogen-bound was developed in functionally active plasmin.

The concentrated culture fractions of HB101 / Pic and HB101 or BSA (10 µg / mL) in microtiter plates were incubated with plasminogen (20 µg / mL). After washing, uPA (3U) and the chromogenic substrate D-valyl-leucyl-lysine-γ-nitroanilide dihydrochloride (25 µg / well) were the latter. The data represent the mean absorbance value at 492 nm standard of three independent experiments, each performed in duplicate. PLG (plasminogen), uPA (plasminogen activator type urokinase), PAI-1 (inhibitor of plasminogen activator type 1) and Subst. (substrate).

**** $p < 0.001$ when comparing the concentrated fractions of culture supernatants with Pic and concentrated fractions of culture supernatants without Pic or BSA.

5. CONSIDERAÇÕES FINAIS

A proteína Pic desempenha um importe papel no processo infeccioso e manutenção da bactéria no hospedeiro uma vez que Pic rompe as barreiras de defesa do organismo humano. Além da sua atuação no sistema complemento, o capítulo I apresenta um estudo *in vitro* em que se avaliou a ação da serinoprotease Pic sobre componentes da matriz extracelular e cascata de coagulação sanguínea.

Foi observado que a proteína Pic foi capaz de se ligar a diversos componentes de matriz extracelular e a mesma é capaz de clivar o plasminogênio, logo a plasmina gerada parece potencializar a clivagem de componentes do sistema complemento.

As próximas etapas deste trabalho serão avaliar de que forma a proteína Pic age com a cascata de coagulação, investigando quais outras moléculas da cascata participam do mecanismo.

6. CONCLUSÃO

- A proteína Pic foi capaz de se ligar a diversos componentes de matriz extracelular, a exemplo da laminina, fibronectina plasmática e colágenos do tipo I e IV, podendo, portanto, contribuir para o processo infeccioso no hospedeiro.

- Pic não foi capaz de se ligar e nem de clivar o fibrinogênio, componente da cascata de coagulação, mas foi capaz de clivar o plasminogênio.

- Plasminogênio ligado a Pic é convertido em sua forma ativa, a plasmina, na presença do ativador exógeno uPA. Desta forma, a geração de plasmina, pode intensificar a clivagem de componentes do sistema complemento, além de resultar em agravamento de muitas das manifestações clínicas da coagulação intravascular disseminada, bem como intensificar o processo de sepse mediada pela proteína Pic.

REFERENCIAS

ABREU, Afonso G. et al. Autotransporter Protein-Encoding Genes of Diarrheagenic Escherichia coli Are Found in both Typical and Atypical Enteropathogenic E. coli Strains. Applied And Environmental Microbiology, [s.l.], v. 79, n. 1, p.411-414, 26 out. 2013. American Society for Microbiology. http://dx.doi.org/10.1128/aem.02635-12.

ABREU, Afonso G. et al. The serine protease Pic as a virulence factor of atypical enteropathogenicEscherichia coli. Gut Microbes, [s.l.], v. 7, n. 2, p.115-125, 3 mar. 2016. Informa UK Limited. http://dx.doi.org/10.1080/19490976.2015.1136775.

ABREU, Afonso G. et al. The Serine Protease Pic From Enteroaggregative Escherichia coli Mediates Immune Evasion by the Direct Cleavage of Complement Proteins. The Journal Of Infectious Diseases, [s.l.], v. 212, n. 1, p.106-115, 12 jan. 2015. Oxford University Press (OUP). http://dx.doi.org/10.1093/infdis/jiv013.

ABREU, Afonso G.; BARBOSA, Angela S.. How Escherichia coli Circumvent Complement-Mediated Killing. **Frontiers In Immunology**, [s.l.], v. 8, 20 abr. 2017. Frontiers Media SA. http://dx.doi.org/10.3389/fimmu.2017.00452.

ALFORD, Andrea I.; RANNELS, D. Eugene. Extracellular matrix fibronectin alters connexin43 expression by alveolar epithelial cells. American Journal of Physiology-lung Cellular And Molecular Physiology, v. 280, n. 4, p.680-688, 2001. http://dx.doi.org/10.1152/ajplung.2001.280.4.l680.

ALMEIDA, Paulo C. et al. Cathepsin B Activity Regulation. Journal of Biological Chemistry, v. 276, n. 2, p.9 44-951, 2000. American Society for Biochemistry & Molecular Biology (ASBMB). http://dx.doi.org/10.1074/jbc.m003820200.

ANDRADE, Z. A. Tecido conjuntivo, reparo, regeneração e cicatrização. In: MONTENEGRO, Mario R.; FRANCO, Marcelo. Patologia processos gerais.4. ed. São Paulo: Ateneu, 2003.

ARCÍA, Jesús et al. Oligomerization and DNA binding of Ler, a master regulator of pathogenicity of enterohemorrhagic and enteropathogenic Escherichia coli. Nucleic Acids Research, [s.l.], v. 40, n. 20, p.10254-10262, 8 set. 2012. Oxford University Press (OUP). http://dx.doi.org/10.1093/nar/gks846.

AYALA-LUJAN, Jorge Luis et al. Broad Spectrum Activity of a Lectin-Like Bacterial Serine Protease Family on Human Leukocytes. Plos One, v. 9, n. 9, p.1-14, 2014. http://dx.doi.org/10.1371/journal.pone.0107920.

BAJZAR L. Thrombin activatable fibrinolysis inhibitor and na antifibrinolytic pathway. Arterioscler Thromb Vasc Biol 20: 2511- 518, 2000. http:// dx.doi.org/ 10.1161/01.ATV.20.12.2511.

BARRETT, Jeffrey C et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. **Nature Genetics**, [s.l.], v. 40, n. 8, p.955-962, 29 jun. 2008. Springer Nature. http://dx.doi.org/10.1038/ng.175.

BHULLAR, Kirandeep et al. The Serine Protease Autotransporter Pic Modulates Citrobacter rodentium Pathogenesis and Its Innate Recognition by the Host. Infection And Immunity, [s.l.], v. 83, n. 7, p.2636-2650, 20 abr. 2015. American Society for Microbiology. http://dx.doi.org/10.1128/iai.00025-15

BHUNIA, A. K. Foodborne microbial pathogens: mecanísms and pathogenesís. New York: Sprínger Science, p. 181-200,2008.

BONNANS, Caroline; CHOU, Jonathan; WERB, Zena. Remodelling the extracellular matrix in development and disease. Nature Reviews Molecular Cell Biology, [s.l.], v. 15, n. 12, p.786-801, dez. 2014. Springer Nature. http://dx.doi.org/10.1038/nrm3904.

BORISSOFF, Julian Ilcheff; SPRONK, Henri M.h.; CATE, Hugo Ten. The Hemostatic System as a Modulator of Atherosclerosis. New England Journal Of Medicine, [s.l.], v. 364, n. 18, p.1746-1760, 5 maio 2011. New England Journal of Medicine (NEJM/MMS). http://dx.doi.org/10.1056/nejmra1011670.

BROWN, Bryan N.; BADYLAK, Stephen F. Extracellular matrix as an inductive scaffold for functional tissue reconstruction. Translational Research, v. 163, n. 4, p.268-285, 2014. http://dx.doi.org/10.1016/j.trsl.2013.11.003

BUERIS, Vanessa et al. Detection of diarrheagenic Escherichia coli from children with and without diarrhea in Salvador, Bahia, Brazil. Memórias do Instituto Oswaldo Cruz, [s.l.], v. 102, n. 7, p.839-844, 30 out. 2007. FapUNIFESP (SciELO). http://dx.doi.org/10.1590/s0074-02762007005000116.

CANIZALEZ-ROMAN, Adrián; NAVARRO-GARCÍA, Fernando. Fodrin CaM-binding domain cleavage by Pet from enteroaggregative Escherichia coli leads to actin cytoskeletal disruption. Molecular Microbiology, [s.l.], v. 48, n. 4, p.947-958, 8 maio 2003. Wiley. http://dx.doi.org/10.1046/j.1365-2958.2003.03492.x.

CELIK N, WEBB CT, LEYTON DL, HOLT KE, HEINZ E, GORRELL R, KWOK T, NADERER T, STRUGNELL RA, SPEED TP, TEASDALE RD, LIKIĆ VA, LITHGOW T. 2012. A bioinformatic strategy for the detection, classification and analysis of bacterial autotransporters. PLoS One 7:e43245. doi:10.1371/journal.pone.0043245.

CHEN, Huiwen Deborah; FRANKEL, Gad. Enteropathogenic Escherichia coli: unravelling pathogenesis. Fems Microbiology Reviews, [s.l.], v. 29, n. 1, p.83-98, jan. 2005. http://dx.doi.org/10.1016/j.femsre.2004.07.002

CLARK, R. A., L. D. NIELSEN, M. P. Welch, and J. M. McPherson. Collagen matrices attenuate the collagen-synthetic response of cultured fibroblasts to TGF-beta. J. Cell Sci. 108:1251–1261, 1995.

CLARK, Richard A.f.. Regulation of Fibroplasia in Cutaneous Wound Repair. The American Journal Of The Medical Sciences, [s.l.], v. 306, n. 1, p.42-48, jul. 1993. Elsevier BV. http://dx.doi.org/10.1097/00000441-199307000-00011.

CROXEN, M. A. et al. Recent Advances in Understanding Enteric Pathogenic Escherichia coli. Clinical Microbiology Reviews, [s.l.], v. 26, n. 4, p.822-880, 1 out. 2013. American Society for Microbiology. http://dx.doi.org/10.1128/cmr.00022-13.

DAUTIN, Nathalie. Serine Protease Autotransporters of Enterobacteriaceae (SPATEs): Biogenesis and Function. Toxins, [s.l.], v. 2, n. 6, p.1179-1206, 28 maio 2010. MDPI AG. http://dx.doi.org/10.3390/toxins2061179.

DENG, W. et al. Dissecting virulence: Systematic and functional analyses of a pathogenicity island. Proceedings Of The National Academy Of Sciences, [s.l.], v. 101, n. 10, p.3597-3602, 26 fev. 2004. Proceedings of the National Academy of Sciences. http://dx.doi.org/10.1073/pnas.0400326101.

DEQUACH, Jessica A. et al. Simple and High Yielding Method for Preparing Tissue Specific Extracellular Matrix Coatings for Cell Culture. Plos One, [s.l.], v. 5, n. 9, p.1-11, 27 set. 2010. Public Library of Science (PLoS). http://dx.doi.org/10.1371/journal.pone.0013039

DUTTA, P. R. et al. Functional Comparison of Serine Protease Autotransporters of Enterobacteriaceae. Infection And Immunity, [s.l.], v. 70, n. 12, p.7105-7113, 1 dez. 2002. American Society for Microbiology. http://dx.doi.org/10.1128/iai.70.12.7105-7113.2002.

DVORAK HF, HARVEY VS, ESTRELLA P, BROWN LF, MCDONAGH J, DVORAK AM. Fibrin containing gels induce angiogenesis. Implications for tumor stroma generation and wound healing. Lab Invest. 1987 Dec; 57(6):673-86.

FIALHO, O. B. Identificação de estirpes de Escherichia coli diarreiogênicas por PCR-multiplex. Curitiba: UFPR, Setor de Ciências da Saúde, 2008. 106 p. Dissertação (Mestrado em Ciências Farmacêuticas).

FRAGA, TATIANA RODRIGUES et al. “Complement Evasion by Pathogenic Leptospira” Frontiers in immunology vol. 7 623. 21 Dec. 2016, doi:10.3389/fimmu.2016.00623

FRANCO RF. Fisiologia da coagulação, anticoagulação e fibrinólise. Medicina (Ribeirão Preto).34(3/4):229-3,2001.

FRANTZ, C.; STEWART, K. M.; WEAVER, V. M. The extracellular matrix at a glance. Journal Of Cell Science, [s.l.], v. 123, n. 24, p.4195-4200, 1 dez. 2010. The Company of Biologists. http://dx.doi.org/10.1242/jcs.023820

FUJIMOTO, Kenta; NAKAJIMA, Keisuke; YAOITA, Yoshio. Expression of matrix metalloproteinase genes in regressing or remodeling organs during amphibian metamorphosis. Development, Growth & Differentiation, [s.l.], v. 49, n. 2, p.131-143, 28 fev. 2007. Wiley. http://dx.doi.org/10.1111/j.1440-169x.2007.00916.x.

GUTIÉRREZ-JIMÉNEZ, J.; ARCINIEGA, I.; NAVARRO-GARCÍA, F. The serine protease motif of Pic mediates a dose-dependent mucolytic activity after binding to sugar constituents of the mucin substrate. Microbial Pathogenesis, v. 45, n. 2, p.115-123, 2008. Elsevier BV. http://dx.doi.org/10.1016/j.micpath.2008.04.006.

HACKER, J.; KAPER, J. B. Pathogenicity islands and the evolution of microbes. **Annu. Rev. Microbiol.**, v. 54, p. 641-679, 2000. http://dx.doi.org/ 10.1146/annurev.micro.54.1.641.

HANSEN, N.u.b. et al. The importance of extracellular matrix for cell function and in vivo likeness. **Experimental And Molecular Pathology**, [s.l.], v. 98, n. 2, p.286-294, abr. 2015. Elsevier BV. http://dx.doi.org/10.1016/j.yexmp.2015.01.006.

HARRINGTON, S. M. et al. The Pic Protease of Enteroaggregative Escherichia coli Promotes Intestinal Colonization and Growth in the Presence of Mucin. **Infection And Immunity**, [s.l.], v. 77, n. 6, p.2465-2473, 6 abr. 2009. American Society for Microbiology. http://dx.doi.org/10.1128/iai.01494-08.

HEDBERG, Craig w. et al. An Outbreak of Foodborne Illness Caused byEscherichia coliO39: NM, an Agent Not Fitting into the Existing Scheme for Classifying DiarrheogenicE. coli. The Journal Of Infectious Diseases, [s.l.], v. 176, n. 6, p.1625-1628, dez. 1997. Oxford University Press (OUP). http://dx.doi.org/10.1086/517342.

HENDERSON I.R., CZECZULIN J., ESLAVA C., NORIEGA F., NATARO J.P. Characterization of Pic, a secreted protease of Shigella flexneri and enteroaggregative Escherichia coli. Infect. Immun. 67:5587–5596, 1999.

HENDERSON, I. R.; NATARO, J. P.. Virulence Functions of Autotransporter Proteins. **Infection And Immunity**, [s.l.], v. 69, n. 3, p.1231-1243, 2001. http://dx.doi.org/10.1128/iai.69.3.1231-1243.2001.

HONARDOUST D, ESLAMI A, LARJAVA H, HÄKKINEN L. Localization of small leucine-rich proteoglycans and transforming growth factor-beta in human oral mucosal wound healing. Wound Repair Regen. 2008;16(6):814-23

HYNES, Richard O and Alexandra NABA. "Overview of the matrisome--an inventory of extracellular matrix constituents and functions" *Cold Spring Harbor perspectives in biology* vol. 4, n1 a004903.Jan 2012. http://dx.doi.org/10.1101/cshperspect.a004903.

IRVING-RODGERS, Helen F. et al. Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. **Cell And Tissue Research**, [s.l.], v. 339, n. 3, p.613-624, 2010. http://dx.doi.org/10.1007/s00441-009-0905-8

LABAT-ROBERT.J. Cell–Matrix interactions, the role of fibronectin and integrins. A survey. Pathologie Biologie. 2012; 60: 15–19.

SCHWARZBAUER J.E, DESIMONE D.W. Fibronectins, their fibrillogenesis, and in vivo functions. Cold Spring Harb Perspect Biol; 3 (7). doi:10.1101/cshperspect.a005041.

KAPER, J. B.; NATARO, J. P.; MOBLEY, H. L. T. Pathogenic Escherichia coli. **Nature Reviews Microbiology**, [s.l.], v. 2, n. 2, p.123-140, fev. 2004. Springer Nature. http://dx.doi.org/10.1038/nrmicro818

KIRKPATRICK, C. A.; SELLECK, S. B. Heparan sulfate proteoglycans at a glance. **Journal Of Cell Science**, [s.l.], v. 120, n. 11, p.1829-1832, 1 jun. 2007. The Company of Biologists. http://dx.doi.org/10.1242/jcs.03432.

KONKEL, ME. et al. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from Campylobacter jejuni. Molecular Microbiology, v. 24, p.953-963, 1997. http://dx.doi.org/10.1046/j.1365-2958.1997.4031771.x

KRESSE H, SCHÖNHER E. Proteoglycans of the extracellular matrix and growth control. J Cell Physiol. 2001;189:266-74.

KUMAR; ABBAS; FAUSTO Robbins & Cotran Patologia Bases Patológicas das Doenças, Ed Saunders, 7 ed, 2005, 1592 p.

LENSELINK SA. Role of fibronectin in normal wound healing. Int Wound J 2013; doi: 10.1111/iwj.12109

LU, P. et al. Extracellular Matrix Degradation and Remodeling in Development and Disease. Cold Spring Harbor Perspectives In Biology, [s.l.], v. 3, n. 12, 14 set. 2011. Cold Spring Harbor Laboratory. http://dx.doi.org/10.1101/cshperspect.a005058.

MATAVELI FD, HAN SW, NADER HB, MENDES A, KANISHIRO R, PINHAL MA, et al. Long-term effects for acute phase myocardial infarct VEGF165 gene transfer cardiac extracellular matrix remodeling. Growth Factors. 2009;27(1):22-31.

MELLIES, J. L. et al. EspC Pathogenicity Island of Enteropathogenic Escherichia coli Encodes an Enterotoxin. Infection And Immunity, [s.l.], v. 69, n. 1, p.315-324, 1 jan.

2001. American Society for Microbiology. http://dx.doi.org/10.1128/iai.69.1.315-324.2001.

MICHELACCI YM, MOURÃO PA, LAREDO J, DIETRICH CP. Chondroitin sulfates and proteoglycans from normal and arthrosic human cartilage. Connect Tissue Res. 1979;7(1):29-36

MIDWOOD, K. S. ; To, W. S. Plasma and cellular fibronectin: distinct and independent functions during tissue repair. Fibrogenesis & Tissue Repair, v. 4, n. 21, p. 1-17, 2011.

MOON HW, BALDINI MM, KAPER JB, LEVINE MM; CANDY DC. Plasmid-mediated adhesion in enteropathogenic Escherichia coli. **J Pediatr Gastroenterol Nutr** 2: 534-538, 1983.

MORENO, Ana Carolina Ramos et al. Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. Diagnostic Microbiology And Infectious Disease, [s.l.], v. 66, n. 1, p.50-57, jan. 2010. Elsevier BV. http://dx.doi.org/10.1016/j.diagmicrobio.2008.03.017.

MUNERA, Diana et al. Autotransporters but not pAA are critical for rabbit colonization by Shiga toxin-producing Escherichia coli O104: H4. **Nature Communications**, [s.l.], v. 5, p.1-20, 20 jan. 2014. Springer Nature. http://dx.doi.org/10.1038/ncomms4080

MURRAY, G.i; CURRAN, S. Matrix metalloproteinases. **European Journal Of Cancer**, [s.l.], v. 36, n. 13, p.1621-1630, ago. 2000. Elsevier BV. http://dx.doi.org/10.1016/s0959-8049(00)00156-8.

MYTHREYE, Karthikeyan; BLOBE, Gerard C. Proteoglycan signaling co-receptors: Roles in cell adhesion, migration and invasion. **Cellular Signalling**, [s.l.], v. 21, n. 11, p.1548-1558, 2009. http://dx.doi.org/10.1016/j.cellsig.2009.05.001.

NATARO, James P.; KAPER, James B.. Diarrheagenic Escherichia coli. Clinical Microbiology Reviews, [s.l.], v. 11, n. 1, p.142-201, 1 jan. 1998. American Society for Microbiology. http://dx.doi.org/10.1128/cmr.11.1.142

NAVARRO-GARCIA, F. et al. Pic, an Autotransporter Protein Secreted by Different Pathogens in the Enterobacteriaceae Family, Is a Potent Mucus Secretagogue. **Infection And Immunity**, [s.l.], v. 78, n. 10, p.4101-4109, 9 ago. 2010. American Society for Microbiology. http://dx.doi.org/10.1128/iai.00523-10.

NAVARRO-GARCIA, F. et al. The Serine Protease Motif of EspC from Enteropathogenic Escherichia coli Produces Epithelial Damage by a Mechanism Different from That of Pet Toxin from Enteroaggregative E. coli. **Infection And Immunity**, [s.l.], v. 72, n. 6, p.3609-3621, 20 maio 2004. American Society for Microbiology. http://dx.doi.org/10.1128/iai.72.6.3609-3621.2004.

NELSON DL, COX MM. Lehninger Principles of Biochemistry. 5 ed. New York: Freeman; 2008. p. 237-40

OTRANTO, Marcela et al. The role of the myofibroblast in tumor stroma remodeling. Cell Adhesion & Migration, [s.l.], v. 6, n. 3, p.203-219, maio 2012. Informa UK Limited. http://dx.doi.org/10.4161/cam.20377.

PARHAM, Nick J. et al. PicU, a second serine protease autotransporter of uropathogenic Escherichia coli. **Fems Microbiology Letters**, [s.l.], v. 230, n. 1, p.73-83, jan. 2004. Oxford University Press (OUP). http://dx.doi.org/10.1016/s0378-1097(03)00862-0.

PATERSON, D. L. Resistance in Gram-negative bacteria: Enterobacteriaceae. **Am. J. Infect. Control**, v. 34, p. 20-28, 2006. http://dx.doi.org/10.1016/j.amjmed.2006.03.013

PUPO, G. M.; LAN, R.; REEVES, P. R.. Multiple independent origins of Shigella clones of Escherichia coli and convergent evolution of many of their characteristics. **Proceedings Of The National Academy Of Sciences**, [s.l.], v. 97, n. 19, p.10567-10572, 22 ago. 2000. Proceedings of the National Academy of Sciences. http://dx.doi.org/10.1073/pnas.180094797.

RAJAKUMAR, K et al. "Use of a novel approach, termed island probing, identifies the Shigella flexneri she pathogenicity island which encodes a homolog of the immunoglobulin A protease-like family of proteins" Infection and immunity vol. 65, n 11, p.4606-14. Nov.1997.

RASKO, David A. et al. Origins of theE. coliStrain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany. New England Journal Of Medicine, [s.l.], v. 365, n. 8, p.709-717, 25 ago. 2011. New England Journal of Medicine (NEJM/MMS). http://dx.doi.org/10.1056/nejmoa1106920.

RODRIGUES, Evandra. Novos conceitos sobre a fisiologia da hemostasia. Revista da Universidade Vale do Rio Verde, [s.l.], v. 10, n. 1, p.218-233, 26 jul. 2012. Universidade Vale do Rio Verde (UninCor). http://dx.doi.org/10.5892/ruvrv.2012.101.218233.

ROMBERGER, D. J. Molecule in focus. Int. J. Biochem. Cell Biol., v. 29, p. 939-943, 1997

ROZARIO T, DESIMONE DW. The extracellular matrix in development and morphogenesis: a dynamic view. Developmental Biology. 2010; 341: 126–140.

RUIZ-PEREZ, F. et al. Serine protease autotransporters from *Shigella flexneri* and pathogenic *Escherichia coli* target a broad range of leukocyte glycoproteins. **Proceedings Of The National Academy Of Sciences**, [s.l.], v. 108, n. 31, p.12881-12886, 18 jul. 2011. Proceedings of the National Academy of Sciences. http://dx.doi.org/10.1073/pnas.1101006108.

RUIZ-PEREZ, F. et al. Serine protease autotransporters from Shigella flexneri and pathogenic Escherichia coli target a broad range of leukocyte glycoproteins. Proceedings Of The National Academy Of Sciences, [s.l.], v. 108, n. 31, p.12881-12886, 18 jul. 2011. Proceedings of the National Academy of Sciences. http://dx.doi.org/10.1073/pnas.1101006108.

RUIZ-PEREZ, Fernando; NATARO, James P. Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence. **Cellular And Molecular Life Sciences**, [s.l.], v. 71, n. 5, p.745-770, 21 maio 2014. Springer Nature. http://dx.doi.org/10.1007/s00018-013-1355-8

RUOSLAHTI E. Proteoglycans in cell regulation. J Biol Chem. 1989;264(23):13369-72

SADER, H. S.; GALES, A. C.; PFALLER, M. A.; MENDES, R. E.; ZOCCOLI, C.; BARTH, A.; JONES, R. N. Pathogen frequency and resistance patterns in Brazilian hospitals: Summary of results from three years of the SENTRY Antimicrobial Surveillance Program. **Braz. J. Infect. Dis.**, v. 5, p. 200-214, 2001. http://dx.doi.org/10.1590/S1413-86702001000400006

SCALETSKY, Isabel C. A. et al. Adherence Factors in Atypical EnteropathogenicEscherichia coliStrains Expressing the Localized Adherence-Like

Pattern in HEp-2 Cells. **Journal Of Clinical Microbiology**, [s.l.], v. 48, n. 1, p.302-306, 28 out. 2009. American Society for Microbiology. http://dx.doi.org/10.1128/jcm.01980-09.

SERINI, Guido et al. The Fibronectin Domain ED-A Is Crucial for Myofibroblastic Phenotype Induction by Transforming Growth Factor-β1. The Journal Of Cell Biology, [s.l.], v. 142, n. 3, p.873-881, 10 ago. 1998. Rockefeller University Press. http://dx.doi.org/10.1083/jcb.142.3.873.

SETTEMBRE C, ARTEAGA-SOLIS E, MCKEE MD, DE PABLO R, AL AWQATI Q, BALLABIO A, et al. Proteoglycan desulfation determines the efficiency of chondrocyte autophagy and the extent of FGF signaling during endochondral ossification. Genes Dev. 2008;22(19):2645-50.

SILVA, Fabiana Fernanda Pacheco da et al. Occurrence of Salmonella spp. and generic Escherichia coli on beef carcasses sampled at a brazilian slaughterhouse. **Brazilian Journal Of Microbiology**, [s.l.], v. 45, p.17-24, 2014. http://dx.doi.org/10.1590/s1517-83822014005000037

SILVA, Ludmila B. da et al. Leptospira interrogans Secreted Proteases Degrade Extracellular Matrix and Plasma Proteins From the Host. Frontiers In Cellular And Infection Microbiology, [s.l.], v. 8, 27 mar. 2018. Frontiers Media SA. http://dx.doi.org/10.3389/fcimb.2018.00092.

SILVA, R. D. R. S. R., & MELO, E. M. D. M. M. A atual teoria da coagulação baseada em superficies celulares. Saúde & Ciência em Ação, 2(1), 79-92, 2016.

SPRONK, H. M. H. et al. Pleiotropic effects of factor Xa and thrombin: what to expect from novel anticoagulants. **Cardiovascular Research**, [s.l.], v. 101, n. 3, p.344-351, 2 jan. 2014. Oxford University Press (OUP). http://dx.doi.org/10.1093/cvr/cvt343.

SUGAHARA, K., KITAGAWA, H., 2002. Heparin and heparan sulfate biosynthesis. IUBMB Life

TRABULSI, L.R.& ALTERTHUM,F. "Microbiologia", Editora Atheneu, 4a. edição, Revista e atualizada em 2005.

TRABULSI, Luiz R. et al. Typical and Atypical Enteropathogenic Escherichia coli. **Emerging Infectious Diseases**, [s.l.], v. 8, n. 5, p.508-513, maio 2002. Centers for Disease Control and Prevention (CDC). http://dx.doi.org/10.3201/eid0805.010385.

VALCARCCELA, J.; CARBALLALC, R.N.; MARTINB, R.I.P.; REISC, R.L.; 39 VÁZQUEZA, J.A. Glycosaminoglycans from marine sources as therapeutic agents. Group of Recycling and Valorisation of Waste Materials (REVAL), Marine Research Institute (IIM-CSIC), R/Eduardo Cabello, 6, CP 36208, Vigo, Pontevedra, Spain. julho de 2017

VOROTNIKOVA, Ekaterina et al. Extracellular matrix-derived products modulate endothelial and progenitor cell migration and proliferation in vitro and stimulate regenerative healing in vivo. **Matrix Biology**, [s.l.], v. 29, n. 8, p.690-700, out. 2010. Elsevier BV. http://dx.doi.org/10.1016/j.matbio.2010.08.007.

WALIA, Baljit et al. Polarized fibronectin secretion induced by adenosine regulates bacterial–epithelial interaction in human intestinal epithelial cells. Biochemical Journal, v. 382, n. 2, p.589-596, 2004. http://dx.doi.org/10.1042/bj20040021

WANG, Hong-jun et al. Stimulation of Skin Repair Is Dependent on Fibroblast Source and Presence of Extracellular Matrix. **Tissue Engineering**, v. 10, n. 7-8, p.1054-1064, 2004. http://dx.doi.org/10.1089/ten.2004.10.1054

WESTERLUND, B.; KORHONEN, T. K.. Bacterial proteins binding to the mammalian extracellular matrix. Molecular Microbiology, [s.l.], v. 9, n. 4, p.687-694, ago. 1993. Wiley. http://dx.doi.org/10.1111/j.1365-2958.1993.tb01729.x.

World Health Organization (WHO). Diarrhoeal disease. Fact sheet n.330. [cited 2014 Dic 3]. Available from: http://www.who.int/ mediacentre/factsheets/fs330/en/.

WU YJ, LA PIERRE DP, WU J, YEE AJ, YANG BB. The interaction of versican with its binding partners. Cell Res. 2005;15:483-94.

YÁÑEZ, Dominique et al. The Role of Fibronectin in the Adherence and Inflammatory Response Induced by Enteroaggregative *Escherichia coli* on Epithelial Cells. **Frontiers In Cellular And Infection Microbiology**, [s.l.], v. 6, p.1-7, 8 dez. 2016. Frontiers Media SA. http://dx.doi.org/10.3389/fcimb.2016.00166.

YATSUYANAGI, J. et al. Characterization of Atypical Enteropathogenic Escherichia coli Strains Harboring the astA Gene That Were Associated with a Waterborne Outbreak of Diarrhea in Japan. **Journal Of Clinical Microbiology**, [s.l.], v. 41, n. 5, p.2033-2039, 1 maio 2003. American Society for Microbiology. http://dx.doi.org/10.1128/jcm.41.5.2033-2039.2003.

YEN, Yihfen T. et al. Common themes and variations in serine protease autotransporters. **Trends In Microbiology**, [s.l.], v. 16, n. 8, p.370-379, ago. 2008. Elsevier BV. http://dx.doi.org/10.1016/j.tim.2008.05.003.

ANEXO A – DIRETRIZES PARA PUBLICAÇÃO DE TRABALHOS NA MEDICAL MICROBIOLOGY AND IMUNOLOGY

MANUSCRIPT SUBMISSION

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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1.1.1 TITLE PAGE

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- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, and telephone number(s) of the corresponding author
- If available, the 16-digit ORCID of the author(s)

1.1.1.2. Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

1.1.1.3. Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.

- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.
Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.
Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

SCIENTIFIC STYLE

- Please always use internationally accepted signs and symbols for units, SI units.
- Genus and species names should be in italics.
- The common names of animals should not be capitalized.

REFERENCES

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:
1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.
The entries in the list should be numbered consecutively.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- ISSN.org LTWA

If you are unsure, please use the full journal title.
For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 2 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

1.1.2 TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

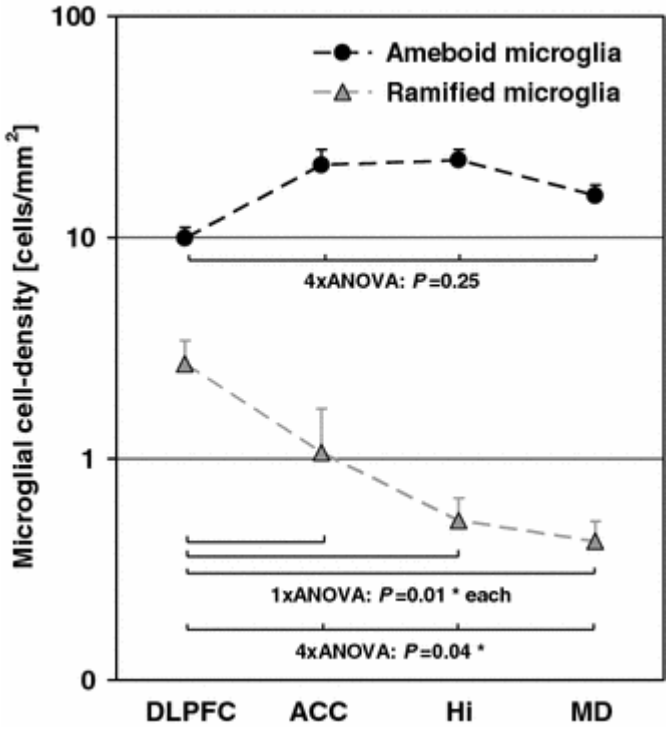
1.1.3 ARTWORK

For the best quality final product, it is highly recommended that you submit all of your artwork – photographs, line drawings, etc. – in an electronic format. Your art will then be produced to the highest standards with the greatest accuracy to detail. The published work will directly reflect the quality of the artwork provided.

1.1.3.1. Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

1.1.3.2. Line Art



- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

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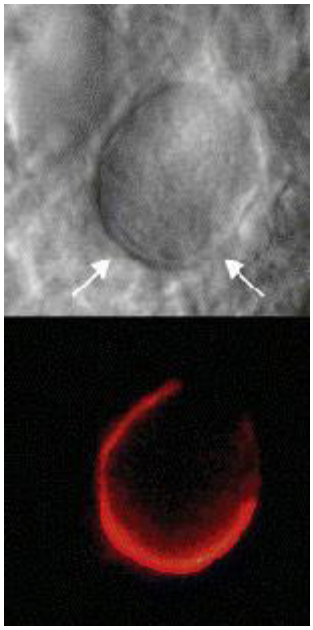
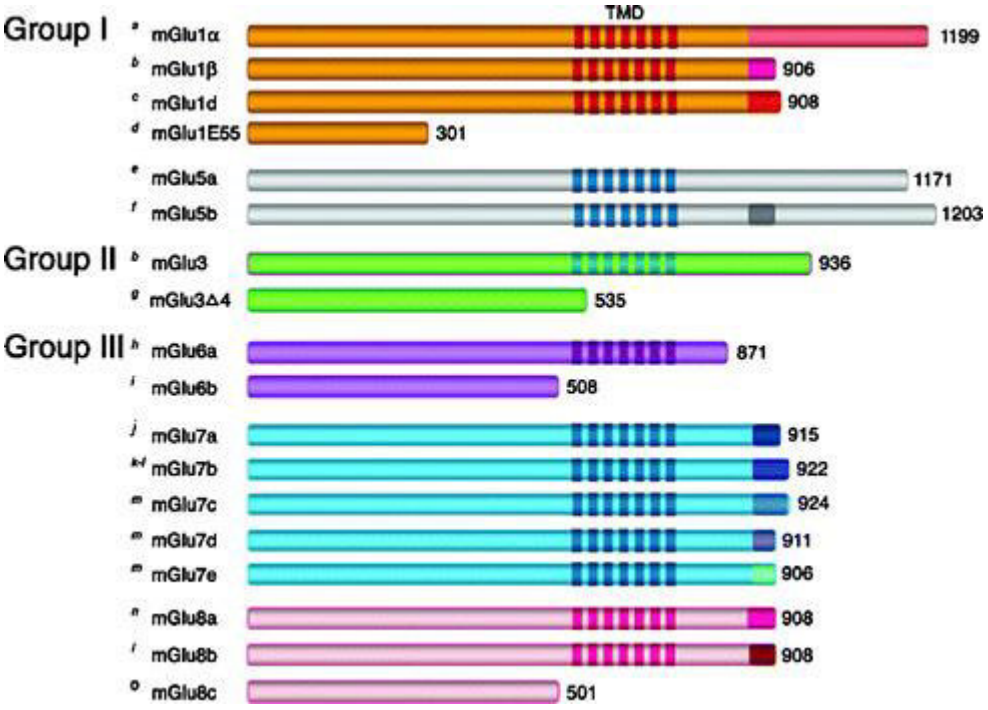


- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

1.1.3.4. Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

1.1.3.5. Color Art

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- Color illustrations should be submitted as RGB.

1.1.3.6. Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

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- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

1.1.3.8. Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
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- It is possible to collect multiple files in a .zip or .gz file.

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- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
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- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

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Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
- Research involving Human Participants and/or Animals
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The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

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- Financial support for attending symposia
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