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Dissertação

Avaliação da atividade do peptídeo antimicrobiano P34 frente a vírus patogênicos aos animais domésticos

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Good, better, best

Never let me rest

Until my good is better

And my better, best.

Tim Duncan

RESUMO

SILVA, Débora Scopel e. **Avaliação da atividade do peptídeo antimicrobiano P34 frente a vírus patogênicos aos animais domésticos.** 2013. 57f. Dissertação (Mestrado) – Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

O desenvolvimento e o uso de fármacos antivirais capazes de prevenir ou combater uma infecção são imprescindíveis para a saúde do homem e dos animais. Entretanto, o arsenal de fármacos antivirais permanece pequeno e apresenta graves restrições de uso, tais como reduzido espectro de atividade, utilidade terapêutica limitada e vários graus de toxicidade. O objetivo desse trabalho foi avaliar atividade antiviral de um peptídeo antibacteriano isolado do conteúdo intestinal do peixe Piau-com-pinta (*Leporinus* sp.), denominado P34. Sua citotoxicidade foi determinada em diferentes linhagens celulares, visando posterior avaliação da atividade antiviral. Foram realizados ensaios antivirais frente a vírus com diferentes características genotípicas e fenotípicas: adenovírus canino tipo 2 (CAV-2), coronavírus canino (CCoV), vírus da cinomose canina (CDV), parvovírus canino tipo 2 (CPV-2), vírus da arterite equina (EAV), vírus da influenza equina (EIV), calicivírus felino (FCV) e herpesvírus felino tipo 1 (FHV-1). Foi observada ação antiviral, *in vitro*, contra EAV e FHV-1, ambos vírus envelopados, com genomas RNA e DNA, respectivamente. Foi observado efeito virucida, contra o EAV, quando o P34 foi incubado por diferentes períodos a 37 °C. A análise por microscopia eletrônica de transmissão sugere que o peptídeo P34 faz ligação e promove lesão do envelope viral. O P34 não possui atividade virucida contra o FHV-1 e embora o mecanismo de ação não tenha sido completamente elucidado, é possível supor que o P34 interfira no processo de adsorção do FHV-1. Dessa maneira, o peptídeo P34 pode representar uma substância com potencial aplicação na prevenção e tratamento das infecções pelo FHV-1 e pelo EAV.

Palavras-chave: peptídeos antimicrobianos. antivirais. doenças infecciosas. herpesvírus felino. arterite viral equina

ABSTRACT

SILVA, Débora Scopel e. **Avaliação da atividade do peptídeo antimicrobiano P34 frente a vírus patogênicos aos animais domésticos.** 2013. 57f. Dissertação (Mestrado) – Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

The development and use of drugs with the ability to prevent or strike an infection are indispensable for human and animal health. However, the antiviral drug arsenal is still small and has serious use restrictions, like small activity range, limited therapeutic usage and several degrees of cytotoxicity. The objective of this work was to evaluate the antiviral activity of an antibacterial peptide isolated from the intestinal contents of the fish Piau-com-pinta (*Leporinus* sp.), named P34. Its cytotoxicity was analyzed in different cell lineages, in order to evaluate its antiviral activity. This study was performed against viruses with different phenotypical and genotypical features like: canine adenovirus type 2 (CAV-2), canine coronavirus (CCoV), canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1). It was observed antiviral activity, *in vitro*, against EAV and FHV-1, both enveloped viruses, with RNA e DNA genomes, respectively. A virucidal effect was observed when P34 was incubated for different periods of time with EAV at 37 °C. Transmission electronic microscopy analysis suggests that the peptide P34 binds and promotes lesions to the viral envelope. The peptide P34 does not have virucidal activity against FHV-1 and although its mechanism of action is not completely elucidated, it is possible to suppose that P34 interferes in the adsorption process of FHV-1. Thus, the peptide P34 may represent an antimicrobial substance with potential application in the prevention and treatment of viral infections caused by FHV-1 and EAV.

Key-words: antimicrobial peptides. antiviral. infectious disease. feline herpesvirus. equine viral arteritis

LISTA DE FIGURAS

Lista de Abreviaturas

AC – *absorbance of control* (absorbância do controle)

AMP – *antimicrobial peptide* (peptídeo antimicrobiano)

AT – absorbance of treated (absorbância dos tratados)

ATCC – *American type culture collection* (Grupo Americano de tipos de cultivo)

BFS – *bovine fetal serum* (soro fetal bovino)

BLS – *bacteriocin-like substance* (substância do tipo bacteriocina)

BoHV-1 – *bovine herpesvirus type 1* (herpesvírus bovino tipo 1)

CAV-2 – *canine adenovirus type 2* (adenovírus canino tipo 2)

CC_{50} – *cytotoxic concentration* (concentração citotóxica 50%)

CCoV – *canine coronavirus* (coronavírus canino)

CDV – *canine distemper virus* (vírus da cinomose canina)

CO_2 – dióxido de carbono

CPE – *cytopathic effect (*efeito citopático)

CPV-2 – *canine parvovirus type 2* (parvovírus canino tipo 2)

CRFK – *Crandell-Rees Feline Kidney* (linhagem de células de rim felino)

CV – *cell viability* (viabilidade celular)

Da – Dalton

DNA – ácido desoxiribonucleico

E – proteína do envelope do vírus da arterite equina

EAV – *equine arteritis virus* (vírus da arterite equina)

EC_{50} – *effective concentration* 50% (concentração eficaz 50%)

EIV – *equine influenza virus* (vírus da influenza equina)

EMEM – *Eagle's minimum essential medium* (meio essencial mínimo com sais de Eagle)

EVA – *equine viral arteritis* (arterite viral equina)

FCV – *feline calicivirus* (calicivírus felino)

FHV-1 – *feline herpesvirus type 1* (herpesvírus felino tipo 1)

GP- glicoproteína

IPVDF – Instituto de Pesquisas Veterinárias Desidério Finamor

kV – kilovolt

LPS - lipopolissacarídeo

MDCK – *Madin-Darby Canine Kidney* (linhagem de células de rim canino)

µg - micrograma

µL – microlitro

mg - miligrama

mL – mililitro

mM - milimolar

MTT – *dimethylthiazolyl diphenyltetrazolium bromide* (brometo tiazolil azul de tetrazólio)

nm - nanômetro

NRU – *neutral red dye uptake* (ensaio de coloração pelo vermelho neutro)

P34 – peptídeo antimicrobiano produzido por uma linhagem de *Bacillus* sp. P34

PI – *percent of inhibition* (percentual de inibição)

RK 13 – *rabbit kidney cells* (linhagem de células de rim de coelho)

RNA – ácido ribonucléico

RPM – *rotations per minute* (rotações por minuto)

$TCID_{50}$ – *tissue culture infective dose* 50% (dose infectante a 50% do cultivo)

TEM – *transmission electron microscopy* (microscopia eletrônica de transmissão)

TI – *therapeutic index* (índice terapêutico)

UFSM – Universidade Federal de Santa Maria

USA – *United States of America* (Estados Unidos da América)

VERO – *African Green Monkey Kidney* (linhagem de células de rim de macaco)

Sumário

1 INTRODUÇÃO

Desde sua descoberta, os peptídeos antimicrobianos vêm ganhando atenção como uma alternativa terapêutica importante no campo da prevenção e tratamento de doenças contra um grande número de micro-organismos (OYSTON et al., 2009). Isso pode ser atribuído principalmente ao aumento da resistência às drogas antimicrobianas, toxicidade e altos custos envolvidos na produção dos fármacos convencionais (MOHAN et al., 2010).

A produção de peptídeos antimicrobianos é uma característica universal dos sistemas de defesa de praticamente todas as formas de vida, com representantes encontrados em organismos como bactérias, plantas, espécies invertebradas e vertebradas, incluindo mamíferos (JENSSEN et al., 2006). Como resultado da intensa pesquisa nesse campo durante a década passada, aproximadamente 20.000 peptídeos antimicrobianos foram listados até agora (OYSTON et al., 2009). Esses peptídeos podem ser derivados da natureza ou sintéticos (ZAIOU, 2007). Os peptídeos antimicrobianos estão localizados geralmente em locais expostos à invasão microbiana, tais como o epitélio de mamíferos, anfíbios e insetos e demonstram uma potente habilidade de combater um amplo espectro de micro-organismos e células, incluindo: parasitas (LÖGFREN et al., 2008), células tumorais (DENNISON et al., 2006; HOSKIN & RAMAMOORTHY, 2008; LU et al., 2008), fungos (THEVISSEN et al., 2007; AERTS et al., 2008), bactérias Gram-positivas (MOTTA et al., 2004), Gram-negativas (LINDE et al., 2008; MOTTA et al., 2008) e vírus (CHERNYSH et al., 2002; SLOCINSKA et al., 2008), na maioria das vezes envelopados (ZASLOFF, 2002).

Peptídeos antimicrobianos produzidos por bactérias, também chamados de bacteriocinas, foram os primeiros a ser isolados e caracterizados (MATTICK & HIRSCH, 1947). Acredita-se que 99% de todas as bactérias possam produzir pelo menos uma bacteriocina (KLAENHAMMER, 1988). Elas possuem ações de pequeno ou amplo espectro, capazes de atingir micro-organismos da mesma espécie ou de

diferentes gêneros (JENSSEN et al., 2006), necessitando de uma molécula-alvo na superfície das células sensíveis para serem ativas (HÉCHARD & SAHL, 2002).

O potencial da espécie *Bacillus* de produzir antibióticos é reconhecido há mais de 50 anos (STEIN, 2005). O gênero *Bacillus* inclui uma variedade de espécies com histórico de uso seguro na indústria (BIZANI & BRANDELLI, 2002). Produtos comerciais que geralmente são obtidos de *Bacillus spp.* incluem enzimas, antibióticos, aminoácidos e inseticidas (GEBHARDT, 2002).

As bacteriocinas constituem um grupo estruturalmente diverso de peptídeos e foram classificadas em duas amplas categorias: as que contêm lantionina (lantibióticos) e as que não contêm lantionina (COTTER et al., 2005). O lantibiótico mais estudado é a nisina, produzida por *Lactococcus lactis*, que tem sido comumente usada há aproximadamente 50 anos como conservante de alimentos (JENSSEN et al., 2006). As bacteriocinas receberam atenção elevada devido ao seu potencial como conservantes na indústria de alimentos, como probióticos na saúde humana e como agentes terapêuticos contra micro-organismos patogênicos (RILEY & WERTZ, 2002). O tratamento com bacteriocinas já foi proposto como uma alternativa para o controle de doenças, visto que ele é potencialmente efetivo e não tóxico ao homem e aos animais (TWOMEY et al., 2000; HEILBORN et al., 2003).

Uma ampla variedade de organismos produz peptídeos antimicrobianos como parte de sua primeira linha de defesa (HANCOCK & LEHRER, 1998). Eles geralmente são pequenos (12 a 100 aminoácidos), têm carga positiva (carga elétrica de +2 a +9), são anfifílicos e foram isolados de micro-organismos unicelulares, insetos e outros invertebrados, plantas, anfíbios, pássaros, peixes e mamíferos, incluindo o homem (MARTIN et al., 1995; WANG & WANG, 2004). Peptídeos antimicrobianos aniônicos são muito raros (PAULMANN et al., 2006) e pensa-se que eles tenham sido desenvolvidos em resposta aos mecanismos de resistência contra os peptídeos catiônicos (LAI et al., 2002), os quais são encontrados em todas as espécies e são agentes antivirais de amplo espectro (ALBIOL MATANIC & CASTILLA, 2004).

A expressão dos peptídeos antimicrobianos pode ser constitutiva ou induzida por infecções ou estímulos inflamatórios (HANCOCK, 2001; CUNLIFFE & MAHIDA, 2004). Eles são capazes de aumentar a fagocitose, estimular a liberação de prostaglandina, neutralizar os efeitos sépticos dos lipopolissacarídeos (LPS), promover o recrutamento e acúmulo de várias células em locais de inflamação (YANG et al., 2002; ELSBACH, 2003), promover angiogênese (KOEZULLA et al., 2003) e induzir a cicatrização de feridas (CHAN & GALLO, 1998).

Numerosos peptídeos foram identificados na hemolinfa e em células fagocíticas ou epiteliais de invertebrados desempenhando um importante papel na proteção contra organismos patogênicos (CHERNYSH et al., 2002; JENSSEN et al., 2006). O papel dos peptídeos antimicrobianos e a regulação de sua expressão, incluindo as cascatas de sinalização envolvidas são bem conhecidos na espécie *Drosophila* (IMLER & BULLET, 2005). Em plantas, acredita-se que os peptídeos antimicrobianos desenvolvam um papel fundamental na defesa contra bactérias e fungos, sendo encontrados principalmente em folhas, flores, sementes e bulbos (GARCÍA-OLMEDO et al., 1998). As glândulas da pele de anfíbios são uma rica fonte de peptídeos, porém eles também são produzidos na mucosa do estômago, indicando sua ação sobre os micro-organismos ingeridos (RINALDI, 2002). As magaininas α-hélice são os peptídeos típicos dos anfíbios, com grande capacidade de permeabilizar membranas de bactérias, fungos, leveduras e vírus (ZASLOFF, 1987).

As catelicidinas são um grande e diverso grupo de peptídeos antimicrobianos de mamíferos (JENSSEN et al., 2006) e foram primeiramente isoladas de neutrófilos de suínos (REDDY et al., 2004). Os grânulos secretórios dos neutrófilos são a principal fonte de catelicidinas, mas elas também podem ser expressas em superfícies mucosas na boca, pulmões, trato genitourinário e nos queratinócitos da pele em desordens inflamatórias (FROHM et al., 1997). As catelicidinas foram isoladas de muitas espécies de mamíferos, tais como ratos, coelhos, ovelhas, cavalos e humanos (JENSSEN et al., 2006). Em um estudo conduzido por Heilborn et al. (2003), o uso da catelicidina humana LL-37 foi capaz de estimular a re-epitelização e cicatrização de feridas, *ex vivo*, em um modelo experimental de células epiteliais humanas. Recentemente, algumas catelicidinas bovinas foram avaliadas com o objetivo de determinar suas atividades contra

bactérias isoladas de animais com mastite e os resultados obtidos demonstraram eficácia em inibir as bactérias presentes no leite, além de ativar as células de defesa do hospedeiro (TOMASINSIG et al., 2010).

Outro grupo importante de peptídeos antimicrobianos de mamíferos são as defensinas (GANZ, 2003; SELSTED & OUELLETTE, 2005), peptídeos cíclicos que são categorizados em três subfamílias: α, β e θ-defensinas (TANG et al., 1999). Dependendo da espécie, as defensinas são encontradas nos grânulos de neutrófilos, macrófagos, células *Natural Killer*, células intestinais de *Paneth*, pele, superfícies mucosas e fluidos corporais (DUITS et al., 2002; FANG et al., 2003).

Embora o mecanismo de ação exato dos peptídeos antimicrobianos ainda não tenha sido completamente estabelecido, acredita-se que a fase inicial seja via interação eletrostática com a célula alvo (OYSTON et al., 2009). Os fosfolipídeos agregam carga às membranas celulares e a distribuição da carga dos peptídeos parece ter um importante papel nas interações peptídeo-membrana (OREN & SHAI, 1998). Peptídeos com carga positiva são atraídos por componentes com carga negativa, tais como o sulfato de heparina, LPS, em membranas externas de bactérias Gram-negativas, e ácidos lipotecóicos de bactérias Gram-positivas (JENSSEN et al., 2006). O modo de ação de muitos peptídeos antimicrobianos inclui atividade microbicida direta ou ação indireta, bloqueando ou inibindo uma etapa importante no ciclo de vida do micro-organismo (ZAIOU, 2007). Ainda que alguns peptídeos possuam um efeito direto no micro-organismo, danificando ou desestabilizando-o, eles parecem estar amplamente envolvidos nas respostas imunes inatas e inflamatórias (HANCOCK & DIAMOND, 2000). Para alguns peptídeos, a lise é causada pela ruptura da membrana citoplasmática, contudo, há dados que sugerem que alguns peptídeos antimicrobianos também atravessam células onde podem haver alvos intracelulares (BROGDEN, 2005). O mecanismo de ação de alguns peptídeos sobre as bactérias está bem esclarecido, porém a ação antiviral é pouco conhecida (LIU et al., 2007).

Representantes de todas as classes estruturais de peptídeos de defesa catiônicos demonstraram habilidade para inibir infecções virais (JENSSEN et al., 2006). O espectro de vírus que são afetados compreende primeiramente os vírus RNA ou DNA envelopados, com exceção do adenovírus (BASTIAN & SCHAFER,

2001; HORNE et al., 2005) e calicivírus felino (McCANN et al., 2003) que não são envelopados. A atividade antiviral dos peptídeos antimicrobianos está frequentemente relacionada aos processos de adsorção e penetração viral (BELAID et al., 2002) ou é resultado de uma ação direta sobre o envelope viral (ABOUDY et al., 1994; ROBINSON et al., 1998).

Apesar de suas estruturas diversas, muitos peptídeos possuem modos de ação antiviral análogos (JENSSEN et al., 2004), indicando que eles são capazes de interagir com seus alvos apesar das diferenças estruturais. Ainda que o alvo viral desses peptídeos varie, os efeitos antivirais demonstrados são similares (JENSSEN et al., 2006). Os peptídeos antimicrobianos podem ainda ter uma ação estágio-específica sobre cada vírus, alguns podem agir sobre a forma intracelular, extracelular ou na liberação da partícula viral (BAI et al., 2007). O efeito dos peptídeos também está relacionado às suas habilidades de inibir a propagação do vírus de uma célula a outra vizinha através de junções estreitas (disseminação célula-a-célula) ou inibir a formação de células gigantes (JENSSEN et al., 2006).

Peptídeos antimicrobianos como a melitina, a cecropina e a magainina são conhecidos por sua capacidade de interagir com membranas lipídicas, resultando em desestabilização, translocação, formação de poros ou lise (DATHE & WIEPRECHT, 1999). Essa propriedade torna o envelope viral um potencial alvo para interação direta (JENSSEN et al., 2006). O bloqueio da entrada do vírus também pode ocorrer por interação específica com receptores celulares ou com glicoproteínas virais (JENSSEN et al., 2006). Os peptídeos podem interagir diretamente com receptores específicos da célula hospedeira (COLE et al., 2002), influenciando na ligação ou entrada do vírus (JENSSEN et al., 2006). O sulfato de heparina, uma das moléculas de glicosaminoglicanos mais importantes da ligação do herpesvírus (SPILLMANN, 2001), pode ser bloqueada por alguns peptídeos antimicrobianos, reduzindo a infecção viral (SHIEH et al., 1992).

As membranas das células hospedeiras estão envolvidas em vários estágios da infecção viral e, devido à habilidade de os peptídeos antimicrobianos interagirem e permeabilizarem membranas, elas devem ser consideradas potenciais alvos, visto que a alteração da membrana celular hospedeira pode afetar a eficiência da entrada do vírus (JENSSEN et al., 2006).

Sabe-se que os peptídeos de defesa do hospedeiro são capazes de atravessar membranas lipídicas, enquanto outros peptídeos estão localizados, como precursores, dentro de vacúolos da célula hospedeira (ANDERSEN et al., 2004). A internalização celular desses peptídeos pode resultar na estimulação de genes e proteínas, influenciando os mecanismos antivirais da célula hospedeira (BOWDISH et al., 2004), ou pode bloquear a expressão de genes e proteínas virais (WACHINGER et al., 1998). A replicação do genoma, ou sua função, pode ser afetada por um agente que se incorpore ou se ligue ao molde de RNA ou DNA (PRUSOFF et al., 1986). Devido à habilidade de os peptídeos antimicrobianos interagirem com o DNA (HSU et al., 2005; SONG et al., 2005), especula-se que eles possam influenciar diretamente a síntese do ácido nucléico viral (JENSSEN et al., 2006). Tossi et al. (2000) referem que alguns peptídeos possuem a capacidade de induzir a degradação de proteínas utilizadas na replicação do DNA.

Estudos relataram atividade antimicrobiana de substâncias produzidas entre várias bactérias isoladas de meios aquáticos da Bacia Amazônica brasileira (MOTTA et al., 2004). Entre elas, uma substância produzida por uma espécie de *Bacillus* sp., isolada do conteúdo intestinal do peixe teleósteo Piau-com-pinta (*Leporinus* sp.) foi purificada, caracterizada e denominada P34. O peptídeo antimicrobiano P34 é uma substância do tipo bacteriocina que inibe bactérias Gram-positivas e Gram-negativas, incluindo micro-organismos patogênicos e deteriorantes (MOTTA et al., 2007a). O efeito do P34 sobre *Listeria monocytogenes* e *Bacillus cereus* demonstrou ser bactericida e bacteriolítico, respectivamente (MOTTA et al., 2007a). Essa substância antimicrobiana é aniônica (MOTTA, 2006), hidrofóbica, tem massa molecular de 1.456 Da, é relativamente estável ao aquecimento e sensível a enzimas proteolíticas, sugerindo ser uma molécula lipopeptídica (MOTTA et al., 2007b). Motta et al. (2008) relataram que o P34 é capaz de provocar a formação de poros, perda do conteúdo protoplásmico e lise da bactéria *Listeria monocytogenes*.

O peptídeo P34 teve sua atividade parcialmente perdida quando tratado com os solventes orgânicos butanol, acetona e metanol e também mostrou-se sensível aos químicos ácido tricloroacético e 2-mercaptoetanol, contudo, ele é estável em pH ácido e básico (MOTTA et al., 2007a). A perda total de sua atividade antimicrobiana só foi observada após 15 minutos de autoclavagem a 121 °C (MOTTA et al., 2007b). Em um estudo realizado por Vaucher et al. (2010) células VERO (*African green*

monkey kidney) foram tratadas com P34 e observou-se perda da integridade da membrana plasmática após 24h, quando utilizadas altas concentrações do peptídeo. Também foram relatadas diminuição da viabilidade e motilidade espermática (VAUCHER et al., 2010). O P34 livre e encapsulado em nanovesículas parece ser eficaz no controle de *Listeria monocytogenes* no leite, especialmente desnatado (MALHEIROS et al., 2012b), e em queijo Minas frescal (MALHEIROS et al., 2012a).

Pouca atenção tem sido destinada à aplicação dos peptídeos como antimicrobianos em estudos clínicos (MOTTA et al., 2007a). Novos tratamentos com os peptídeos poderiam ser usados em conjunto com as drogas já existentes, como parte de uma terapia combinatória para obter efeitos sinérgicos ou adicionais (JENSSEN et al., 2006). Progressos foram feitos no campo da terapia com peptídeos contra o vírus da imunodeficiência humana (LIU et al., 2005; SUN et al., 2005), herpesvírus simples, rotavírus, adenovírus (CARRIEL-GOMES et al., 2007), citomegalovírus humano (LUGANINI et al., 2010), vírus da hepatite C (YAN et al., 2011), entre outros.

O rápido aumento e disseminação de micro-organismos multiresistentes obrigaram os pesquisadores a considerar métodos alternativos para combater as infecções (MOTTA et al., 2007a). Dada a diversidade de peptídeos antimicrobianos produzidos na natureza, eles podem ser considerados como uma alternativa no combate a infecções contra micro-organismos específicos (RILEY & WERTZ, 2002). O desenvolvimento de fármacos derivados de estudos clínicos e laboratoriais a respeito dos peptídeos antimicrobianos promete revolucionar o tratamento de muitas doenças inflamatórias e infecciosas (GALLO et al., 2002).

Diante do desafio de buscar novos compostos antivirais que sejam eficazes no tratamento e na prevenção das infecções no ramo da medicina veterinária, o presente trabalho teve como objetivo a avaliação da atividade exercida pelo peptídeo P34 contra o adenovírus canino tipo-2, coronavírus canino, vírus da cinomose canina, parvovírus canino tipo-2, vírus da arterite equina, vírus da influenza equina, calicivírus felino, herpesvírus felino tipo-1.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o potencial do peptídeo antimicrobiano P34, produzido pelo *Bacillus* sp. linhagem P34, quanto a sua atividade antiviral frente a alguns vírus patogênicos aos animais domésticos e determinar o seu mecanismo de ação.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a citotoxicidade do peptídeo antimicrobiano P34 em linhagens celulares CRFK, MDCK e RK 13.

- Avaliar o possível efeito antiviral e virucida do P34 frente ao CAV-2, CCoV, CDV, CPV-2, EAV, EIV, FCV e FHV-1.

3 ARTIGOS

3.1 Artigo 1

Screening for antiviral activity of a novel antimicrobial peptide produced by *Bacillus* sp. P34 against pathogenic viruses of domestic animals

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Screening for antiviral activity of a novel antimicrobial peptide produced by *Bacillus* sp. P34 against pathogenic viruses of domestic animals

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Abstract

P34 is an antimicrobial peptide produced by a species of *Bacillus* isolated from the intestinal contents of a fish in the Amazon basin with reported activity against bacteria. The aim of this work was to evaluate the peptide P34 for its antiviral properties *in vitro* against canine adenovirus type 2 (CAV-2), canine coronavirus (CCoV), canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivírus (FCV) and feline herpesvirus type 1 (FHV-1). The results showed that the peptide P34 exhibited *in vitro* antiviral activity against EAV and FHV-1. P34 inhibited the replication of EAV by 99,9% and FHV-1 by 94,4%. Virucidal activity was detected only against EAV. When P34 and EAV were incubated for 6 h at 37 °C the viral titer reduced from $10^{4.5}$ $TCID_{50}$ to $10^{2.75}$ $TCID_{50}$, showing a percent of inhibition of 98.6%. In conclusion, we demonstrated that P34 inhibited EAV and FHV-1 in infected cell cultures and showed virucidal activity against EAV. Since there is documented resistance to the current drugs used against herpesviruses and there is no treatment for equine viral arteritis, it is advisable to search for new antiviral compounds to overcome those infections.

Key words: antimicrobial peptides; antiviral activity; herpesvirus, equine viral arteritis

1. Introduction

The impact of the increasing resistance of microorganisms to drugs and specific substances has been motivating several research groups. Since their discovery, the antimicrobial peptides (AMPs) are conquering special attention as an important therapeutic alternative in the field of prevention and treatment of infections against a large number of microorganisms [26]. AMPs are a universal feature of the defense systems of all forms of life, with representatives found in organisms ranging from bacteria, plants, invertebrate and vertebrate species, including mammals [14]. Studies about antiviral compounds date from 1950 [11], but for several reasons such as serious side effects, just a few drugs were approved for clinical use [8].

Antimicrobial activity was reported among several bacteria isolated from the aquatic environments of Brazilian Amazon basin [22]. Among them, a species of *Bacillus* producing an antimicrobial peptide was isolated from the intestinal contents of the fish Piau-com-pinta (*Leporinus* sp.) [24]. This peptide was purified and named P34 and its antimicrobial activity was characterized as a bacteriocin like substance [21]. Its inhibitory activity was detected against Gram-positive bacteria, like *Listeria monocytogenes* and *Bacillus cereus* [24], and Gram-negative bacteria like *Escherichia coli* [20] and *Salmonella enteritidis* [23]. While some studies on P34 have shown its importance as a food preservative [21], little attention has been addressed to its application as an antimicrobial substance in clinical studies.

So far, there is no data regarding P34 antiviral activity, thus the aim of the present work was to evaluate the activity exerted by the peptide P34 against the canine adenovirus type 2 (CAV-2), canine coronavirus (CCoV), canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1).

2. Material and methods

2.1. Antimicrobial peptide (P34), cells and viruses

The peptide P34 was produced as described elsewhere [24]. After purification, total protein concentration was measured in triplicate by the Lowry method according to the manufacturer's protocol (Total Protein Kit, Micro Lowry, Peterson's Modification – Sigma Aldrich, USA). The peptide was stored at -20 °C until used for antiviral assays.

Madin-Darby Canine Kidney (MDCK - ATCC® Number: CCL-34^{TM}, USA), Crandell-Rees Feline Kidney (CRFK - ATCC® Number: CCL-94^{TM}, USA) and Rabbit Kidney (RK13 - ATCC® Number: CCL-37^{TM}, USA) cells were cultivated in Eagle's minimum essential medium (E-MEM – Sigma Aldrich, USA) supplemented with 10% of bovine fetal serum (BFS – Gibco, USA), penicillin (Sigma Aldrich, USA), streptomycin (Vetec, Brasil), amphotericin B (Cristália, Brasil) and enrofloxacin (Bayer, Brasil), in an incubator at 37 °C.

The antiviral activity of the AMP P34 was evaluated against viruses with different phenotypic and genotypic features. FCV [37], CCoV (MAV 795 strain), EAV (Bucyrus strain) and EIV (local isolate) were kindly provided by the Virology Laboratory of the Federal University of Santa Maria (UFSM), Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) provided CAV-2 (Toronto A26/61 strain), CDV (Lederle VR128 strain), CPV-2 (Cornell strain) and FHV-1 (B927 strain). These viruses were propagated on MDCK, CRFK or RK13 cell cultures.

2.2. Cytotoxicity assays

MDCK, CRFK and RK13 cells grown in 96-well tissue culture plates (TPP, Switzerland) were incubated with different concentrations of P34 for 72 h at 37 °C and 5% CO_2. Cell viability was measured by the neutral red dye uptake (NRU) assay [4] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) procedure [19]. The percentage of cell viability (CV) was calculated as: CV = AT/ AC x 100, where AT and AC were the absorbances of treated and control cells, respectively [35]. The cytotoxicity of P34 was expressed as the 50% cytotoxic concentration (CC_{50}).

2.3. Antiviral assays

2. 3. 1. Cytopathic effect inhibition (CPE) assay

The inhibition of CPE assays were performed on confluent MDCK, CRFK and RK13 cell monolayers, in the presence or absence of P34. End-point titrations were performed as described elsewhere [16] and titers were expressed in Tissue Culture Infective Dose 50% ($TCID_{50}$/100µL). The cells were observed for CPE daily for 72 h at an Olympus CK-2 inverted microscope.

The viral percents of inhibition (PI) were calculated by PI= [1- (Titer of treated/ Titer of controls)] x 100, adapted from Felipe et al. [11].

2.3.2. P34 virucidal effect

Virus strains were incubated at 37 °C for 6 h with E-MEM in the presence or absence of P34. After the incubation period, the infectivity was immediately determined by virus titrations on cell cultures.

2.4. Statistical analysis

All assays were performed in triplicate and mean values were calculated using Microsoft Excel®. Statistical analysis were performed using a two-tailed Student's t-test and values were considered significant when $P < 0.05$.

3. Results

3.1. P34 cytotoxicity

In order to distinguish selective antiviral activity from cytotoxicity, the peptide was evaluated on MDCK, CRFK and RK13 cells by the NRU and MTT assays. CC_{50} was quite similar in both NRU and MTT tests for each cell lineage. CC_{50} values were 2,11µg/mL, 2,5µg/mL and 3,92 µg/mL for MDCK, CRFK and RK13 cells, respectively. Cytotoxicity was not observed at 1,37 µg/mL of the peptide P34 for MDCK, 0,92 µg/mL for CRFK and 2,29 µg/mL for RK13 cell cultures. These concentrations were then used in all the subsequent assays.

3.2 Antiviral assays

Titrations showed that the presence of the peptide P34 had no statistically significant effect ($P > 0.05$) against the production of viral particles of CAV-2, CCoV, CDV, CPV-2, EIV and FCV. However, a significant reduction on viral titers occurred when P34 was incubated with EAV and FHV-1. The titer of EAV was expressively reduced from 10^7 $TCID_{50}$ to $10^{1,75}$ $TCID_{50}$ in the presence of P34, presenting a PI of 99,9%. The titer of FHV-1 was $10^{4,5}$ $TCID_{50}$ in the presence of P34 and $10^{5,75}$ $TCID_{50}$ in its absence, resulting in a PI of 94,4%. The peptide P34 had only a direct inactivating effect against EAV infectious particles. A potent virucidal effect was observed and EAV infectivity was reduced by 98.6%. After 6 h of incubation, EAV titer was reduced from $10^{4.5}$ $TCID_{50}$ to $10^{2.75}$ $TCID_{50}$ in the presence of P34 ($P > 0.05$).

4. Discussion

A great number of biological activities from peptides isolated from different sources have been studied for antiviral activities [3]. In the last years several AMPs were and continue to be tested in laboratories, but just a few of them have reached the clinical routine [25, 36].

Ideally, to be the most useful, any antimicrobial agent has to exhibit a broad-spectrum antimicrobial activity [17]. P34 is an anionic [20], thermostable, hydrophobic, lipidic, bacteriocin-like substance produced by a *Bacillus* sp. with antimicrobial properties against bacteria [24] and viruses, according to the present study. However, anionic antimicrobial peptides are very rare [27] and it is thought that these peptides were developed in response to the resistance mechanisms toward cationic antimicrobial peptides [15], which are found in all species and are potential broad-spectrum antiviral agents [2].

In order to evaluate the anionic peptide P34 as an antiviral substance *in vitro*, CAV-2, CPV-2 and FHV-1 were exposed to the AMP, being all DNA viruses, only FHV-1 having an envelope [18, 6, 11]. The RNA viruses tested were CCoV, CDV, EAV, EIV and FCV, all enveloped viruses except for FCV [7, 30, 12, 1, 10]. According to the assays performed it is clear that the peptide P34 does not have a broad antiviral activity, since it only inhibited EAV and FHV-1.

Peptides have demonstrated their ability to kill rapidly a broad range of microorganisms including multidrug resistant bacteria, fungi and viruses by their lytic membrane properties [28]. AMPs like magainin, mellitin and cecropin are known for their ability to interact with lipid membranes resulting in destabilization, translocation, pore formation or lysis [32]. Blocking viral entry may occur by specific interactions with cellular receptors or viral envelope compounds, apart from viral glycoproteins [14]. A possible mechanism proposed to explain the activity of P34 against FHV-1 would be its interaction with cellular receptors like heparan sulfate, or the blocking of certain viral glycoproteins. Heparan sulfate is the most important glycosaminoglycan molecule with respect to herpesvirus attachment [33], consequently, blocking of heparan sulfate can reduce the viral infection [31]. Thus, it is possible that the P34 peptide interferes in the adsorption, penetration or viral replication, or even exerts a competition with the viral particles for the cellular receptors used for EAV and FHV-1 infections.

The virucidal activity of P34 seems to be virus-specific as no viral inactivation was detected against all viruses tested, except for EAV. Although antiviral activity against enveloped viruses has been attributed to direct lysis of viral membranes, we hypothesize that the peptide P34 inactivates the virus through an interaction with a non-lipidic structural component since no activity was detected against other enveloped viruses analyzed.

EAV is a member of the family *Arteriviridae*, and belongs to the order *Nidovirales*, along with porcine respiratory and reproductive syndrome virus, simian hemorrhagic fever virus and lactate dehydrogenase elevating virus [9, 12]. Although equine viral arteritis (EVA) causes severe economic losses to the equine industry, there is no specific treatment [34], so it seems advisable to develop antiviral drugs for the treatment of the disease. Herpesviruses are cosmopolite agents causing several infections to humans and animals, especially in immunocompromised individuals [11]. An important feature of the members of this family is their ability to cause and reactivate latent infections in their hosts, and this is important for the control of the disease [13]. Among the drugs that possess inhibitory action against herpesvirus replication, the most used in the human medicine are the nucleoside analogues [5] and there is evidence of resistance to some of them [8], so it is necessary to develop antiviral drugs with alternative mechanisms of action.

The current antiviral drug armamentarium comprises about 40 compounds that have been officially approved for clinical use; however, most of the approved drugs are used for the treatment of human immunodeficiency virus infections [11]. The fast and increased pathogen dissemination and resistance to drugs have forced the scientists to consider alternative methods to overcome infections [21]. Therefore, as many AMPs are produced in nature, they may become an alternative to control specific pathogen infections [29] and, according to the present study, the peptide P34 may be an interesting therapeutic prospection for the treatment of horses and cats affected by the equine arteritis virus and feline herpesvirus type-1, respectively. However, more detailed studies must be performed to elucidate the P34 antiviral mechanism of action in order to apply it *in vivo* in the future.

In summary, from the results obtained we can conclude that the peptide P34 has antiviral activity against EAV and FHV-1, a virucidal effect was only observed against EAV. Nevertheless no antiviral activity was detected against CAV-2, CCoV, CDV, CPV-2, EIV and FCV.

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3.2 Artigo 2

Antiviral properties of a novel antimicrobial peptide derived from *Bacillus* sp. P34 against equine arteritis virus

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Abstract

P34 is a novel antimicrobial peptide produced by a species of *Bacillus* isolated from the intestinal contents of a fish in the Amazon basin. This peptide showed antibacterial properties against Gram-positive and Gram-negative bacteria and was classified as a bacteriocin like substance. The aim of this work was to evaluate P34 for its antiviral properties *in vitro* against the equine arteritis virus. The results obtained show that P34 exerts antiviral and virucidal properties against equine arteritis virus, acting probably in the viral envelope. The antiviral assays performed showed that P34 reduces significantly the viral titers of treated cell cultures. The mechanism of action of P34 seems to be time/temperature-dependent. This peptide tends to be a promising antiviral compound for the prevention and treatment of arteriviral infections.

Key words: arterivirus, antiviral activity, antimicrobial peptide

1. Introduction

Equines are often infected by the equine arteritis virus (EAV) resulting in the development of panvasculitis, edema, hemorrhage and abortion (Jeronimo and Archambault, 2002). EAV belongs to the order *Nidovirales*, which consists of a broad group of viruses with glycosylated envelopes containing linear, single-stranded RNA genomes of positive polarity, as the toroviruses, arteriviruses, and roniviruses (Gorbalenya et al., 2006). EAV is a member of the family *Arteriviridae*, along with porcine respiratory and reproductive syndrome virus, simian hemorrhagic fever virus and lactate dehydrogenase elevating virus (Cavanagh, 1997; de Vries et al., 1997). It was first isolated from a fetal lung collected during an epizootie of abortion in Ohio, in the United States (Bryans et al., 1957). Meanwhile, reports of a respiratory disease similar to the equine viral arteritis (EVA), which presented abortion and was transmitted venereally occurred still at the end of the XIX century (Pottie, 1888), suggesting that EAV has been present as an infectious agent inside the equine population for a long time (Glaser et al., 1996).

EVA may be confused with other equine diseases and must be considered in sporadic and epizootic respiratory syndromes and foal death associated with respiratory and/or enteric signs (Del Piero, 2000). Virus persistence is maintained in the male reproductive tract within the accessory sex glands and it is testosterone-dependent (Glaser et al., 1996). Persistently infected stallions shed EAV continuously in the semen and the virus survives chilling and freezing (Timoney and McCollum, 1993). The mechanism by which the persistent infection is established is not known, but sexual maturity may be a contributing factor (Glaser et al., 1996).

Although EVA may cause prominent economic losses for the equine industry, there is no specific treatment (Timoney and McCollum, 1993), except for castration to prevent persistent infection (Glaser et al., 1996). Therefore, it seems advisable to develop safe and effective antiarteriviral strategies to control and prevent EAV infections. EAV was not isolated in Brazil yet, but there are serological studies proving the presence of this agent in our country (Souza et al., 1999; Heinemann et al., 2002; Diel et al., 2006; Lara et al., 2006). Moreover there are reports about the isolation of EAV next to our boundaries, in Argentine (Echeverria et al., 2003; Metz et al., 2008; Metz et al., 2010).

Natural peptides have a variety of interesting biological activities including antibacterial, antifungal, antiparasitic, antitumoral, and antiviral properties (Gallo et al., 2002). These

peptides, either inducible or constitutive, have been found in almost all groups of animals (Andreu and Rivas, 1998). Motta et al. (2004) reported the isolation of a species of *Bacillus*, producing an antimicrobial peptide (AMP), from the aquatic environments of Brazilian Amazon basin. This peptide was purified and named P34 and its antimicrobial activity was classified as a bacteriocin like substance (Motta et al., 2007b). Its inhibitory activity was detected against Gram-positive bacteria, like *Listeria monocytogenes* and *Bacillus cereus* (Motta et al., 2007b), and Gram-negative bacteria like *Escherichia coli* (Motta, 2006) and *Salmonella enteritidis* (Motta et al., 2008). Previous studies have evaluated the peptide P34 for its antiviral properties *in vitro* against some viruses of veterinary importance, revealing P34 is active against EAV and FHV-1. In this report, we described the evaluation of the activity exerted by P34 against EAV *in vitro* and determined its antiviral mechanism of action.

2. Material and methods

2.1. Antimicrobial peptide (P34), virus and cells

The peptide P34 was isolated from a lineage of *Bacillus* detected in the intestinal contents of the fish Piau-com-pinta (*Leporinus* sp.) (Motta et al., 2007b). P34 was produced as described by Motta et al. (2007a). After purification, total protein concentration was measured in triplicate by the Lowry method according to the manufacturer's protocol (Total Protein Kit, Micro Lowry, Peterson's Modification – Sigma Aldrich, USA). The peptide was stored at -20 °C until used for antiviral assays.

The Bucyrus strain of EAV, kindly provided by the Virology Laboratory of the Federal University of Santa Maria, was used in the experiments and propagated in Rabbit Kidney (RK13 - ATCC® Number: CCL-37^{TM}, Rockville, USA) cell cultures, as described by Snijder and Meulenberg (1998).

Cells were cultured in Eagle's minimum essential medium (E-MEM – Sigma Aldrich, USA) supplemented with 10% bovine fetal serum (BFS – Gibco, USA), penicillin (Sigma Aldrich, USA), streptomycin (Vetec, Brazil), enrofloxacin (Bayer, Brazil) and amphotericin B (Cristália, Brazil) in a 5 % CO_2 incubator at 37 °C.

2.2. Cytotoxicity assays

Monolayers of RK13 cells grown in 96 well tissue culture plates (TPP, Switzerland) were incubated with different concentrations of P34 for 72 h at 37 °C. Cell viability was measured by the neutral red dye uptake (NRU) assay (Borenfreund and Puerner, 1984) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) procedure (Mosmann, 1983). The percentage of cell viability (CV) was calculated as: CV = AT/ AC x 100, where AT and AC were the absorbances of treated and control cells, respectively. The cytotoxicity of P34 was expressed as the 50% cytotoxic concentration (CC_{50}).

2.3. Antiviral assays

The peptide P34 was evaluated for its antiviral potential at different stages of viral infection. The antiviral activity was expressed as the percent of inhibition (PI), adapted from Felipe et al. (2006), using antilogarithmic tissue culture infective dose ($TCID_{50}$) values as follows: PI = [1- (titer of treated/titer of controls)] x 100. The peptide concentration preventing the cytopathic effect induced by the virus in 50% was defined as the 50% effective concentration (EC_{50}). The therapeutic index (TI) was determined by the ratio between CC_{50} and EC_{50}. Infection without the peptide P34 was taken as the control.

2. 3. 1. Cytopathic effect (CPE) inhibition assay

Initially, an inhibition of CPE assay was performed by titrations on confluent RK13 cell monolayers, in the presence or absence of P34. The statistical method of Behrens and Kärber was used to determine the 50% end-point (Mahy and Kangro, 1996). The cells were observed for CPE daily for 72 h at an Olympus CK-2 inverted microscope. Titers were expressed as $TCID_{50}$/100µL.

2.3.2. Virus yield reduction assay

This assay was conducted as described by Luganini et al. (2010) with modifications. RK13 cells were infected with 100 $TCID_{50}$ of EAV and incubated at 37 °C for 1 h. Following virus binding, the inoculum was aspirated and fresh E-MEM was added, in the presence or in the absence of P34. After 24, 48, 72 and 96 h post infection, plates were frozen-thawed and the supernatants were used for further titrations.

2.3.3. Receptor competition assay

Confluent RK13 cell cultures grown in 96 well microplates were infected with 100 $TCID_{50}$ of EAV or with 100 $TCID_{50}$ of EAV mixed with the peptide P34 (final concentration 2,29 μg/mL) and incubated at 37 ºC for 1 h. Following incubation, the virus or the mixture were aspirated, cells were washed and fresh E-MEM was added. Similarly, the effect of the peptide P34 on RK13 cells was analyzed by treating cells individually with this peptide for 1 h in the same concentration above. Virus infectivity was assessed by inoculating 100 $TCID_{50}$ of EAV for 1 h at 37 ºC onto those treated cells. After 72 h the plates were frozen-thawed and viral titers were measured.

2.3.4. P34 virucidal effect

Suspensions of EAV or EAV with P34 (2,29 μg/mL) were incubated at 4 ºC, 20 °C and 37 °C for 30 min, 1 h, 2 h, 3 h, 4 h, 6 h and 12 h. To verify the activity derived from an interference of the peptide P34 with a lipidic structural component, other than the proteins from the envelope, an incubation of the bovine herpesvirus type 1 (BoHV-1) was also performed with P34 at 37 °C during 6 h (data not shown). Immediately after incubation, viral titers were determined and the virucidal activity was expressed as $TCID_{50}$ and PI.

2.4. Transmission electron microscopy (TEM)

RK13 cells were infected with EAV and after 48 h the infected cultures were frozen-thawed to extract virus particles. The cleared supernatant was incubated on a plate shaker for 12 h at 37 °C, in the presence or absence of P34 (2,29 μg/mL). The samples were layered onto a 25% sucrose cushion made up in TM buffer (20mM Tris-HCl [pH 7,6] – 20mM $MgCl_2$) and centrifuged in an SW 50.1 rotor at 28 000 rpm for 3 h at 4 ºC as described elsewhere (Wieringa et al., 2004). The resultant pellets were resuspended in 100 μL of phosphate saline buffer and stored at 4 °C. Both samples were fixed with 2.5% (v/v) glutaraldehyde/20mM sodium cacodylate and then postfixed in 1% (w/v) osmium tetroxide. Dehydration was done in a graded ethylic alcohol (30-100%) and acetone series. After dehydration, samples were embedded in Araldite-Durcupan (Durcupan ACM, Fluka, USA) for 72 h at 60 °C. Ultrathin sections (Ultramicrotome UPC 20, Leica, Germany) were mounted on grids and poststained with 2% uranyl acetate. Preparations were observed with a Zeiss EM 109 (Germany) transmission electron microscope operating at 80 kV.

2.5. Statistical analysis

All assays described were performed in triplicate. Statistical analysis was performed using mean values by a two-tailed Student's t-test. Values were considered significant when $P < 0.05$.

3. Results

3.1. P34 cytotoxicity

To distinguish selective antiviral activity from cytotoxicity, the peptide was evaluated on RK13 cells by the NRU and MTT assays. CC_{50} was 3,92 µg/mL in both tests. Cytotoxicity was not observed at 2,29 µg/mL of the peptide P34, and this concentration was used in all the assays performed. The EC_{50} median value obtained for the antiviral effect of P34 against EAV in cell culture was 0,28 µg/mL.

3.2. Antiviral assays

3.2.1. CPE inhibition assay

The ability of P34 to inhibit CPE caused by EAV was evaluated and the results demonstrated a significant reduction on virus titer. In the presence of P34 a titer of $10^{1,75}$ $TCID_{50}$ was obtained, while in its absence the titer was 10^{7} $TCID_{50}$, resulting in a PI of 99,9%.

3.2.2. P34 inhibits EAV during the post-infection stage

Virus yield reduction assays were performed in order to evaluate at which stage of the viral infection the peptide P34 demonstrated antiviral activity. The titrations performed with the aliquots from the plate frozen after 24 h demonstrated median titers of $10^{3,51}$ $TCID_{50}$ in the absence of peptide and $10^{1,86}$ $TCID_{50}$ in the presence of the peptide P34, resulting in a PI of 97,7%. After 48 h a reduction from $10^{5,04}$ $TCID_{50}$ to $10^{2,63}$ $TCID_{50}$ was observed in the presence of the peptide P34 (PI of 99,6%) and after 72 h, from $10^{5,85}$ $TCID_{50}$ to $10^{3,11}$ $TCID_{50}$ (PI of 99,8%). No significant difference was observed between viral titers in the presence or absence of the peptide P34 when cells were infected with 100 $TCID_{50}$ of EAV for 96 h.

3.2.3. P34 inhibits EAV adsorption on RK13 cells

Once we observed that P34 was able to inhibit EAV, the next step was to address whether P34 would still inhibit EAV during its adsorption on RK13 cells. When the peptide P34 and 100

$TCID_{50}$ of EAV were both added to the cells, no infectious virus was detected even after 72 h, indicating an impeachment of EAV. The infection of RK13 cells just with 100 $TCID_{50}$ of EAV resulted in a titer of $10^{6,5}$ $TCID_{50}$.

3.2.4. P34 peptide does not bind on RK13 cell receptors

RK13 cells treated for 1 h with the peptide P34, before the addition of 100 $TCID_{50}$ of EAV, did not influence virus infectivity since there was no significant titer reduction when compared with the EAV control inoculation. This suggests that P34 does not interact with RK13 cell surfaces and the hindrance of cellular receptors and/or of viral attachment proteins are not involved in its antiviral mechanism.

3.2.5. P34 virucidal activity

The peptide P34 showed virucidal activity; however, temperature had a strong influence on the inactivation rate. The incubation performed with EAV mixed with P34 at temperatures of 4 °C and 20 °C did not result in statistically significant difference in viral titers ($P > 0.05$). Virucidal effect was detected when EAV and P34 were incubated at 37 °C. Thus, to determine the effective virucidal time needed for P34 to act on EAV, different treating times were tested (Fig. 1).

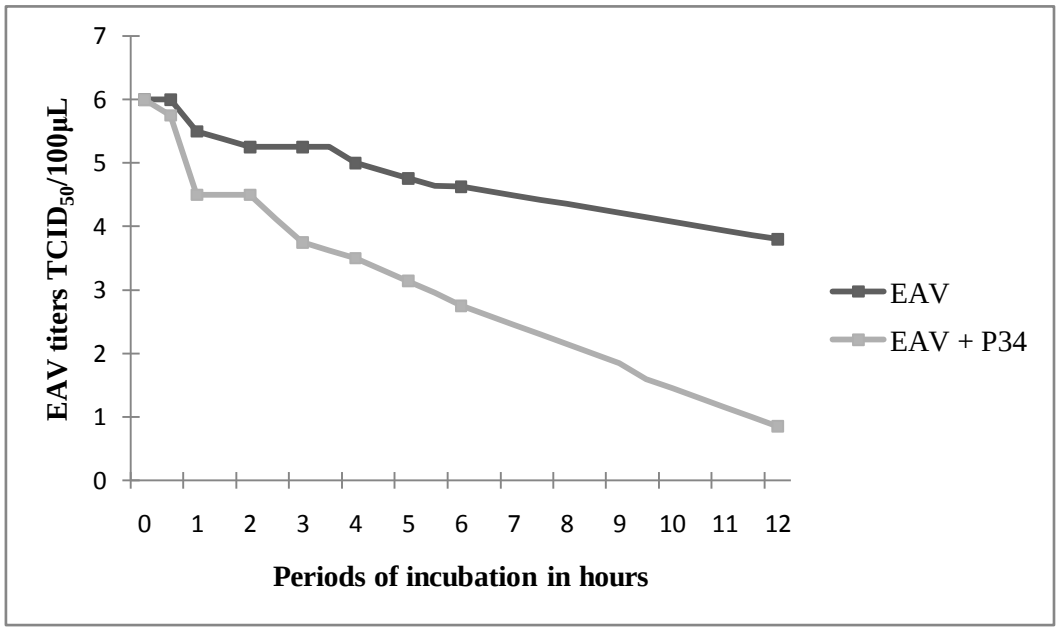

Fig. 1. EAV titers ($TCID_{50}$/100µL) after different periods of incubation at 37 °C in the absence or presence of the peptide P34 (2,29 µg/mL).

The virucidal effect was first detected with 1 h of incubation with P34. When EAV and P34 were incubated for 6 h at 37 °C, the virus titer reduced from $10^{4.5}$ $TCID_{50}$ in the absence of P34 to $10^{2.75}$ $TCID_{50}$ in its presence, showing a PI of 98.6%. Furthermore, incubation for 12 h resulted in viral titers even more significantly reduced ($P < 0.01$) in the presence of P34 ($10^{0.85}$ $TCID_{50}$) when compared to the control ($10^{3.80}$ $TCID_{50}$), resulting in a PI of 99.8%.

3.2.6. TEM

Round viral particles with about 50 nm in diameter were observed in the control sample (Fig.2A), while the EAV particles treated with P34 were damaged (Fig. 2B). Lysis and loss of the original shape were observed in the EAV treated particles. Besides, the images obtained suggest the release of the inner contents of the treated viral particles (perhaps viral nucleic acid).

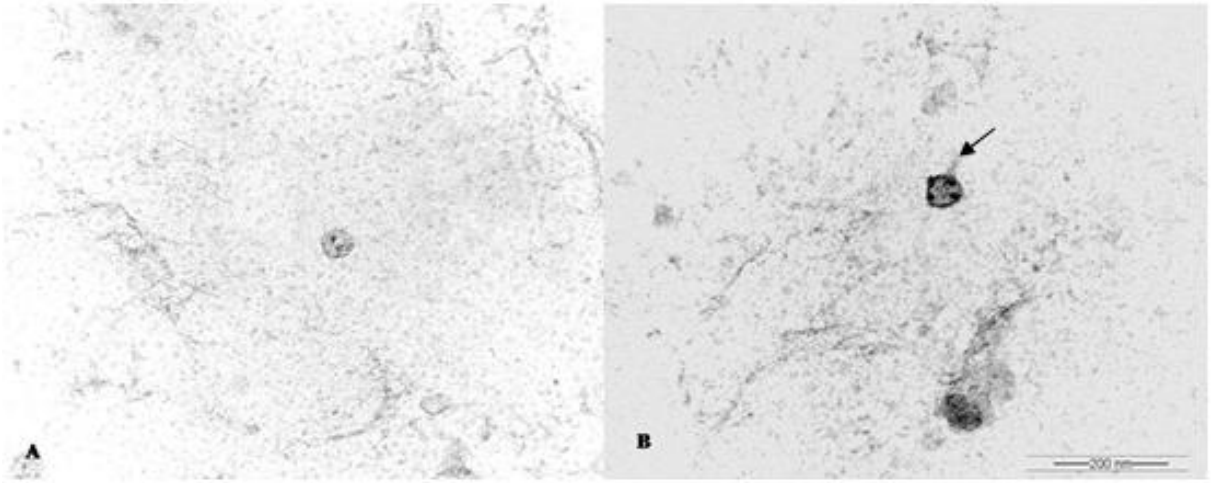

Fig. 2. Transmission electron micrographs. (A) Spherical morphology of a normal EAV particle with about 50 nm in diameter. (B) EAV particle treated with the peptide P34 showing loss of shape, vacuolization and probable release of the inner contents (arrow). 85.000 x magnification. Scale bar = 200 nm.

4. Discussion

Although the antibacterial and antifungal activities of antimicrobial peptides have been the main focus of studies to date, some of these molecules have also shown to be effective against viral pathogens (Albiol Matanic and Castilla, 2004; Carriel-Gomes et al., 2007; Sun et al., 2005; Yan et al., 2011). In the present study we evaluated the inhibitory and virucidal activities of the peptide P34, produced by *Bacillus* sp. P34, against EAV. The results obtained

were promising and indicated that P34 reduces EAV yields without toxic effects to the cells. The P34 exerted its antiviral effect by preventing the viral adsorption on the cells, probably by binding to the virus particles. Furthermore, tests performed to evaluate the virucidal activity of P34 demonstrated that the peptide had a directed inhibitory effect against the extracellular virus.

Cytotoxicity assays are necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity or programmed cell death (Eisenbrand et al., 2002). According to Al-Khayat and Ahmad (2012) if TI is one or less, the drug has significant side effects, but if the index is larger than one, the margin of safety is large. Thus, our results about the TI showed that P34 tends to be a promising therapeutic drug, considering its TI value was 14.

P34 is an anionic (Motta, 2006), thermostable, hydrophobic, lipidic peptide, with antimicrobial properties described against bacteria (Motta et al., 2007b). Bacteria treated with P34 suffered cytoplasmic membrane alteration, resulting in vacuolization of the protoplasm, pore formation and disintegration of the cells, demonstrating thus a bactericidal effect (Motta et al., 2008). As many AMPs show the ability to interact with lipid membranes (Jenssen et al., 2006) the viral envelope could be a possible target for direct interaction with the peptide P34. The incubations of EAV with P34 at 37 °C in different periods of time resulted in significant reduction of virus titers, indicating that the peptide is able to inactivate the EAV particles. When transmission microscopy analysis was performed, the images obtained suggested destabilization, changes in shape, lysis of the particles and, possibly, release of the viral nucleic acids into the surrounding medium. Based on these statements, samples observed by EM suggest that P34 exerts its virucidal activity by interacting with the viral envelope.

The P34 virucidal potential can be attributed to the membrane lytic properties described about other peptides (Reddy et al., 2004) and this ability can be related to their hydrophobic or hydrophilic helicoidal components (Matsuzaki et al., 1997). It was also observed that this virucidal effect was time/temperature-dependent, since the effect increased with higher incubation times. The same event did not occur at 4 °C and 20 °C. As demonstrated in Fig. 1, the virucidal effect of P34 against EAV was more effective with 12 h of incubation, reaching a PI of 99,8%. Furthermore, the virucidal activity of P34 appears to be virus-specific as no viral inactivation was detected when it was incubated with BoHV-1, another enveloped virus. Then we hypothesize that the peptide P34 inactivates the virus through an interaction with a

non-lipidic structural component. The peptide P34 was able to inhibit the entry of EAV into RK13 cells when they were both added to the cells during the adsorption step (1 h at 37 °C), apparently influencing viral binding or entrance. The addition of P34 for 1 h onto the cells, before the inoculation of EAV, did not interfere with viral attachment and posterior infection. Therefore, we suppose there is no direct interaction of P34 with EAV specific receptors from the host cell.

Arterivirus entry relies on receptor-mediated endocytosis, but the identities of the cellular receptor(s) and the viral attachment protein(s) have remained controversial (Darwich et al., 2010; Kreutz and Ackermann, 1996; Nauwynck et al., 1999; Van Breedam et al., 2010). Recent studies provide genetic evidence that the minor envelope proteins GP2, GP3, GP4, and E together play a key role in the virus entry into cultured cells (Tian et al., 2012; Zevenhoven-Dobbe et al., 2004). The small unglycosylated envelope protein (E) (Snijder et al., 1999) and three minor envelope glycoproteins were recently demonstrated to exist as a covalently associated heterotrimer in the virion (Wieringa et al., 2003). Probably these envelope proteins are the sites of P34 binding. Although P34 also inhibited EAV after cellular infection, the exact moment when it happened could not be addressed yet.

Because treatment with some AMPs is potentially effective and non-toxic to human and animals, they have already been proposed as an alternative for disease control (Twomey et al., 2000). The AMP produced by strain P34 may represent an antimicrobial substance with potential application for the prevention and treatment of EAV infection, however further investigations are needed to make its application *in vivo* possible.

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4 CONCLUSÃO GERAL

- O peptídeo P34 apresenta baixa citotoxicidade, e esta manifestou-se em diferentes intensidades para cada linhagem celular analisada.

- O peptídeo P34 parece não ter ação contra os vírus não envelopados avaliados (CAV-2, CPV-2, FCV).

- A ação inibitória do peptídeo P34 supostamente não está relacionada com a presença de envelope viral, uma vez que não foi detectada atividade sobre o CCoV, CDV e EIV.

- O peptídeo antimicrobiano P34 possui atividade antiviral contra o FHV-1 e o EAV.

- O peptídeo P34 não possui atividade virucida sobre o FHV-1.

- O P34 exerce ação virucida sobre o vírus da arterite equina, sendo ela tempo/temperatura dependente.

- O mecanismo pelo qual o peptídeo P34 exerce sua ação virucida sobre o EAV parece estar relacionado à ligação e lesão do envelope viral.

- O P34 não demonstrou efeito virucida em temperaturas abaixo de 37°C.

- O peptídeo P34 possui um alto índice terapêutico, indicando ser uma substância com grande potencial para uso em estudos clínicos.

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