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Inhibition of equine arteritis virus by an antimicrobial peptide produced by *Bacillus* sp. P34

[*Inibição do vírus da arterite equina por um peptídeo antimicrobiano produzido pelo* Bacillus *sp. P34*]

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ABSTRACT

P34 is an antimicrobial peptide produced by Bacillus sp. P34, isolated from the intestinal contents of a fish from the Amazon basin. This peptide showed antibacterial properties against Gram-positive and Gram-negative bacteria and was characterized as a bacteriocin like substance. It was demonstrated that the peptide P34 exhibited antiviral activity against feline herpesvirus type 1 in vitro. The aim of this work was to evaluate P34 for its antiviral properties in vitro, using RK 13 cells, against the equine arteritis virus, since it has no specific treatment and a variable proportion of stallions may become persistently infected. The results obtained show that P34 exerts antiviral and virucidal activities against equine arteritis virus, 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 since it has a high therapeutic index. However, more detailed studies must be performed to address the exact step of viral infection where P34 acts, in order to use this peptide as an antiviral drug in vivo in the future.

Keywords: equine arteritis virus, antiviral therapy, antimicrobial peptide, virucidal activity

RESUMO

P34 é um peptídeo antimicrobiano produzido pelo Bacillus *sp. P34, isolado do conteúdo intestinal de um peixe na Bacia Amazônica. Esse peptídeo demonstrou propriedades antibacterianas contra bactérias Gram-positivas e Gram-negativas e foi caracterizado como uma substância do tipo bacteriocina. Foi demonstrado que o peptídeo P34 exibiu atividade antiviral contra o herpesvírus felino tipo 1* in vitro. *O objetivo deste trabalho foi avaliar o P34* in vitro, *em cultivo de células RK 13, contra o vírus da arterite equina, uma vez que não há tratamento específico e uma variável proporção de garanhões pode permanecer persistentemente infectada. Os resultados obtidos mostram que o peptídeo exerce atividade antiviral e virucida contra o vírus da arterite equina, agindo provavelmente no envelope viral. Os ensaios antivirais realizados demonstraram que o P34 reduz significativamente os títulos do vírus nas células infectadas e tratadas. O mecanismo de ação do P34 parece ser tempo/temperatura dependente. Esse peptídeo tende a ser um antiviral promissor para o tratamento e a prevenção das infecções por arterivírus, tendo em vista que ele possui um alto índice terapêutico. Contudo, estudos mais detalhados devem ser realizados para precisar a etapa exata da infecção viral em que o P34 age, para que ele possa ser usado como antiviral* in vivo *no futuro.*

Palavras-chave: vírus da arterite equina, terapia antiviral, peptídeo antimicrobiano, atividade virucida

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INTRODUCTION

Equines can be infected by the equine arteritis virus (EAV) resulting in the development of panvasculitis, edema, hemorrhage and abortion (Del Piero, 2000). 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 (Balasuriya *et al*., 2013). 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 (Timoney and McCollum, 1993). EAV was first isolated from a fetal lung collected during an epizooty of abortion in Ohio, in the United States (Bryans *et al*., 1957). Meanwhile, reports of a respiratory disease similar to 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). Although EAV has still not been isolated in Brazil, there is serological evidence of its presence in different regions of the country (Bello *et al*., 2007; Diaz *et al*., 2015; Sartori *et al*., 2016).

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). The virus can be present in the male reproductive tract within the accessory sex glands and it is testosterone-dependent (Balasuriya et al., 2013). 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 antiviral strategies to control and prevent EAV infection.

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 a species of *Bacillus* (*Bacillus* sp. P34), producing an antimicrobial peptide (AMP), from the aquatic environments of Brazilian Amazon basin. This peptide was purified, named P34 and its antimicrobial activity was detected against Gram-positive bacteria, like *Listeria monocytogenes* and *Bacillus cereus* (Motta *et al*., 2007), Gram-negative bacteria like *Escherichia coli* and *Salmonella enteritidis* (Motta *et al*., 2008) and some viruses (Silva *et al*., 2014).

The aim of this work was to evaluate the activity exerted by P34 against EAV *in vitro* and determine its antiviral mechanism of action.

MATERIAL AND METHODS

The peptide P34 was isolated from a lineage of *Bacillus* (*Bacillus* sp. P34) detected in the intestinal contents of the fish Piau-com-pinta (*Leporinus* sp.). The AMP P34 was produced as described by Motta *et al*. (2007). After purification, total protein concentration was measured 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 its use for the antiviral assays.

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

Cells were cultured in Eagle's minimum essential medium (E-MEM – Sigma Aldrich, USA) supplemented with 10% of bovine fetal serum (BFS – Gibco, USA), penicillin (200UI/mL, Sigma Aldrich, USA), streptomycin (0,2µg/mL, Vetec, Brazil), enrofloxacin (10mg mL^{-1}, Bayer, Brazil) and amphotericin B (0,025 µg mL^{-1}, Cristália, Brazil) in an incubator at 37°C.

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 (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}).

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-(antilogarithmic test value/antilogarithmic control value)] 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 was taken as the control.

Initially, an inhibition of CPE assay was performed 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 titration. The cells were observed for CPE daily for 3 days at an Olympus CK-2 inverted microscope. Titers were expressed as $TCID_{50}$/100µL.

To assess how P34 would interfere in the viral yield, a virus yield reduction assay was performed. Following virus binding, the inoculum was aspirated and fresh E-MEM was added, in the presence or absence of P34. After 24, 48 and 72 h post infection, plates were frozen-thawed for further titration.

A receptor competition assay was performed on subconfluent RK13 cell cultures grown in microplates. The cells were infected with 100 $TCID_{50}$ of EAV or with 100 $TCID_{50}$ of EAV mixed with the peptide P34 (final concentration 2.3µg/mL) and incubated at 37ºC for 1h. Following incubation, the virus or the mixture were aspirated, cells were washed and fresh E-MEM was added. 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 cited above. Virus infectivity was assessed by inoculating 100 $TCID_{50}$ of EAV for 1 h at 37°C onto those treated cells. After 72 h the plates were frozen-thawed and viral titers were measured.

Suspensions of EAV or EAV with P34 (2.3µg/mL) were incubated at 4°C, 20°C and 37°C for 30 min, 1h, 2h, 3h, 4h, 6h and 12 h in FBS-free E-MEM to assess P34 virucidal effect. After incubation, the viruses' titers were determined and the virucidal activity was expressed as $TCID_{50}$ and PI.

To perform an electron microscopic study RK13 cells were infected with EAV and after 48 h the infected cultures were frozen-thawed to extract viral particles. The cleared supernatant was incubated on a shaker plate for 12h at 37°C, in the presence or absence of P34. 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 3h at 4°C as described elsewhere (Wieringa *et al*., 2004). The resultant pellets were resuspended in 100µL of phosphate saline buffer and stored at 4°C. Both samples were fixed with 2.5% glutaraldehyde/20mM sodium cacodylate and then fixed in 1% osmium tetroxide. Dehydration was made 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 post-stained with 2% uranyl acetate. Preparations were observed with a Zeiss EM 109 transmission electron microscope operating at 80 kV.

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

RESULTS

To distinguish selective antiviral activity from cytotoxicity, the peptide was evaluated on RK13 cells by the NRU and MTT assays. CC_{50} was 3.92µg/mL in both tests. Cytotoxicity was not observed at 2.3µg/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 0.28µg/mL.

The ability of P34 to inhibit the CPE caused by EAV was evaluated and the results demonstrated a significant reduction on the viral titers. 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%.

The titrations performed with the aliquots from the plate frozen after 24h demonstrated median EAV titers of $10^{3.51}$ $TCID_{50}$ in the absence of peptide and $10^{1.86}$ $TCID_{50}$ with 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%) (Fig. 1), showing that the peptide acted during the post-infection stage.

Once we observed that P34 was able to inhibit EAV, the next step was to address whether P34 would still inhibit EAV after the virus binding step. When the peptide P34 and 100 $TCID_{50}$ of EAV were both added to the cells (treatment "b" in the table 1), no infectious virus was detected even after 72 h, indicating an impeachment of EAV. The infection of RK13 cells with just 100 $TCID_{50}$ of EAV resulted in a titer of $10^{6.5}$ $TCID_{50}$ ("a" in the table 1).

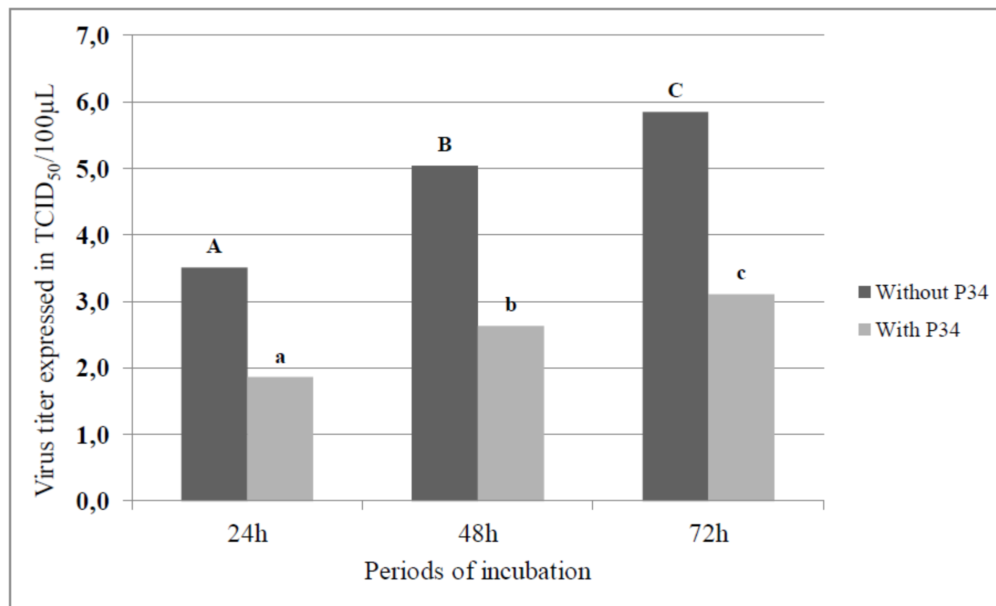

Fig. 1. Virus yield reduction assay. Demonstration of EAV titers in the absence (■) or in the presence (■) of the peptide P34 in different periods of incubation. Different letters represent statistical significance among the treatments (P<0.05).

RK13 cells treated for 1h with the peptide P34, before the addition of 100 $TCID_{50}$ of EAV (treatment "c" in the table 1), 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 (Table 1).

Peptide P34 showed virucidal properties; however, temperature had a strong influence on the inactivation rate. The incubation of EAV mixed with P34 performed at temperatures of 4°C and 20°C did not result in statistically

significant differences in viral titers (*P*>0.05). Virucidal activity 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 (Figure 2). 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 12h 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%.

The appearance of EAV particles was compared with those of the EAV incubated with P34 by electron microscopy. Rounded viral particles with about 50nm in diameter were observed in the control sample; however, the EAV particles treated with P34 were damaged, exhibiting irregular shape (Figure 3).

Table 1. Receptor competition assay results. Viral titers of EAV according to the treatment performed. The control (letter a) represents the EAV titer of non-treated cells. Letter b demonstrates that P34 added with EAV onto the RK 13 cells resulted in inhibition of the virus (no viral titer). Letter c demonstrates that the treatment of RK 13 cells with P34 pre-infection with EAV does not result in viral inhibition, since the virus titer is equal to the control

Receptor Competition Assay	Viral Titer ($TCID_{50}$/100µL)
a) EAV (Control)	$10^{6,5}$
b) EAV + P34 (Together)	0
c) P34 1h + EAV 1h	$10^{6,5}$

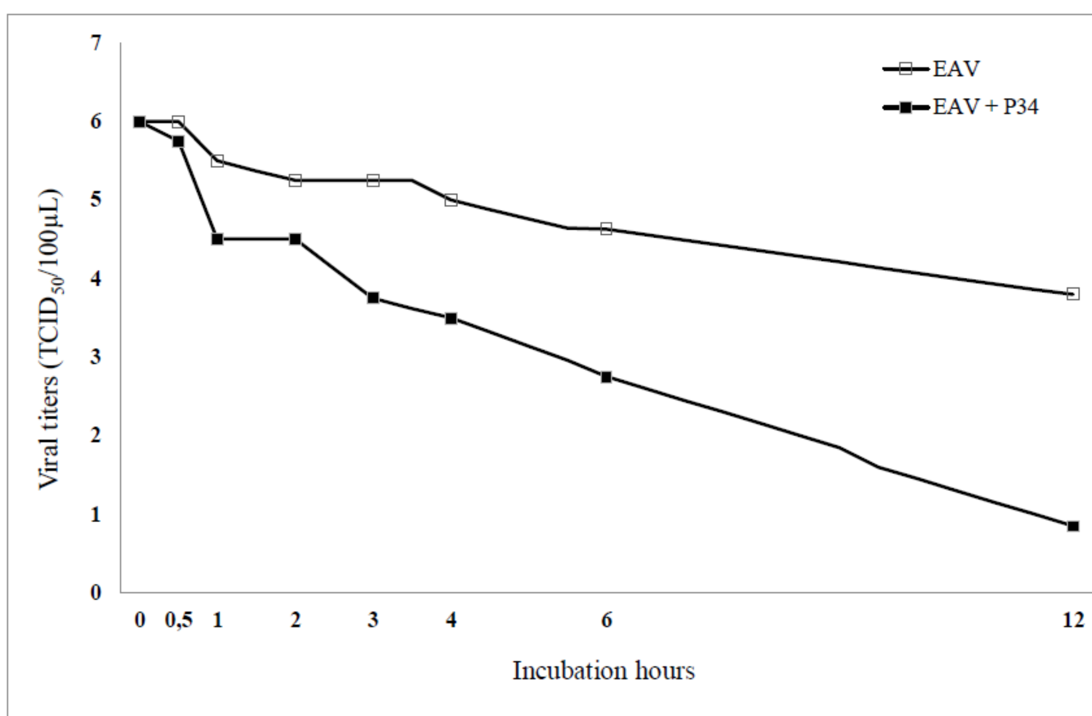

Figure 2. P34 virucidal effect. EAV titers ($TCID_{50}$/100µL) after different periods of incubation at 37°C in the absence or presence of the peptide P34 (2,3µg/mL).

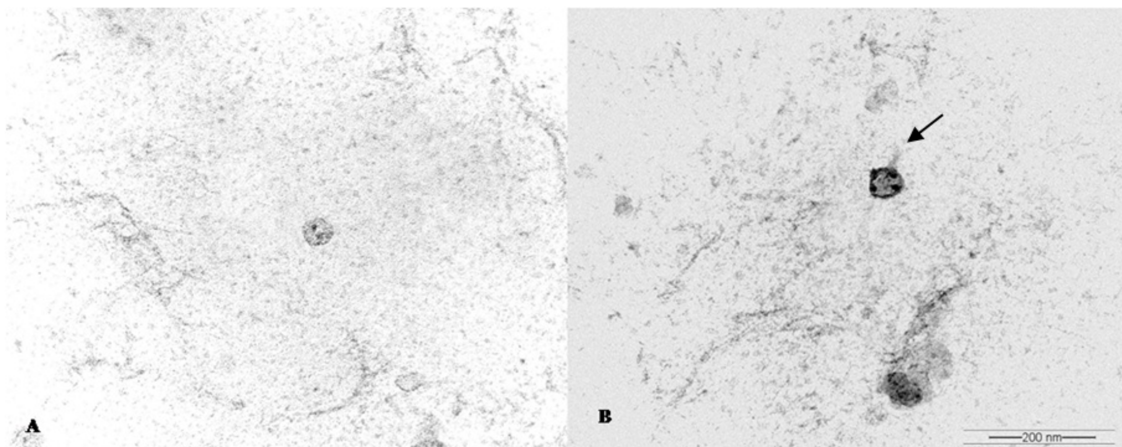

Figure 3. Transmission electron microscopy. Transmission electron micrographs showing: (A) spherical morphology of EAV particle with about 50nm in diameter. (B) EAV particle treated with the peptide P34 showing irregular shape (arrow). 85.000 x magnification. Scale bar = 200nm.

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 (Yan *et al*., 2011). In the present study, the inhibitory and virucidal activities of the peptide P34 against EAV were evaluated. P34 exerted its antiviral effect by preventing viral entry, in part by binding to virus particles. Furthermore, tests performed to evaluate the virucidal effect of P34 demonstrated that the peptide had a directed effect against free virus particles, since the interaction of P34 directly with the virus showed an inhibitory effect (Figure 2).

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). Considering that treatment with AMPs is potentially effective and non-toxic to humans and animals, it has already been proposed as an alternative for disease control (Galdiero *et al*., 2013). 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 or non-ionic thermostable, hydrophobic, lipidic peptide, with antimicrobial properties described against bacteria (Motta *et al*., 2007). 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). The ability of AMPs to interact with lipid membranes (Jenssen *et al*., 2006) suggests that the viral envelope could be a possible target for direct interaction of 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 (Figure 2). When transmission microscopy analysis was performed, the images obtained suggested destabilization of the viral particle, which demonstrated irregular shape (Figure 3). 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 its membrane lytic properties as described for other peptides (Reddy *et al*., 2004) and this ability can be related to the hydrophobic or hydrophilic helicoidal components of the peptide (Matsuzaki *et al*., 1997). It was observed that the virucidal effect was time/temperature dependent, since the event did not occur at 4°C and 20°C. Besides, the effect increased with higher incubation times indicating that a minimum level of membrane fluidity may be required for P34 to

inactivate the virus. As demonstrated in Figure 2, the virucidal effect of P34 against EAV was more effective at 12h of incubation, reaching a PI of 99.8%. Presumably, these effects are due to the incorporation of P34 into the viral envelope inducing disorder and damage of the lipid layer. 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 (Table 1). The incubation of P34 for 1 h onto the cells, before the addition of EAV, did not interfere with viral attachment and posterior infection (Table 1). Therefore, we suppose there is no direct interaction of P34 with specific viral 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). 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 demonstrated to exist as a covalently associated heterotrimer in the virion (Wieringa *et al*., 2004). Possibly these envelope proteins are the sites of P34 binding and posterior inactivation. Although P34 also inhibited EAV after cellular infection, the exact moment when it happened could not be addressed yet.

CONCLUSIONS

The results obtained were promising and indicate that P34 exerts virucidal activity and reduces EAV yields without toxic effects for the cells. The AMP 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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