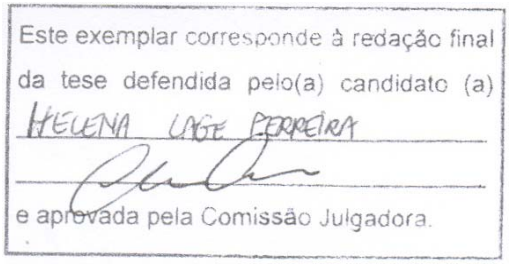
UNIVERSIDADE ESTADUAL DE CAMPINAS

HELENA LAGE FERREIRA

"ESTUDO DO EFEITO DA INTERFERÊNCIA POR RNA (RNAi) NA REPLICAÇÃO DO METAPNEUMOVÍRUS AVIÁRIO (AMPV) SUBTIPO A *IN VITRO*"



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Orientadora: Profa. Dra. Clarice Weis Arns
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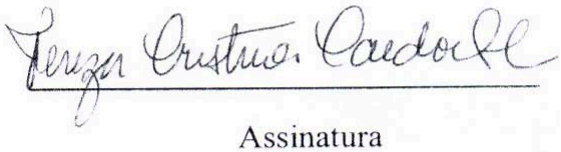

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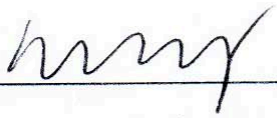

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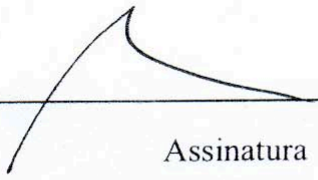

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Lista de Siglas e Abreviaturas

AMPV (avian metapneumovirus): Metapneumovírus Aviário

CEL (chicken embryo liver): Cultivo de fígado de embrião de galinha

CER: chicken embryo related

ECP- Efeito citopático

ELISA (enzyme linked immuno sorbent assay): ensaio imunoenzimático

F (fusion): proteína de fusão

FEG: Fibroblasto de embrião de galinha

G (attachment): glicoproteína

HMPV (human metapneumovirus): Metapneumovírus Humano

L (large protein): Polimerase

M (matriz): Proteína Matriz

miRNA: micro RNA

mRNA (messenger RNA): RNA mensageiro

N (nucleoprotein): Nucleoproteína

NNR (negative-strand nonsegmented RNA): fita RNA negativa não segmentada

P (phosphoprotein): Fosfoproteína

PCR (Polymerase Chain Reaction): Reação em cadeia da polimerase

RISC: Complexo de silenciamento induzido por RNA

RNA- Ácido ribonucleico

RNAi (RNA interference): Interferência por RNA

RRT-PCR: Retrotranscrição- Reação em cadeia da polimerase em tempo real

RSV (respiratory syncytial virus): vírus respiratório sincicial

RT (reverse transcriptase): Transcriptase reversa

SH (small hydrophobic): proteína pequena hidrofóbica

SHS (swollen head syndrome): Síndrome da cabeça inchada

siRNA (short interfering RNA): RNA pequeno interferente

SN: Soroneutralização

TOC (tracheal organ culture): Cultivo de anel traqueal

TRT (turkey rhinotracheitis): Rinotraqueíte em perus

VBI: vírus da bronquite infecciosa

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Resumo

O metapneumovírus aviário (AMPV) é o agente primário da rinotraqueíte dos perus (TRT). O AMPV pertence à família *Paramyxoviridae*, subfamília *Pneumovirinae*, gênero *Metapneumovirus.* Também está associado à síndrome da cabeça inchada (SHS) em galinhas e é responsável por significativas perdas econômicas em sua produção. O presente estudo foi dividido em três partes. A primeira parte do trabalho consistiu em avaliar a beta-actina, gene utilizado como controle interno das técnicas moleculares de detecção viral, das células chicken embryo related (CER). Para isso, foi realizado o sequenciamento dos amplicons gerados pelo PCR do gene da beta-actina. A beta-actina das células BHK21 e CER foram detectadas utilizando oligonucleotídeos hamster-específicos. Além disso, pela análise filogenética as células CER e BHK_{21} apresentaram uma alta similaridade genética ($p>0.996$). Estes resultados sugerem que as células CER não deveriam ser mais consideradas como células aviárias. A segunda parte do estudo consistiu em comparar a especificidade e limite de detecção de duas novas técnicas de RT-PCR convencional (genes da nucleoproteína (N) e da proteína de fusão -F) e de duas novas técnicas de real time RT-PCR (RRT-PCR; genes F e N) com um RT-PCR (gene da glicoproteína -G) previamente estabelecido para a detecção do AMPV. Todos estes métodos foram capazes de detectar os isolados AMPV subtipo A (AMPV/A). As técnicas RRT-PCR (genes F e N) foram capazes de amplificar os maiores limites de detecção (diluições 10^{-5} e 10^{-5}, respectivamente). Além disso, o RRT-PCR gera resultados rápidos e sensíveis, o que o torna uma ferramenta alternativa para o isolamento viral. Na terceira parte, foi realizado o silenciamento gênico de AMPV pela aplicação de seqüências curtas e específicas de RNA (siRNAs, do inglês short interfering RNA)

para regiões alvo do genoma viral. Assim, foram desenhadas moléculas de siRNA contra os genes N e F do AMPV. Três dias após a infecção viral, o efeito do siRNA na replicação viral foi verificado por titulação viral, RRT-PCR e RT-PCR. Os títulos virais das células CER transfectadas com o siRNA/N apresentaram queda de até 99,9% em relação ao controle. A produção de mRNAs para os genes N, F e G do AMPV também apresentou uma redução de até 99,7%. Desta forma, a molécula de siRNA contra o gene N foi capaz de inibir a replicação do AMPV *in vitro*. Em estudos futuros, a associação de siRNAs tendo como alvo o complexo da RNA polimerase deve ser avaliada como uma eficiente ferramenta para evitar o escape viral na terapia antiviral.

"**THE EFFECT OF RNA INTERFERENCE IN AVIAN METAPNEUMOVIRUS SUBTYPE A IN VITRO REPLICATION"**

Abstract

Avian metapneumovirus (AMPV) is the primary causative agent of severe rhinotracheitis in turkeys. AMPV belongs to the Paramyxoviridae family, Pneumovirinae subfamily, within the genus *Metapneumovirus*. It is associated with swollen head syndrome in chickens and is the source of significant economic losses to animal food production. The present study is divided in three parts. In the first part, the chicken embryo related (CER) cells beta-actin was evaluated. The CER beta actin gene was amplified by RT-PCR, and the amplicon was sequenced. The BHK_{21} and CER beta-actins were detected using hamster-specific primers. The results showed that such cells are closely related to BHK_{21} ($p > 0.966$), having a p-distance of 0.7 from chicken embryo fibroblasts. This confirms that CER cells are phylogenetically closely related to BHK_{21} cells. The second part of the study, we compared the specificity and detection limits of two newly designed conventional RT-PCRs (F and N genes) and two newly defined real time RT-PCR (RRT-PCR; (F and N genes), with an established RT-PCR (G gene) for AMPV detection. All the RT-PCR tested assays were able to detect the six isolates. The higher detection limits were observed at 10^{-5}-fold and 10^{-5}-fold dilutions of the N- and F- based RRT-PCR, respectively. Important to note that RRT-PCR assays generate fast and sensitive results, becoming a feasible alternative for virus isolation. In the third part, the silencing of AMPV by targeting its viral regions was promoted. We designed specific short interfering RNA (siRNA) targeting the nucleoprotein (N) and fusion (F) genes. Three days after the

virus infection, the effect of siRNA in the virus replication was verified by virus titration, real time RT-PCR, and RT-PCR assays. AMPV titers presented reduction by 99.9%, when compared to the siRNA/F and siRNA/GFP treated samples. Also, real time RT-PCR results presented reduction of AMPV N, F and G mRNAs by 99.7%, when transfected with siRNA/N. Therefore, an siRNA sequence targeting the N gene was able to inhibit the AMPV production in vitro. In future studies, a combination of siRNAs targeting the RNA-polymerase complex may be used as a tool to study AMPV-infected cells or as an antiviral therapy.

1. Introdução

A importância econômica e social da avicultura brasileira coloca o setor em evidência no âmbito nacional e internacional, visto que o Brasil é o maior exportador de carne de frango em receita cambial. Somando-se as produções norte-americana e a brasileira, suplementadas ainda pela produção de alguns outros países latino-americanos, como o México, o continente americano concentra atualmente 47% da produção mundial. Por essa razão, a importância dos países da América Latina vem crescendo no comércio mundial do produto (Uba, 2003).

Atualmente, as doenças respiratórias são responsáveis por grandes perdas econômicas na criação de aves em todo o mundo, tanto na produção de carne, quanto na de ovos. Tais perdas são representadas por gastos com medicamentos, aumento dos índices de mortalidade, elevação dos índices de condenação de carcaças, quedas da produção e incubabilidade de ovos férteis, diminuição da espessura da casca de ovos e diminuição da viabilidade das ninhadas. Com a criação intensiva de aves, ou seja, grandes populações vivendo em espaços confinados, a entrada de um patógeno virulento, como vírus respiratórios, pode causar alta mortalidade e grandes perdas (Morley e Thomson, 1984; O'brien, 1985).

Assim, a necessidade de estudos sobre as enfermidades respiratórias é de grande importância considerando, sobretudo, a crescente projeção da indústria avícola brasileira no comércio mundial.

1.1. INTRODUÇÃO

O AMPV é o agente etiológico da rinotraqueíte em perus (TRT) e está associado à síndrome da cabeça inchada (SHS) em aves comerciais (Dani, Durigon *et al.*, 1999). O AMPV está associado aos principais patógenos de doenças respiratórias em aves e pertence à família *Paramyxoviridae*, subfamília *Pneumovirinae*, gênero *Metapneumovirus* (Randhawa, Marriott *et al.*, 1997; Bäyon-Auboyer, Jestin *et al.*, 1999; Munir, Kaur *et al.*, 2006). Os metapneumovírus são envelopados e apresentam o genoma de aproximadamente 13.000 nucleotídeos de comprimento (Ling, Easton *et al.*, 1992; Fenner, Gibbs *et al.*, 1993). Este agente causa uma doença aguda e altamente contagiosa do trato respiratório em aves comerciais e populações de aves silvestres em todas as partes do mundo (Munir e Kapur, 2003). A forma mais grave da doença em galinhas é causada, provavelmente, pela associação do AMPV com infecções bacterianas secundárias, como pela *Escherichia coli* (Gough, 2003).

1.2. GENOMA VIRAL

O AMPV, que era classificado no gênero *Pneumovirus*, foi recentemente designado como um Metapneumovírus aviário. Estes vírus apresentam o genoma de RNA fita simples negativa, e contém oito genes organizados de forma diferente dos 10 genes dos pneumovirus (Easton, Domachowske *et al.*, 2004). No genoma dos pneumovirus, o cassete F-M2 situa-se entre os genes G e L, enquanto que, nos metapneumovírus, ele se localiza entre os genes M e SH

(Figura 1) (Collins, Hill *et al.*, 1996; Randhawa, Marriott *et al.*, 1997; Pringle, 1998).

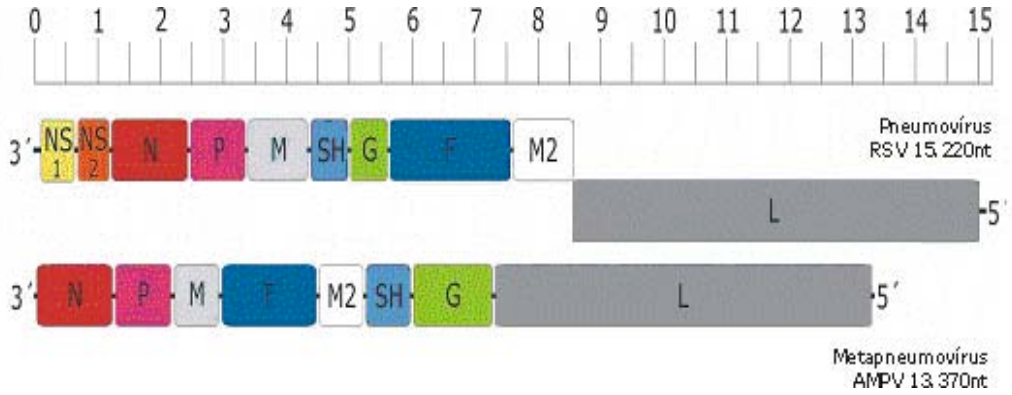

Figura 1: Comparação entre os genomas dos gêneros pneumovírus e metapneumovírus. (Adaptado de Easton, 2004).

O genoma do AMPV é constituído por oito genes virais dispostos na seguinte ordem: nucleoproteína - fosfoproteína- matriz - fusão- segunda matriz- proteína hidrofóbica – glicoproteína G- polimerase (3´-M-P-M-F-M2-SH-G-L-5'), flanqueado pelas sequências *leader* e *trailer* nas posições 3' e 5', respectivamente (Easton, Domachowske *et al.*, 2004).

O RNA genômico está associado às proteínas N, L e P, que formam o complexo da RNA-polimerase. Este complexo é necessário para a replicação e transcrição viral (Barik, 2004; Easton, Domachowske *et al.*, 2004). A proteína L é o principal componente deste complexo e se liga na porção 3´ das seqüências promotoras do RNA genômico. Entretanto, a proteína L é incapaz de iniciar a transcrição ou replicação na ausência da proteína P, que oferece para a proteína L o prolongamento necessário para promover sua liberação e produção de

transcritos inteiros (Dupuy, Dobson *et al.*, 1999). A proteína N está intimamente associada com o RNA genômico, que oferece resistência à ação das RNAses (Barik, 2004) e induz a formação de uma estrutura hélica ao genoma viral. Ela é sintetizada em grandes quantidades, e uma vez sintetizada, se liga aos RNAs genômicos virais recentemente transcritos. No fim da replicação viral, os genomas de fita negativa encapsidados, junto com outras proteínas estruturais, são empacotados dentro de partículas virais (Easton, Domachowske *et al.*, 2004).

A proteína M nos vírus de RNA fita simples negativa (NNR) possui duas funções: inibir a transcrição viral durante a montagem pela associação com a proteína N, e mediar a associação da proteína N com o envelope nascente. A proteína M dos metapneumovírus parece apresentar estas funções, ou, talvez, estas funções sejam divididas entre as proteínas M e M2 (Collins, Hill *et al.*, 1996).

A partícula viral apresenta duas principais glicoproteínas de superfície na membrana viral: a proteína G, que promove a ligação do vírus com o receptor celular (Levine, Klaiber-Franco *et al.*, 1987), e a proteína F, essencial para a fusão das membranas viral e celular (Walsh e Hruska, 1983). A proteína F é sintetizada primeiramente como F0, que se torna ativa por uma clivagem em F1 e F2 assim que a proteína chega ao complexo de Golgi (Collins, Huang *et al.*, 1984; González-Reyes, Ruiz-Arguello *et al.*, 2001). Portanto, a clivagem de F0 é determinante para a infectividade e patogenicidade viral. A terceira proteína de superfície, SH, com função desconhecida, é altamente expressa na superfície

das células infectadas, mas é incorporada em pequenas quantidades na partícula viral (Olmsted e Collins, 1989).

1.3. REPLICAÇÃO VIRAL

Todas as fases da replicação dos metapneumovírus ocorrem no citoplasma e estão esquematicamente demonstradas na figura 2. Na adsorção viral com o receptor celular, a glicoproteína G liga-se ao receptor da superfície celular. Após esta ligação, ocorre uma mudança conformacional da glicoproteína F para a liberação do peptídeo de fusão. Em seguida, o complexo da RNA-polimerase é liberado no citoplasma (Lamb e Kolakofsky, 1996). O complexo da RNA-polimerase é composto pelo RNA genômico em associação com as proteínas N, L e P. Este complexo é responsável por toda a síntese do RNA viral (Easton, Domachowske *et al.*, 2004). O RNA genômico dos vírus de NNR possui duas funções: serve como molde para síntese de RNA mensageiro (mRNA) e também para a síntese da fita antigenômica (+). Os vírus NNR codificam e empacotam a sua própria RNA polimerase, enquanto que o mRNA é sintetizado apenas quando ocorre o desnudamento viral na célula infectada. A replicação viral ocorre após a síntese do mRNA e requer a síntese contínua de proteínas virais. O antigenoma sintetizado é utilizado como molde para as cópias de RNA genômico (-). Como todos os eventos da replicação viral, a montagem também ocorre no citoplasma. A montagem do complexo RNA-polimerase ocorre em duas etapas: a primeira consiste na associação da nucleoproteína com o RNA viral (RNA-N) para formar uma estrutura hélica e, a segunda, na associação do

complexo RNA-N com as proteínas P e L. A montagem do envelope viral ocorre na superfície celular. As proteínas de membrana são sintetizadas no retículo endoplasmático e sofrem, gradualmente, uma maturação conformacional antes de serem transportadas. Apenas as proteínas corretamente montadas são transportadas para o complexo de Golgi. Lá, as cadeias de carboidrato podem ser modificadas e as proteínas F, com múltiplos sítios de clivagem, têm seus sítios clivados. Por fim, as glicoproteínas são transportadas à membrana citoplasmática. Não se conhece, até o momento, o mecanismo que provoca a montagem da partícula viral na membrana plasmática. Acredita-se que as caudas das glicoproteínas têm grande importância no contato com a proteína M, que, por sua vez, se encontra associada ao complexo RNA-polimerase (Lamb e Kolakofsky, 1996).

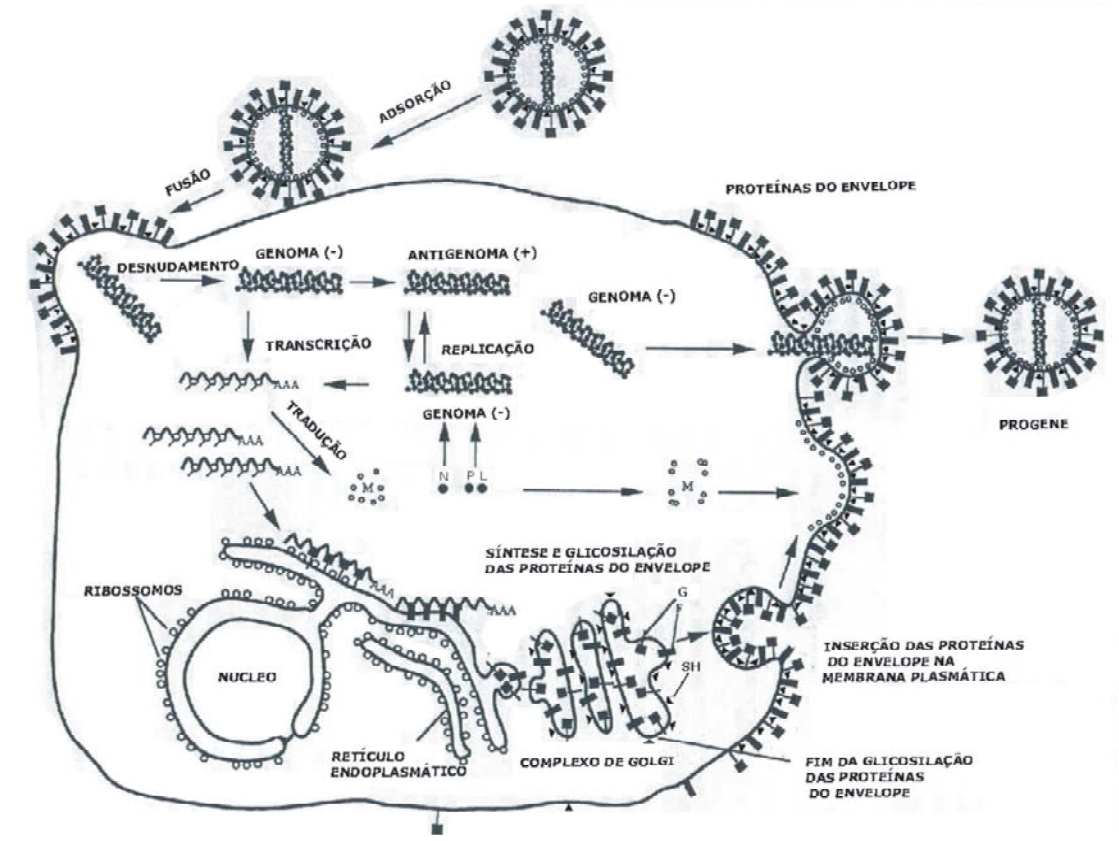

Figura 2: Representação esquemática da replicação do AMPV (adaptado de Lamb e Kolakofsky, 1996)

1.4. VARIABILIDADE DE AMOSTRAS DO AMPV

Inicialmente, acreditava-se que havia apenas um sorotipo de AMPV, contendo dois subtipos diferenciados entre si por anticorpos monoclonais (Collins, Gough *et al.*, 1993) . Estudos posteriores verificaram variações no gene da glicoproteína G e confirmaram a presença de dois subtipos distintos, classificados como subtipos A e B (Juhasz e Easton, 1994). Até o final da década de 90, acreditava-se que apenas os subtipos A e B eram presentes no continente europeu (Van De Zande, Nauwynck *et al.*, 1998; Hafez, Hess *et al.*, 2000). Após quase 20 anos do primeiro isolamento de AMPV, o subtipo C foi

descrito nos Estados Unidos (isolado proveniente do Colorado), com o seqüenciamento dos genes das proteínas M (Seal, 1998), F (Seal, Sellers *et al.*, 2000; Tarpey, Huggins *et al.*, 2001), N e P (Dar, Tune *et al.*, 2001). Um isolado viral a partir de um pato infectado na França foi classificado nesse mesmo subtipo por imunofluorescência indireta, pois apresentou maior reatividade com um anti-soro produzido contra o subtipo C quando comparado àqueles produzidos contra os subtipos A e B (Toquin, Bäyon-Auboyer *et al.*, 1999). Na França, um relato recente da existência de uma estirpe de AMPV geneticamente distinta dos subtipos A, B e C foi referida como tipo D (Bäyon-Auboyer, Arnauld *et al.*, 2000). As seqüências dos isolados brasileiros apresentaram, até o momento, identidade de 95,8 a 99% com aquelas do subtipo A disponíveis no GenBank (Ferreira, Spilki *et al.*, 2007).

As seqüências de AMPV disponíveis no GenBank revelam diferenças entre os quatro subtipos descritos. Estas sequências demonstram uma grande heterogeneidade genômica entre os subtipos, o que dificulta o desenvolvimento de métodos moleculares capazes de detectá-los simultaneamente (Bäyon-Auboyer, Jestin *et al.*, 1999; Cook e Cavanagh, 2002). A seqüência de aminoácidos da proteína F do subtipo C (Seal, Sellers *et al.*, 2000; Tarpey, Huggins *et al.*, 2001) tem aproximadamente 72% de identidade com as mesmas seqüências dos subtipos A e B, identidade esta inferior àquela encontrada entre os subtipos A e B (83%) (Naylor, Britton *et al.*, 1998). Achados similares são observados na comparação das seqüências de aminoácidos das proteínas M (Randhawa, Pringle *et al.*, 1996; Seal, 1998) e N (Li, Ling *et al.*, 1996; Dar, Munir

et al., 2001) entre esses três subtipos. Recentemente, a análise filogenética das seqüências de aminoácidos das proteínas G, SH e L do subtipo C revelou que este apresenta maior similaridade com os isolados de metapneumovírus humano (hMPV) do que com os outros subtipos de AMPV (Toquin, De Boisseson *et al.*, 2003), corroborando os resultados de análises prévias das seqüências das proteínas F, N, P, M e M2 (Njenga, Lwamba *et al.*, 2003).

1.5. DISTRIBUIÇÃO GEOGRÁFICA DO AMPV

Os primeiros relatos da presença do AMPV em produções avícolas de galinhas e perus foram realizados no final dos anos 70, na África do Sul (Buys e Du Preez, 1980; Buys, Du Preez *et al.*, 1989b). Logo após foram relatados surtos em frangos na Espanha (Diaz De Espada e Perona, 1984), em galinhas e galinhas d'Angola na França (Drouin, Toux *et al.*, 1985), em frangos de corte na Inglaterra (O'brien, 1985; Wyeth, Chettle *et al.*, 1987), em poedeiras e matrizes na Holanda (Goren, 1985) e em matrizes em Israel (Perelman, Meroz *et al.*, 1988). Sorologia positiva para AMPV também foi descrita no Chile e América Central (Jones, 1996; Toro, Hidalgo *et al.*, 1998), em avestruzes no Zimbabwe (Cadman, Kelly *et al.*, 1994) e em gaivotas no mar Báltico (Heffels-Redmann, Neumann *et al.*, 1998). Em nenhuma delas houve isolamento viral. Nos EUA, as primeiras confirmações de surtos por estes vírus em perus ocorreram em 1997 (Seal, 1998). No Brasil, seu isolamento foi realizado a partir de material proveniente de matrizes antes da introdução da vacina no país (Arns e Hafez, 1995). Embora não existam dados disponíveis sobre a circulação do AMPV em

diversas áreas geográficas, dois países se declararam como livres destes vírus: Austrália (Bell e Alexander, 1990) e Canadá (Heckert e Myers, 1993).

A disseminação viral da África para Europa e o seu primeiro aparecimento na costa leste da Inglaterra sugere que as aves migratórias desempenham um importante papel na transmissão global do AMPV (Stuart, 1989). Esta hipótese é corroborada pelo aparecimento dos vírus em Minnesota, EUA, onde há uma grande população de aves migratórias (Anon, 1998).

1.6. SINAIS CLÍNICOS DA METAPNEUMOVIROSE AVIÁRIA

O AMPV causa infecção respiratória severa em perus. O nome original desta doença era rinotraqueíte em perus (TRT). A enfermidade é caracterizada por espirros, estertores traqueais, edema dos seios infraorbital, nasal e muitas vezes frontal, com descarga ocular. A descarga nasal pode se tornar mucopurulenta devido infecção bacteriana secundária. Apesar da replicação dos vírus ocorrer na traquéia e pulmões, ela é muito mais limitada ao trato respiratório superior, onde as partículas virais podem ser detectadas mais facilmente (Cook, 2000).

O aparecimento dos sinais clínicos é rápido e a infecção pode se disseminar em 24 horas (Stuart, 1989). Normalmente, ocorre recuperação total das aves dentro de 14 dias. Porém, como em outras infecções respiratórias em aves domésticas, o manejo precário ou ocorrência de infecção bacteriana secundária podem provocar o agravamento dos sinais clínicos, como aerossaculite, pericardite, pneumonia, e também aumento da morbidade e da

mortalidade (Cook, Ellis *et al.*, 1991). Observações experimentais e de campo sugerem que o AMPV pode facilitar a infecção pelo *Ornithobacterium rhinotracheale* e exacerbar infecções pelo *Mycoplasma gallisepticum* (Hafez, 1988; Naylor e Jones, 1993).

Em poedeiras e matrizes de perus, a infecção pelo AMPV provoca queda na qualidade e na produção de ovos (Stuart, 1989; Cook, Orthel *et al.*, 1996). A recuperação pode ser alcançada em até três semanas e um aumento na incidência de ovos com casca fina pode ser vista durante esse período. Peritonite por ruptura de ovos também já foi relatada (Jones, Williams *et al.*, 1988). Ainda não está esclarecido se o tecido do oviduto é susceptível à infecção pelos vírus, ou se as alterações no trato reprodutivo são causados por efeitos sistêmicos (Cook, 2000). Tanto as amostras de AMPV atenuadas como as virulentas produzem altos títulos virais nos tecidos nasais e sinusais de perus jovens, mas somente até dez dias após a inoculação (Cook, Ellis *et al.*, 1991; Van De Zande, Nauwynck *et al.*, 1999).

O papel do AMPV em enfermidades em galinhas ainda não está totalmente esclarecido. Sabe-se que estes vírus podem infectar galinhas e induzir resposta sorológica específica (Wyeth, Chettle *et al.*, 1987). Porém, nem sempre associada à enfermidade (Cook, Dolby *et al.*, 1988). Estes vírus já foram isolados de frangos e galinhas em todas as idades (Picault, Giraud *et al.*, 1987; Buys, Du Preez *et al.*, 1989b). Infecções experimentais têm sido realizadas em aves dessa mesma espécie (Jones, Baxter-Jones *et al.*, 1987; Majó, Allan *et al.*,

1995; Catelli, Cook *et al.*, 1998). Por isso, o AMPV também é freqüentemente associado à SHS em galinhas.

A SHS é caracterizada por apatia, edema de face e seios infraorbitais. Desorientação cerebral, torcicolo e opistótono freqüentemente são relatados com a progressão da doença (O'brien, 1985; Hafez, 1993). A morbidade pode chegar a 10%, a produção de ovos de matrizes é freqüentemente afetada e a mortalidade raramente excede 2%. A SHS é relatada tanto em frangos de corte como em matrizes e, nessas últimas, a evidência do papel do AMPV como agente etiológico primário da enfermidade é maior (Cook, 2000). Porém, ele não é o único agente associado à SHS. Outros também foram descritos, como o vírus da bronquite infecciosa- VBI (Morley e Thomson, 1984) e *Escherichia coli* (Droual e Woolcock, 1994).

Em frangos de corte questiona-se o papel do AMPV como patógeno primário (Cook, 2000). Porém, a distribuição do vírus nos tecidos e seus sítios de replicação são muito similares à infecção em perus. Isolamento viral e técnicas imunohistoquímicas demonstraram a presença do vírus apenas em tecidos do trato respiratório superior, particularmente nos turbinados nasais, e por um curto período de tempo após inoculação (Catelli, Cook *et al.*, 1998).

1.7. DIAGNÓSTICO DO AMPV

A) CLÍNICO

O diagnóstico baseado nos sinais clínicos pode ser realizado em perus (Stuart, 1989). Em galinhas não é recomendado, pois o AMPV está associado à

SHS. Desta forma, é extremamente difícil estabelecer uma boa conexão entre o agente viral e a enfermidade em frangos e galinhas (Cook, 2000).

B) ISOLAMENTO VIRAL

A metodologia comumente utilizada para o diagnóstico de rotina de AMPV inclui isolamento viral em cultivos primários de células de embrião de galinha (Picault, Giraud *et al.*, 1987), em cultivo de anel de traquéia (TOC) (Mcdougall e Cook, 1986; Wyeth, Gough *et al.*, 1986), em cultivos de linhagem de células CER (chicken embryo related) (Arns e Hafez, 1995) e inoculação em ovos embrionados (Buys, Du Preez *et al.*, 1989a; Cook e Ellis, 1990). Os embriões com seis dias de idade são inoculados através do saco da gema e, após oito dias, o líquido alantóide e a membrana do saco da gema são coletados. Depois de duas ou três passagens, os embriões podem apresentar hemorragias e também mortalidade (Cook, 2000). O material inoculado em cultivos celulares pode apresentar efeito citopático (ECP) caracterizado por áreas dispersas com arredondamento celular e formação de sincícios após poucas passagens (Buys, Du Preez *et al.*, 1989a; Cook, Huggins *et al.*, 1999). A identificação do AMPV pode ser confirmada por microscopia eletrônica (Giraud, Bennejean *et al.*, 1986; Mcdougall e Cook, 1986; Buys, Du Preez *et al.*, 1989a) ou através de métodos imuno químicos (Baxter-Jones, Wilding *et al.*, 1986; Jones, Williams *et al.*, 1988; Catelli, Cook *et al.*, 1998).

Embora os primeiros isolados de AMPV tenham sido obtidos após passagens em ovos embrionados (Buys, Du Preez *et al.*, 1989a), TOCs foram utilizados por muitos anos para o isolamento desses vírus. Acreditava-se que

todos os subtipos de AMPV fossem ciliostáticos. De fato, as estirpes dos subtipos A e B podem demorar até 10 dias para apresentar ciliostase, embora o pico dos títulos virais ocorra em 3-5 dias (Cook, Ellis *et al.*, 1991). Entretanto, o subtipo C não é ciliostático (Cook, Huggins *et al.*, 1999).

O isolamento do AMPV a partir de amostras clínicas é extremamente difícil, uma vez que estes vírus persistem por um período muito curto após a infecção. Deve ser realizado já no primeiro sinal clínico da doença. Por isso, se os sinais clínicos forem muito evidentes, as aves sem sintomatologia clínica presentes no mesmo lote das aves doentes devem ser selecionadas (Cook, 2000).

C) DETECÇÃO VIRAL

A transcriptase reversa-reação em cadeia da polimerase (RT-PCR) tem sido amplamente utilizada para o diagnóstico das infecções por AMPV. Ela oferece resultados mais rápidos que o isolamento viral (Mase, Asahi *et al.*, 1996; Naylor, Worthington *et al.*, 1997; Bäyon-Auboyer, Jestin *et al.*, 1999; Dani, Durigon *et al.*, 1999). Além disso, ela é importante para a caracterização de isolados virais e para a realização de estudos moleculares e epidemiológicos (Jing, Cook *et al.*, 1993). O real time RT-PCR (RRT-PCR) também já foi desenvolvido para o diagnóstico do AMPV para todos os subtipos. Esta técnica já demonstrou promover um resultado mais rápido e oferecer a quantificação do mRNA presente na amostra (Guionie, Toquin *et al.*, 2007a).

D) SOROLOGIA

O diagnóstico das infecções provocadas pelo AMPV também pode ser realizado por sorologia. O teste da soroneutralização (SN) pode ser produzido em uma variedade de sistemas, como cultivos de TOC, fibroblasto de embrião de galinha (FEG), cultivo de fígado de embrião de galinha (CEL) ou células VERO. Os testes de SN e ELISA demonstram sensibilidades similares, mas o ELISA é o teste mais comumente utilizado (Grant, Baxter-Jones *et al.*, 1987; Chettle e Wyeth, 1988; Baxter-Jones, Grant *et al.*, 1989; O'loan, Allan *et al.*, 1989; Eterradossi, Toquin *et al.*, 1995). Diversos kits de ELISA comerciais para o diagnóstico sorológico estão disponíveis. No entanto, a sensibilidade entre eles pode variar bastante (Mekkes e De Wit, 1998). Além disso, os kits comerciais de ELISA não são capazes de detectar anticorpos específicos para os isolados do subtipo C (Cook, Huggins *et al.*, 1999).

1.8. CONTROLE DO AMPV

As boas práticas de manejo e uma boa biosegurança são importantes para prevenir e minimizar os efeitos da infecção pelo AMPV. Embora a infecção por estes vírus não possa ser tratada diretamente, a utilização de antibióticos é importante para controlar infecções bacterianas secundárias (Stuart, 1989; Hafez e Weiland, 1990). A forma de utilização dos antibióticos empregados pela indústria avícola tem mudado consideravelmente na última década. Isso se deve, principalmente, às preocupações sobre as possíveis conseqüências negativas à saúde humana causadas pela sua utilização excessiva. A noção de que antibióticos utilizados na indústria avícola possam causar riscos à saúde não

é novidade (Walton, 1971). Diversos isolados de *Escherichia coli* já apresentaram resistência a múltiplos agentes antimicrobianos (Hinton, 1986). Estratégias para minimizar os impactos causados pela utilização de antibióticos precisam ser desenvolvidas (Singer e Hofacre, 2006).

As infecções pelo AMPV podem ser prevenidas pela vacinação (Giraud, Bennejean *et al.*, 1986; Buys, Du Preez *et al.*, 1989a). As vacinas oferecem uma excelente proteção em perus e também em galinhas quando administradas corretamente. Uma única vacinação pode oferecer proteção aos perus durante toda a vida. Entretanto, pode ocorrer reinfecção na fase tardia da vida. Por isso, em alguns casos, os perus são revacinados depois de aproximadamente 10-12 semanas (Cook, 2000). Em frangos, a proteção durante toda a vida também pode ser obtida com uma única dose de vacina. Em galinhas, a administração de vacina inativada oferece uma redução dos efeitos da infecção pelo AMPV na produção de ovos. Desta forma, o programa completo de vacinação, composto por uma dose da vacina viva seguida da vacina inativada, é necessário para uma proteção completa de aves adultas (Cook, Orthel *et al.*, 1996). As vacinas contra os subtipos A e B oferecem uma excelente proteção cruzada entre eles (Cook, Huggins *et al.*, 1995; Eterradossi, Toquin *et al.*, 1995; Toquin, Eterradossi *et al.*, 1996). Estas vacinas também oferecem proteção contra o isolado Colorado, subtipo C (Cook, Huggins *et al.*, 1999) e contra os isolados do subtipo D (Toquin, Bäyon-Auboyer *et al.*, 1999). No Brasil, são utilizadas vacinas contra os subtipos A e B.

1.9. RNAi

A interferência por RNA é um processo pelo qual moléculas de RNA fita dupla (dsRNAs) promovem um silenciamento gênico, tanto pela degradação de seqüências-específicas complementareas ao RNA mensageiro (mRNA), quanto pela supressão da tradução protéica (Huppi, Martin *et al.*, 2005). Este fenômeno foi observado pela primeira vez no nematódeo *Caenorhabditis elegans* (Fire, Xu *et al.*, 1998) e, posteriormente, em outros organismos, como plantas, invertebrados (Drosófila) e vertebrados (Bitko, Musiyenko *et al.*, 2005; Buckingham, 2006; Ronemus, Vaughn *et al.*, 2006). Em mamíferos, moléculas de dsRNAs menores que 30 nucleotídeos (nt) geram uma resposta seqüência-específica pela RNAi sem induzir resposta imune celular (Caplen, Parrish *et al.*, 2001; Elbashir, Harborth *et al.*, 2001). O uso de moléculas exógenas de siRNAs (do inglês, short interfering RNA) para bloquear a expressão gênica representa uma potente ferramenta para estudos da função gênica e identificação de novas drogas. O mecanismo da RNAi é ativado por dois principais tipos de seqüências específicas: os siRNAs e os micro RNA (miRNAs). Estes são sintetizados quimicamente pela Dicer, uma enzima da família RNase III, a partir dos precursores dsRNA e precursor miRNA (pre-miRNA), respectivamente. Uma vez sintetizados, os siRNAs/miRNAs têm uma fita selecionada para ser incorporada ao complexo de silenciamento induzido por RNA (RISC). Este complexo causa a degradação do mRNA complementar às seqüências-específicas de siRNA/miRNA e supressão da tradução, Figura 3 (Elbashir, Harborth *et al.*, 2001).

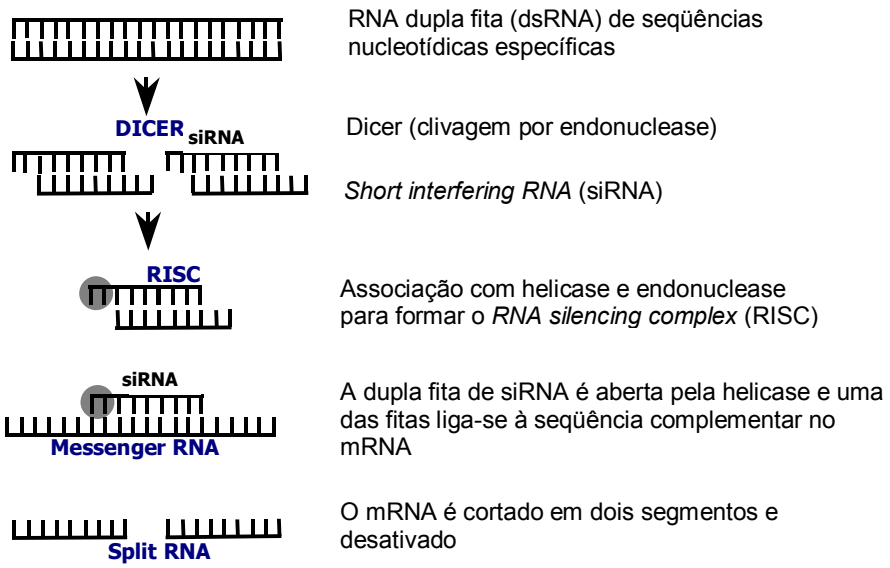

Figura 3: Silenciamento pós-transcricional de mRNA por RNAi usando siRNA (Hilleman, 2003).

O silenciamento de mRNA em mamíferos é um mecanismo de defesa natural realizado pelos miRNAs fita simples. Eles são pequenos RNAs que bloqueiam a tradução, acarretando breve sobrevida dos mRNAs alvos.

Nos últimos anos, o número de miRNAs identificados nos humanos aumentou intensamente (Berezikov, Guryev *et al.*, 2005; Lewis, Burge *et al.*, 2005). Os siRNAs e miRNAs dividem a associação das proteínas em RISC. Os miRNAs derivam dos RNA transcritos que contêm uma extensa alça e se acoplam no RISC como uma fita simples de RNA. Geralmente, os miRNAs têm nucleotídeos não complementares aos mRNAs alvos. Já os siRNAs, fitas duplas de RNA, necessitam ser introduzidos nas células de mamíferos e requerem a seleção da fita antisenso para a sua incorporação ao RISC. Embora os miRNA

sejam diferentes dos siRNA na sua biogênese, suas funções são muito semelhantes (Aronin, 2006).

Muitos trabalhos na literatura apresentam siRNAs com graus de eficiência diferentes. O desenho de siRNAs não se limita a escolher uma seqüência qualquer de 21pb baseado na seqüência do RNA mensageiro. Por exemplo, seqüências com a mesma composição podem exibir diferentes taxas de atividade. Em estudos anteriores, diversos algoritmos sobre o desenho de siRNAs funcionais foram propostos (Amarzguioui e Prydz, 2004; Ding, Chan *et al.*, 2004; Levenkova, Gu *et al.*, 2004; Reynolds, Leake *et al.*, 2004; Ui-Tei, Naito *et al.*, 2004; Arziman, Horn *et al.*, 2005; Patzel, Rutz *et al.*, 2005; Yiu, Wong *et al.*, 2005). Em alguns casos, estes algoritmos tiveram a eficácia comprovada e, em outros casos, falharam em distinguir siRNAs funcionais dos não-funcionais. Outros estudos relatam que a acessibilidade do mRNA alvo pode ser bloqueada pela formação de estruturas secundárias (Bohula, Salisbury *et al.*, 2003; Heale, Soifer *et al.*, 2005; Overhoff, Alken *et al.*, 2005; Westerhout, Ooms *et al.*, 2005; Yiu, Wong *et al.*, 2005).

A tecnologia RNAi apresenta muitas possibilidades de aplicação em biologia molecular, como na identificação da função de genes, no uso terapêutico no combate a vírus, prions e outros patógenos, e para criação de modelos animais para desordens genéticas. Este impacto na biologia molecular causado pela descoberta da tecnologia do RNAi foi publicado na revista *Science* na reportagem *Breakthrough of the Year* 2002. Esta metodologia já foi aplicada na inibição da replicação *in vitro* de um grande número de vírus, incluindo

poliovírus, vírus da imunodeficiência humana, vírus da hepatite C, vírus da hepatite B, influenzavírus e vírus da Síndrome Respiratória Aguda Grave (Capodici, Kariko *et al.*, 2002; Coburn e Cullen, 2002; Gitlin, Karelsky *et al.*, 2002; Surabhi e Gaynor, 2002; Ge, Mcmanus *et al.*, 2003; Kapadia, Brideau-Andersen *et al.*, 2003; Randall, Grakoui *et al.*, 2003; Shlomai e Shaul, 2003; Wilson, Jayasena *et al.*, 2003; Zhang, Li *et al.*, 2004). A inibição do AMPV subtipo C também já foi descrita (Munir, Kaur *et al.*, 2006). Outros estudos utilizaram siRNAs em camundongos adultos para silenciar um gene do vírus da hepatite C (Mccaffrey, Meuse *et al.*, 2002). A RNAi está sendo amplamente aplicada *in vivo* e, com sucesso, em camundongos, ratos, suínos e cobaios (Bitko, Musiyenko *et al.*, 2005; Zhang, Yang *et al.*, 2005; Chen, Liu *et al.*, 2006). Além disso, foram realizados estudos em ovos embrionados de aves para inibir a replicação de retrovírus (Hu, Bushman *et al.*, 2004).

Pelos aspectos discutidos, é possível identificar que a tecnologia RNAi abre um leque de oportunidades para a pesquisa em vírus: primeiro como uma ferramenta para analisar interações entre vírus e hospedeiro (Saleh, Van Rij *et al.*, 2004) e, segundo, como uma possível ferramenta terapêutica contra infecções virais.

2. Objetivos

2.1. OBJETIVO GERAL

- Verificar a inibição na replicação de metapneumovírus aviários (AMPV) subtipo A *in vitro* pela interferência por RNA.

2.2. OBJETIVOS ESPECÍFICOS

- Detectar e sequenciar para determinar a origem do gene da beta-actina, controle interno das técnicas moleculares utilizadas na detecção do AMPV *in vitro*, em células CER;
- Padronizar e comparar as técnicas de RT-PCR convencional e real time RT-PCR para a detecção de AMPV;
- Utilizar seqüências de siRNAs para o gene N do AMPV para estudos de inibição da replicação viral.

3. Artigos

ARTIGO I

BETA-ACTIN OF CHICKEN EMBRYO-RELATED (CER) CELLS IS CLOSELY RELATED TO BABY HAMSTER KIDNEY CELLS

Helena Lage Ferreira, Fernando Rosado Spilki, Tereza Cristina Cardoso, Clarice Weis Arns

Manuscrito em preparação

BETA-ACTIN OF CHICKEN EMBRYO-RELATED (CER) CELLS IS CLOSELY RELATED TO BABY HAMSTER KIDNEY CELLS

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Abstract

The chicken embryo-related (CER) cells were first reported as being able to support rabies virus replication. Afterwards, it was frequently used to isolate and replicate other animal viruses. CER cells are frequently classified as being originated from a mixture of chicken embryo fibroblast (CEF) and baby hamster kidney cells (BHK_{21}). The aim of this study was evaluated the CER cells beta-actin and verify if it is phylogenetically closer to BHK_{21} or CEF cells. The CER beta actin gene was amplified by RT-PCR, and the amplicon was sequenced. In addition, the CER chromosomes number was established. The CER chromosome number ranges from 2n=36 to 40. The BHK_{21} and CER beta-actins were detected using hamster-specific primers and CEF cells were positive to Gallus gallus beta-actin amplification. The results showed that such cells are closely related to BHK_{21} ($p > 0.966$), having a p-distance of 0.7 from chicken embryo fibroblasts. This confirms that CER cells are phylogenetically closely related to BHK_{21} cells.

Keywords: chicken embryo fibroblast cells; baby hamster kidney cells; chick embryo-related; beta-actin

Short Communication

Chicken embryo-related (CER) cells are frequently classified as being originated from a mixture of chicken embryo fibroblast (CEF) and baby hamster kidney cells (BHK_{21}) (Smith, Tignor *et al.*, 1977). BHK_{21} cells (ATCC number of CCL-10, clone 13) are defined as hamster (Golden syrian) fibroblast morphology derived from 1 day old newborn kidney cells with 2n chromosomes number of 44 (2n=44) (Atcc, 1994). CEF cells present a diploid number of 2n=78, as they have a high percentage of microchromosomes (Schmid, Nanda *et al.*, 2005).

CER cells were first used to isolated rabies virus and are frequently used today to isolate and replicate several other animal viruses. CER cells were used to isolate avian metapneumovirus (AMPV) from turkey flocks in Germany and from chicken flocks in Brazil (Arns e Hafez, 1992). It was also used to propagate infectious bursal disease virus (IBDV), and infectious bronchitis virus (IBV) Massachusetts serotype (Cardoso, Rahal *et al.*, 2000; Ferreira, Pilz *et al.*, 2003; Cardoso, Teixeira *et al.*, 2005; Cardoso, Teixeira *et al.*, 2005).

CER cells were previously classified as heteroploid and antigenically related to BHK_{21} cell by using fluorescent antibodies test. Nowadays, it is possible to establish relationships among different organisms by using the sequence of an actin gene or protein structure (Da Silva Vaz, Imamura *et al.*, 2005). Actins are usually encoded by multigene family and remarkably conserved through organisms evolution (particularly animal evolution) (Da Silva Vaz, Imamura *et al.*, 2005). There are three main actin isotypes (alpha, beta and gamma) which show >90% amino-acid homology among isotypes and >98%

homology within members of a particular isotypic group (Bork, Sander *et al.*, 1992; Da Silva Vaz, Imamura *et al.*, 2005).

The purpose of this study was to verify if CER cells are genotype closer related to BHK_{21} or CEF cell by amplifying the beta-actin gene from *Gallus gallus* and hamster (Golden Syrian) in CER, CEF and BHK_{21}, and after compared these sequences with those previously obtained from others species.

BHK_{21} cells (C-13) (ATCC CCL10) were purchased from American Type Culture collection (Rockville, MD). CER cells were kindly supplied by Dr Hafez M. Hafez, from Institute for Poultry Diseases, Free University Berlin, Germany. Both cell lines were cultured in Eagle's minimal essential medium (E-MEM) with 10% fetal bovine serum at 37°C. Chicken embryo fibroblast (CEF) cells were cultured as previously described (Ferreira, Pilz *et al.*, 2003).

CER cells chromosome counting was performed by 22h post-seeding, placed in $25cm^2$ flasks with 20μL colchicine (1mg/mL), and incubated for 2h at 37ºC. 10mL of 0.52% KCl were added to the flasks, and the suspension was incubated at room temperature for 2h. The cells were collected by centrifugation at 1000*g*, and the pellet re-suspended three times in 10mL cold methanol:acetic acid solution (3:1). Chromosomes were then counted (Davies, 1993).

DNA was isolated from CER, CEF, and BHK_{21} cells to evaluate beta-actin amplification, using High Pure Viral acid nucleic kit (Roche, Germany), following manufacturer's recommendations. Primers were selected based on the conserved regions of the available nucleotide sequences of Gallus gallus beta-actin: Gg β-actin F (5´-AAGATCTGGCACCACACTTTC-3`), Gg β-actin R (5´-

ACAGCTTCTCCTTGATGTCAC-3´); and of Hamster beta-actin: Hm β-actinF (5´-TGCGTGACATCAAAGAGAAGC-3´), Hm β-actinR (5`-TCTTGATCTTCATGGTGCTGG-3´). PCR amplification of beta actin genes was performed using Taq DNA Polymerase Recombinant (Invitrogen, USA), with final concentrations of 1X PCR buffer, 0.3mM dNTP mixture, 0.125mM MgCl2, 0.4 µM of each primer in a total reaction volume of 25 µL, containing 1 µL of DNA. Amplification involved an initial denaturation step at 94°C for 30min, followed by 35 cycles at 94 C for 30sec, 54°C for 30 sec, 72°C for 2min. After the last cycle, a final extension at 72°C for 2 min was carried out. Beta-actin PCR products (Gallus gallus – 400 bp; hamster – 360 bp) were fractionated by 1% agarose gel electrophoresis, and stained with ethidium bromide. PCR products were then directly sequenced using BigDye Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Japan) with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Japan).

Seven nucleotide sequences were obtained by searching the GenBank Database through the Nucleotide sequences search page (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). The beta-actin nucleotide sequences of *Fucus vesiculosus*, two *Cricetinae* gen sp, *Mesocricetus auratus*, *Mus musculus, Rattus norvergicus, Gallus gallus* (GenBank accession numbers X98885, AF014363, AF014364, (Cricetinae gen.sp. Golden Syrian) AJ312092, X03672, BC063166, X00182), and CER cells and CEF cells (GenBank accession number provided by the authors **DQ207608,** and **DQ207609)** were used. The respective access numbers are shown for each

sequence with the name of the strain in brackets (Figure 2). The nucleotide sequence of the beta-actin gene from alga (*Fucus vesiculorum*) was included as out-group for phylogenetic analysis.

BioEdit software, version 7.01 (Hall, 1999), was used to manipulate the nucleotide and amino acid retrieved sequences. The alignment of the sequences was performed with ClustalW software, version 1.83 (Thompson, Higgins *et al.*, 1994), using full alignment and a number of 2000 total replications in the bootstrap (Efron, Halloran *et al.*, 1996). Phylogenetic relationships between these nucleotide sequences were performed in MEGA 3. (Kumar, Tamura *et al.*, 1994). Neighbor joining trees were constructed from Kimura-2 parameters (p distance). Bootstrap re-sampling was performed for each analysis (1000 replications).

The CER chromosome number ranges from 2n=36 to 40. The analysis was performed in 8 different days and a standard deviation of $\pm$ 0.23 was observed ($p<0.001$). The morphology was compatible to macrochromosomes. No evidence of microchromosmes was observed (data not shown).

As shown by Figure 1A, the BHK_{21} and CER beta-actins were detected using hamster-specific primers (360bp). Only CEF cells were positive to *Gallus gallus* beta-actin amplification, generating a 400bp product, (Figure 1B). Phylogenetic analysis was performed using beta-actin CER cells nucleotide sequences. Its similarity with *Mesocricetus auratus and Cricetinae (Golden Syrian)* beta-actin sequence ranges from 92 to 96% (table 1). The CER beta actin sequence was classified in a separate cluster, distant from *Gallus gallus* and CEF beta-actin (Figure 2).

The chicken karyotype (2n=78) has a typical organization comprising a few machromosome pairs and several tiny michromosomes (Stock e Bunch, 1982). In our study, the number of CER chromosomes ranged from 2n=36 to 40, which is close to the BHK_{21} chromosome number. Same result was described by Bussеareau et al. (1982). Another study reported CER chromosomes ranging from 4 to 180 (Smith, Tignor *et al.*, 1977). This difference may be due to the fact that certain chromosomes were masked or by the fact that our CER cell line was more homogeneous.

Phylogenetic analysis was performed to demonstrate the relationship between rodents, avian, CEF, and CER beta actins. It showed high similarity of CER cells with Mesocricetus auratus and Cricetinae (Golden Syrian). Important to note that Golden Syrian is the origin of BHK_{21} cells.

Those results suggest that CER cells should be re-classified as BHK_{21}-like mammalian cell, as the chicken cells apparently died completely, while the BHK_{21} cells became immortal. This may be due to the continuous expression of endogenous telomerase activity, also observed in other rodent cells (Prowse e Greider, 1995; Holt, Wright *et al.*, 1996). These findings are important for the validation of quantitative mRNA assays. Indeed, beta-actin could be used as housekeeping gene.

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Figure Captions

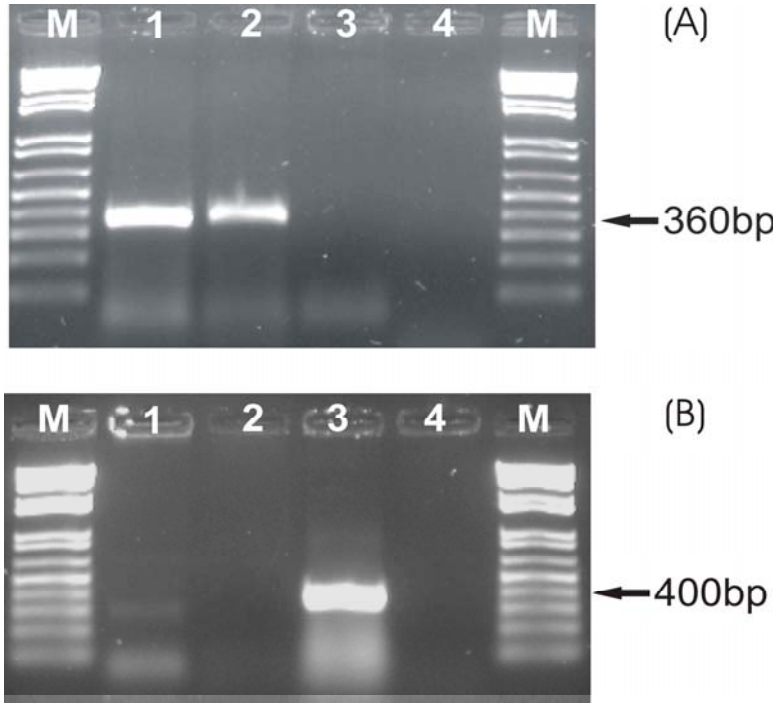

Figure 1 - Detection of (A) hamster beta-actin (360bp) and (B) *Gallus gallus* beta-actin (400bp) in different culture cells, as indicated: M: Leader 1kb plus; 1- BHK_{21} cells; 2- CER cells;.3- CEF; 4- Negative control, respectively.

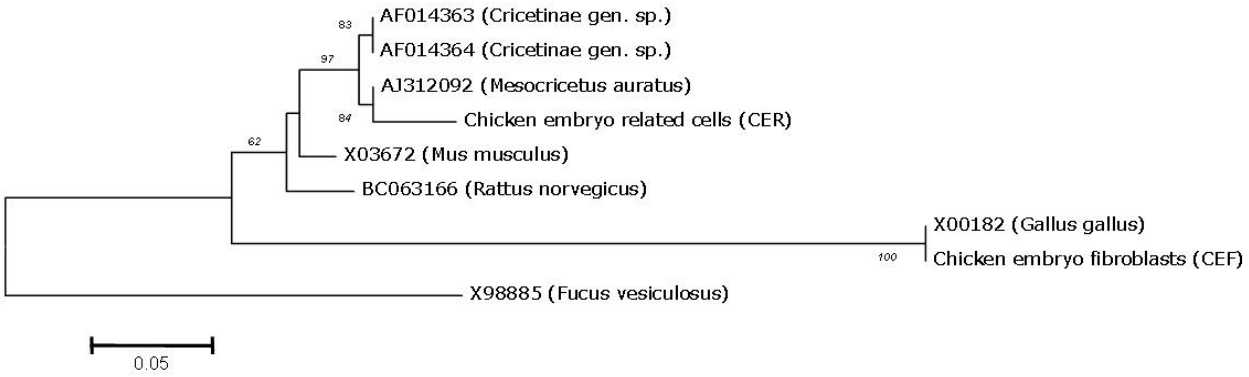

Figure 2 - Phylogenetic tree prepared with beta actin gene nucleotide sequences (from position 3068 to 3264) of CER cell, rodent, and avian sequences. For the parameters applied to measure genetic relationships, please refer to the text. GenBank accession numbers are given in each taxon and the correspondent species of the sequence is shown in brackets. The tree was rooted using a *Fucus vesiculosus* sequence. The bar indicates the number of substitutions per site.

Table 1: Genetic p-distances between beta actin of CER cells, rodent, and avian sequences, as determined by partial gene sequences

	X03672	**BC063166**	**AF014363**	**AF014364**	**AJ312092**	**DQ207608**	**X00182**	**DQ207609**
X03672(*Mus_musculus*)								
BC063166(*Rattus_norvegicus*)	0.961							
AF014363 (*Cricetinae*_gen._sp.)	0.955	0.938						
AF014364 (*Cricetinae*_gen._sp.)	0.955	0.938	1.000					
AJ312092 *(Mesocricetus_auratus*)	0.955	0.938	0.988	0.988				
DQ207608 (CER cells)	0.927	0.905	0.955	0.955	0.966[A]			
X00182(*Gallus_gallus*)	0.727	0.733	0.727	0.727	0.727	0.700[B]		
DQ207609 (CEF cells)	0.727	0.733	0.727	0.727	0.727	0.700	1.000[C]	
X98885 (*Fucus_vesiculosus*)	0.733	0.727	0.727	0.727	0.727	0.705	0.605	0.605

A = identity between CER and *Mesocricetus auratus*
B = identity between CER and *Gallus gallus*
C = identity between CEF and *Gallus gallus*

ARTIGO II

COMPARATIVE EVALUATION OF CONVENTIONAL RT-PCR AND REAL-TIME RT-PCR (RRT-PCR) FOR DETECTION OF AVIAN METAPNEUMOVIRUS SUBGROUP A (AMPV/A)

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Comparative evaluation of conventional RT-PCR and real-time RT-PCR (RRT-PCR) for detection of avian metapneumovirus subtype A (AMPV/A)

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RT-PCR and RRT-PCR comparison for AMPV/A detection

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Abstract

Avian metapneumovirus (AMPV) belongs to the genus Metapneumovirus of the Paramyxoviridae family. Virus isolation, serology, and detection of genomic RNA are used as diagnostic methods for AMPV. The objective of the present study was to compare the detection of AMPV isolates genome fragments from the viral RNA extraction product using different conventional and real time RT-PCR methods. Two new RT-PCR test detecting fusion (F) gene, nucleocapsid (N) gene and two news real time RT-PCR tests detecting F and N genes were compared with an established test in the attachment (G) gene. In order to evaluate the detection limits, RNA extraction was performed from two different isolates, previously serially diluted. Each dilution of RNA was submitted in duplicate to reverse transcription into cDNA by using hexamer primers. The cDNA products were then used in conventional RT-PCR and real time RT-PCR. All the RT-PCR tested assays were able to detect the six isolates. The higher detection limits were observed at 10^{-5}- fold and 10^{-5}-fold dilutions of the N- and F- based RRT-PCR, respectively. The present study suggests that the conventional F- based RT-PCR and N- and F- based RRT-PCR can be successfully use for AMPV/A. It also suggests that the established conventional G- based RT-PCR can be used for rapid AMPV/A and AMPV/B detection. Due to its capacity to deal with large number of samples in a rapid, sensitive, and specific manner, RRT-PCR can be used for AMPV virus diagnostic and screening as an alternative tool to virus isolation.

Keywords: avian metapneumovirus; G;F;N genes; real time RT-PCR; RT-PCR

1. Introduction

The avian metapneumovirus (AMPV), previously called avian pneumovirus (APV) or Turkey rhinotracheitis virus (TRTV), was the only recognized member of *Metapneumovirus* genus, until the isolation of Human metapneumovirus (hMPV) in the Netherlands, in 2001 (Van Den Hoogen, De Jong *et al.*, 2001). AMPV is a member of the Paramyxoviridae family, *Pneumovirinae* subfamily, within the new genus *Metapneumovirus* (Cook, 2000; Cook e Cavanagh, 2002; Alkhalaf e Saif, 2003; Njenga, Lwamba *et al.*, 2003; Easton, Domachowske *et al.*, 2004; Harnden, 2005), which contains a non segmented, negative-sense RNA genome of approximately 13,000 nt length. The AMPV genome is composed by eight viral genes arranged in the order: nucleocapsid–phosphoprotein–matrix–fusion–second matrix–small hydrophobic–glycoprotein–large polymerase (‘3–N–P–M–F–M2–SH–G–L–5’). It is flanked by a leader and a trailer at the ‘3 and 5’ ends, respectively (Easton, Domachowske *et al.*, 2004).

AMPV causes acute rhinotracheitis characterized by coughing, nasal discharge and conjunctivitis in turkeys. In chickens, AMPV plays a role, in association with bacteria, on the development of swollen head syndrome (Cook, 2000; Njenga, Lwamba *et al.*, 2003; Turpin, Lauer *et al.*, 2003; Velayudhan, Lopes *et al.*, 2003). AMPV infection is also associated to egg drop production in laying hens (Cook, 2000). The virus was first described causing clinical evident disease in South Africa. Nonetheless, major outbreaks of the disease were later reported in Europe (Hafez, Hess *et al.*, 2000), United States (US), United Kingdom, Middle East, Asia (Njenga, Lwamba *et al.*, 2003), and in other parts of the world (Buys, Du Preez *et al.*, 1989b; 1989a; Cook e Cavanagh,

2002). AMPV is also present in Brazilian flocks at least since 1992 (Arns e Hafez, 1992).

Initially, the first analysis of AMPV isolates by ELISA and virus neutralization showed that this virus should be divided in two different groups (Collins, Gough *et al.*, 1993; Cook, Jones *et al.*, 1993), named subtypes A and B (AMPV/A, AMPV/B) (Juhasz e Easton, 1994). Further characterization of isolates from United States showed the emergence of a third subtype, C named AMPV/C (Seal, Sellers *et al.*, 2000; Lwamba, Bennett *et al.*, 2002; Shin, Cameron *et al.*, 2002; Alvarez, Lwamba *et al.*, 2003; Njenga, Lwamba *et al.*, 2003). This last subtype is more related to human metapneumovirus (hMPV) isolates than to the other AMPV subtypes (Toquin, De Boisseson *et al.*, 2003). A fourth subtype (AMPV/D) was detected in France (Bäyon-Auboyer, Arnauld *et al.*, 2000).

Diagnosis of AMPV infection can be achieved by virus isolation in chicken or turkey tracheal organ cultures (TOC). Alternatively, it can be obtained from cell cultures (Giraud, Bennejean *et al.*, 1986; Mcdougall e Cook, 1986; D'arce, Coswig *et al.*, 2005). Other methods allow the identification and characterization of AMPV, such as immunofluorescence staining or virus neutralization of the isolate with polyclonal or monoclonal antibodies (Baxter-Jones, Wilding *et al.*, 1986; Grant, Baxter-Jones *et al.*, 1987; Otsuki, Hirai *et al.*, 1996). The observation of cytopathogenic effect may require several passages in cell cultures and has to be confirmed afterwards by immunostaning. Therefore, several days are required for virus isolation and identification. On the other hand, new molecular methods, such as polymerase chain reaction (PCR), allow the development of rapid, sensitive and specific detection of

AMPV (Juhasz e Easton, 1994; Bäyon-Auboyer, Jestin *et al.*, 1999; Dani, Durigon *et al.*, 1999; D'arce, Coswig *et al.*, 2005; Guionie, Toquin *et al.*, 2007b).

In the present study, two newly defined RT-PCR assays, two newly defined real time RT-PCR tests detecting the F and N genes, and an established test in the attachment (G) gene (Bayon-Auboyer et al., 1999) were performed and compared for detection of six Brazilian isolates. The detection limits of RT-PCR tested assays were also compared.

2. Materials and Methods

2.1 Virus Strains: Six Brazilian AMPV strains were isolated from trachea and nasal exsudated in Chicken embryo-related cell line, CER (Smith, Tignor *et al.*, 1977; Ferreira, Pilz *et al.*, 2003). These isolates named as: chicken/A/BR/119/95, chicken/A/BR/121/95, SHSBR/662/03, SHSBR/668/03, SHSBR/669/03 and TRTBR/169, were described as AMPV/A (Dani, Durigon *et al.*, 1999; D'arce, Coswig *et al.*, 2005). One strain (STG SHS-1439, AMPV/B) from Germany was included in the analysis. In addition, 10-fold serial dilutions of isolates chicken/A/BR/121/95 and SHSBR/669/03 were performed. The titers were calculated by the Reed-Muench method (Reed e Muench, 1938).

2.2. RNA extraction and reverse transcription (RT): Total RNA was extracted from 200μL of infected cell cultures using High Pure Viral RNA kit (Roche, Mannheim, Germany), according to manufacturer's recommendations. A 5μL RNA sample was used for the generation of cDNA using 60 ng of a hexamer primer (Invitrogen, Carlsbad, CA, USA) and Superscript III reverse transcriptase enzyme (Invitrogen, Carlsbad, CA,

USA) with final volume of 20µL according to manufacturer's recommendations. Specificity tests were performed on RNA isolated from stocks of other RNA viruses, including, infectious bronchitis virus (IBV), respiratory syncytial virus (hRSV).

2.3. Primer selection: Four different pairs of primers AMPV-specific targeting the N, F genes and two different probes located in the N and F genes (Table 1) were selected based on the conserved regions of the nucleotide sequences available for the F and N genes of AMPV/A. For the RRT-PCR, the forward primer, reverse primer and probe were defined using the Primer Express Version 2.0 software (Applied Biosystems). Also, primers AMPV-specific targeting the G gene previous described by Bäyon-Auboyer et al. (1999) were selected.

2.4. Conventional RT-PCR : PCR amplification of N and F genes was performed using the Taq DNA Polymerase Recombinant (Invitrogen, Carlsbad, USA), with final concentrations of 1X PCR buffer, 0.3mM of dNTP mixture, 0.125mM of $MgCl_2$, 0.2 µM of each primer in a total reaction volume of 25 µL containing 1 µL of cDNA. For the reaction G gene amplification, the reaction used has been described by Bäyon-Auboyer et al. (1999). PCR products (N gene– 698 bp; F gene- 698 bp; G gene- 448bp) were observed in 1% agarose gel electrophoresis, stained with ethidium bromide.

2.5. Real time RT-PCR (RRT-PCR): Real-time PCR amplification of N and F genes was performed using the Quantitec Probe PCR kit (Qiagen, Hilden, Germany), with final concentrations of 900 nM of each primer, and 300 nM of the Taqman probe in a total

reaction volume of 25 µL containing 1 µL of cDNA. An external standard curve was created using spectrophotometrically determined copy number standards of purified PCR product for each gene. After an initial reverse transcription step and an initial denaturation step at 95°C for 15min, 50 cycles (95°C 15 sec – 60°C 1 min) were performed with fluorescence detection at the end of the annealing-extension step. Amplification and fluorescence detection were carried out in an Applied Biosystems 7500 real time PCR cycler. All samples were run in duplicate. For absolute quantification, a PCR product containing the target sequence was used as DNA standard. C_t (threshold cycle) values were used, as C_t indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold.

In order to convert threshold cycles in a copy number, an external standard curve was created with known copy numbers of F gene and N gene of AMPV. Copy number was calculated using the following formula:

Y molecules/µL = (Xg/µL DNA/ [Length of PCR product in base pairs x 660]) x 6.022 x 10^{23}).

2.6. Sequencing: Nucleotide sequences were determined by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Perkin-Elmer Applied Biosystems, Foster city, USA) using AMPV F, N and G products purified with the GFX PCR DNA and gel Band Purification kit (GE Healthcare, Buckingham, UK). The sequence was performed using BigDye Dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Foster city, USA).

2.7. Nucleotide sequences accession numbers: AMPV sequences presented in this paper have been deposited in GenBank under accession numbers according to nucleotide in segments of the N, G, and F protein genes (Table 2). LAH strain (accession number AY640317) was taken from the full genome available at the same database.

2.8. Sequences analysis: The BioEdit software, version 7.01(Hall, 1999), was used to manipulate the retrieved amino acid sequences. Sequence alignments were performed using the ClustalW software, version 1.83 (Thompson, Higgins *et al.*, 1994). In order to ensure a higher level of confidence in our analysis, full alignment and a number of 2000 total replications on the bootstrap were used (Efron, Halloran *et al.*, 1996). Phylogenetic relationships between these protein sequences were performed in MEGA 3 (Kim, Yokoyama *et al.*, 2004), neighbor joining trees were constructed from Kimura-2 parameters and calculated using pair-wise deletion. Bootstrap was resampled as a test of phylogeny using 250 replications.

3. Results

3.1. Conventional RT-PCR: All the six isolates were detected using conventional G, F-, and N-based, RT-PCR (Figure 1). The RT-PCR products had the appropriated size on ethidium bromide stained agarose gels. All negative and blank controls were negative using conventional RT-PCR (data not shown).

3.2. RRT-PCR: The N- and F- based RRT-PCR assays were also able to detect all isolates. A standard curve for N gene AMPV quantification was established using a PCR product containing a target sequence serially diluted from 8 $x10^7$ to 8 $x10^0$. The standard curve showed an efficacy of 98.71%, a slope of -3.353247, a regression coefficient of 0.993317, and an intercept of 45.66.

The standard curve of F gene AMPV quantification was generated using F target sequence serially diluted from 10^8 to 10^0. RRT-PCR efficiency was 99.95%, slope was -3.3229, a regression coefficient was 0.998116, with an intercept of 49.621..

The F- and N- based RRT-PCR were able to detect 3,000 and 100 copies, respectively. The six tested isolates showed copy numbers ranged from 6.89x 10e8 to 2.89 x 10e7 (C_t values: 19.69 to 24.64). For the N- based RT-PCR, copy numbers detected were 1.40 x 10e8 to 3.23 x10e6 (C_t values: 18.51 to 23.84).

F- based RRT-PCR was able to detect 2.51 x 10e7 (C_t: 24.86) and 7.91 x10e7 (C_t: 22.92) copy numbers, from the 10^{-1}- fold dilution of the isolate chicken/A/BR/121/95 and SHSBR/669/03, respectively. It was also able to detect 4,301 (C_t: 38.23) and 7,052 (C_t: 37.76) copy numbers, from the 10^{-5}- fold dilution of the isolate chicken/A/BR/121/95 and SHSBR/669/03, respectively.

N- based RRT-PCR was able to detect 1.44 x 10e6 (C_t: 24.94) and 6.74 x10e6 (Ct: 22.77) copy numbers from the 10^{-1}- fold dilution of the isolate chicken/A/BR/121/95 and SHSBR/669/03, respectively. And it was also able to detect 131 (C_t: 38.60) and 134 (C_t: 38.59) copy numbers from the 10^{-5}- fold dilution of the isolate chicken/A/BR/121/95 and SHSBR/669/03, respectively.

3.3 Detection Limit: In order to evaluate the detection limit, two different isolates (chicken/A/BR/121/95 and SHSBR/669/03) were 10-fold serially diluted, and RNA was extracted. The chicken/A/BR/121/95 titer was $10^{5.3}$ $TCID_{50}$/mL and the SHSBR/669/03 titer was 10^{6} $TCID_{50}$/mL. Each RNA dilution was submitted in duplicate to reverse transcription into cDNA by using hexamer primers. The cDNA products were then used in conventional RT-PCR and/or real time RT-PCR. The detection limit results using G-, F- and N- based RT-PCR and F- and N- based RRT-PCR assays are described in Table 3.

The detection limits for the two isolates were visualized at 10^{-4}- fold, 10^{-5}- fold, and 10^{-1}-fold dilutions using the G-, F- and N- based conventional RT-PCR, respectively (Figure 2). The detection limits of the N- and F- based RRT-PCR were observed at 10^{-5}- fold and 10^{-5}-fold dilutions from both isolates, respectively (Figure 3). Although virus titer of the isolates were different, no distinction could be observed in the detection limits by using RT-PCR assays.

3.4. Specificity: The specificity of RT-PCR detection methods was evaluated using different RNA viruses. The developed methods were found to be specific for AMPV/A. No specific amplification was detected for other RNA viruses, such as IBV, hRSV and AMPV/B (data not shown). The conventional RT-PCR for the G gene was also able to detect AMPV/B, as previously described by Bäyon-Auboyer et al. (1999).

3.5. Sequences analysis: High levels of nucleotide identity were found among the 6 isolates (AMPV/A= 100% for the G gene, 98.5 to 100% for the F gene; 95.8 to 100 % for the N gene). All the Brazilian strains are clustered among the AMPV/A.

4. Discussion

Accurate AMPV diagnosis based on clinical signs is not recommended as such virus can be impacted by antigenic and genetic variation, as well as by other disease manifestations. Therefore, a differential diagnosis is extremely important (Cook e Cavanagh, 2002). Virus isolation is normally the ideal method for confirming AMPV infection. Nonetheless, once the clinical signs appear, it can be very difficult to isolate the virus. Also, this kind of procedure is very time consuming (Buys, Du Preez *et al.*, 1989a; Cook, 2000; Cook e Cavanagh, 2002). Previous studies demonstrated that conventional RT-PCR could be effective in AMPV detection as well. (Juhasz e Easton, 1994; Bäyon-Auboyer, Jestin *et al.*, 1999; Shin, Rajashekara *et al.*, 2000). As RRT-PCR is considered less variable than conventional RT-PCR (Bustin e Mueller, 2005), it became the benchmark for detection and quantification of RNA targets and is being increasingly used in new diagnostic assays (Bustin, 2000). In the present study, we compared the specificity and detection limits of two newly designed conventional RT-PCRs and two newly defined RRT-PCRs with an established RT-PCR for AMPV detection. Our emphasis was to compare the molecular assays and its evaluation by using the same extracts –diluted RNA and reverse transcribed cDNA. We analyze

those methods in the absence of any variable which could influence the assay reproducibility.

Our results showed that the totality of the conventional RT-PCR and RRT-PCR tested assays was able to detect all six isolates. Juhasz & Easton (1994) confirmed the presence of two distinct groups, named AMPV/A and AMPV/B. Bayon-Auboyer et al. (1999) described the ability of the G- based RT-PCR assay to detect AMPV/A and AMPV/B in field samples. Our results go in the same sense, as the G-based RT-PCR was able to detect the AMPV subtype B. Also, the conventional F-based RT-PCR and the RRT-PCR tested assays specifically detected AMPV/A.

Bayon-Auboyer et al. (1999) also reported that the G-based RT-PCR method was sensitive enough to detect AMPV in swabs without requiring previous virus propagation. In this study, the findings suggested that the F- and N-based RRT-PCR and the conventional F-based RT-PCR are more sensitive than other assays. These assays presented a detection limit 10^{-5}-fold, while the G-based RT-PCR presented 10^{-4}-fold.

Real-time quantitative PCR is extremely accurate and less labor-intensive than current quantitative PCR methods (Heid, Stevens *et al.*, 1996). It is also able to provide DNA and RNA exact quantification. Quantitative results can be obtained by comparing the Ct values with the standard curve generated by known copy numbers of a standard curve (Bustin, 2000). In our study, the F- and N- based RRT-PCR was able to detect 3,000 and 100 copies, respectively. The sensitivity of the N-based RRT-PCR seemed to be higher than the recently reported G-based RRT-PCR (Guionie, Toquin *et al.*, 2007b). Also, the N-based RT-PCR, previous described by Bayon-Auboyer et al. (1999), showed more sensitivity than other tests targeting different genes. This fact could be

explained by the polarity exhibited during the transcription process. The genes closer to the promoter (3´end of the negative-strand genome) are most abundantly transcribed in nonsegmented negative-strand RNA viruses (Barik, 1992). The N gene is the promoter closest gene, thus, the transcription process produces more N mRNA than F and G genes.

According to a recent report, some positive signals (C_t value higher than 37) are occasionally observed with, non-target controls, which corresponds to the one copy range, that are apparently due to non-specific amplification and/or probe disruption at the end of the amplification process in absence of target cDNA (Loisy, Atmar *et al.*, 2005). We considered thus that C_t values higher than 39 may indicate either a problematic sample, or RNA purification, or RRT-PCR reaction.

The present study suggests that the conventional F-based RT-PCR and N- and F-based real time RT-PCR can be successfully used for AMPV/A detection. It also suggests that the established conventional G- based RT-PCR can be used for rapid detection of AMPV/A and AMPV/B. Moreover, real time RT-PCR assays can offer targeted mRNA detection, generating quantitative data. Due to its capacity to deal with large number of samples in a rapid, sensitive, and specific manner, RRT-PCR can be used for AMPV virus diagnostic and screening, as an alternative tool to virus isolation. It is therefore an important tool for AMPV monitoring in populations.

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Tables

Table 1- Primers or probes and characteristics for each amplified gene by RT-PCR and real time RT-PCR

Molecular Method	Gene	Primers or probe	Positions*	Sequence (5'- 3')	Cycle Profile	Ref.
RT-PCR	N	Nf	215-235	GCAAAACACACCGACTATGAG	94°C, 30 s; 53°C, 30 s; 72°C, 60 s**	this study
		Nr	892-912	TAGACCTCAGATACTTGCCTC		
Real time RT-PCR	N	AMPVN+494	494-514	CAAAAGCCGTCTGCCTTGGAT	95°C, 15 s; 60°C, 60 s***	this study
		AMPVN-567	547-567	GAGGCCAACTTGGTGAAAATG		
		AMPVN+516FAMTAMRA	516-545	CTCCCGTTATTCTATTATGCATTGGTGCCC		
RT-PCR	F	Ff	3178-3198	AGGGAGCTCAAAACAGTGTCA	94°C, 30 s; 54°C, 30 s; 72°C, 60 s**	this study
		Fr	3855-3875	CAGTACCACCCTTGATCTTCT		
Real time RT-PCR	F	AMPVF+3643	3643-3663	ATGCCAACTTCATCAGGACAGA	95°C, 15 s; 60°C, 60 s***	this study
		AMPVF-3721	3700-3721	TCAATATACCAAACCCCTTCCTTCT		
		AMPVF+3667FAMTAMRA	3367-3394	AGTTTGATGTTGAACAATCGTGCCATGGT		
RT-PCR	G	Ga	5944-5964	CCGGGACAAGTATCTCTATGG	94°C, 20 s; 54°C, 45 s; 72°C, 45 s****	Bäyon-Auboyer et al. 1999
		Gy	6390-6412	TCTCGCTGACAAATTGGTCCTGA		

* Nucleotide numbering based on avian metapneumovirus genome (GenBank accession no. AY640317)

** There were 35 cycles. They were preceded by a denaturation step (94°C, 3 min) and followed by an elongation step (72°C, 7 min).

***There were 50 cycles. They were preceded by a denaturation step (95°C, 15 min) and followed by an elongation step (72°C, 3 min).

***** There were 30 cycles. They were preceded by a denaturation step (94°C, 15 min) and followed by an elongation step (72°C, 10 min).****

Table 2 - Origins of virus strains and GenBank accession number according to nucleotide in segments of the N, G, and F protein genes

GenBank accession number			Virus	Subtype	Strain	Host	Year	State-BRAZIL
G gene	F gene	N gene						
AAW29976	DQ207607	DQ175635	AMPV	A	chicken/A/BR/119/95	Chicken	1995	MG
AAW29977	DQ175630	DQ175636	AMPV	A	chicken/A/BR/121/95	Chicken	1995	MG
AAU87838	DQ175632	DQ175638	AMPV	A	SHSBR/662/03	Chicken	2003	SP
AAU87839	DQ175633	DQ175639	AMPV	A	SHSBR/668/03	Chicken	2003	SP
AAU87840	DQ175634	DQ175640	AMPV	A	SHSBR/669/03	Chicken	2003	SP
AAU43593	DQ175631	DQ175637	AMPV	A	TRTBR/169	Turkey	2003	SC

Table 3 - Comparisons of detection limit of conventional RT-PCR (G, F, and N gene) and real time RT-PCR (F and N gene) molecular methods in detecting serially diluted of AMPV. x

Sample	Dilution	G	F	F	N	N
		RT-PCR gel (448bp)	RT-PCR gel (698bp)	RRT-PCR	RT-PCR gel (698bp)	RRT-PCR
chicken/A/BR/121/95	ND[a]	+	+	+	+	+
	10^{-1}	+	+	+	+	+
	10^{-2}	+	+	+		+
	10^{-3}	+	+	+		+
	10^{-4}	+	+	+		+
	10^{-5}		+	+		+
	10^{-6}					
	10^{-7}					
SHSBR/669/03	ND[a]	+	+	+	+	+
	10^{-1}	+	+	+	+	+
	10^{-2}	+	+	+		+
	10^{-3}	+	+	+		+
	10^{-4}	+	+	+		+
	10^{-5}		+	+		+
	10^{-6}					
	10^{-7}					

Figure captions

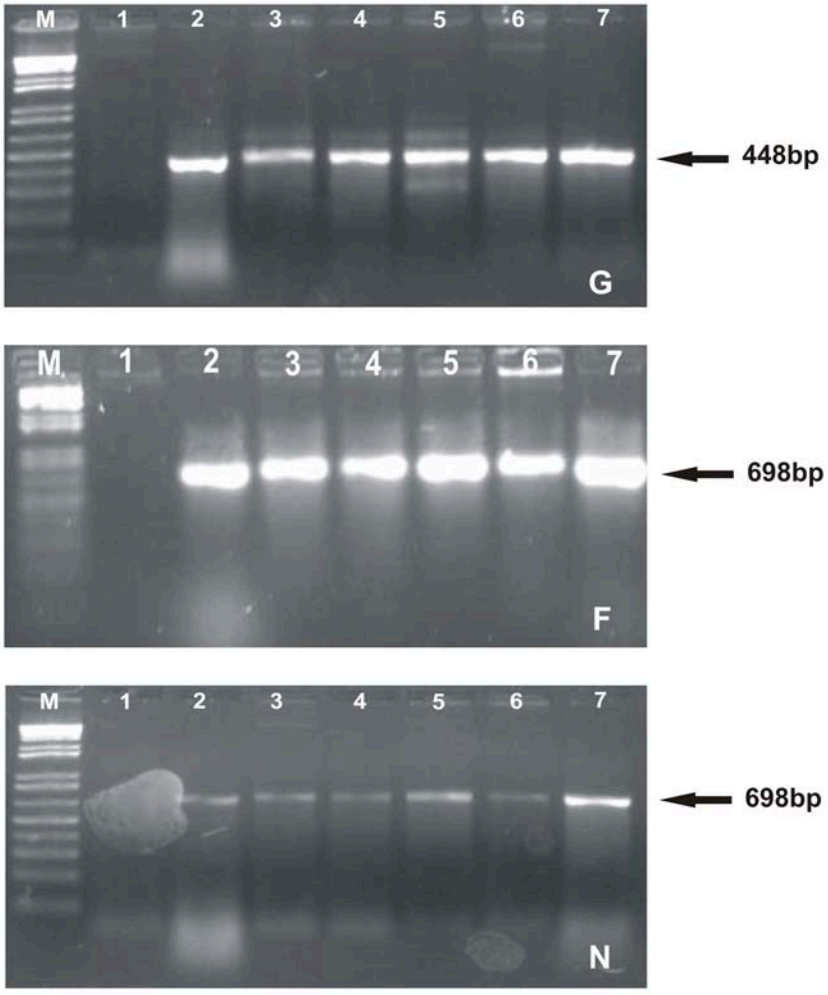


Figure 1- Detection of AMPV/A six isolates by conventional RT-PCR for G, F and N gene. All RT-PCR products had the appropriate size on ethidium bromide stained agarose gels (G=448bp; F= 698bp and N=698bp). M: Leader 1kb plus; 1: negative control; 2: chicken/A/BR/119/95; 3: chicken/A/BR/121/95; 4: SHSBR/662/03; 5: SHSBR/668/03; 6: SHSBR/669/03; 7: TRTBR/169.

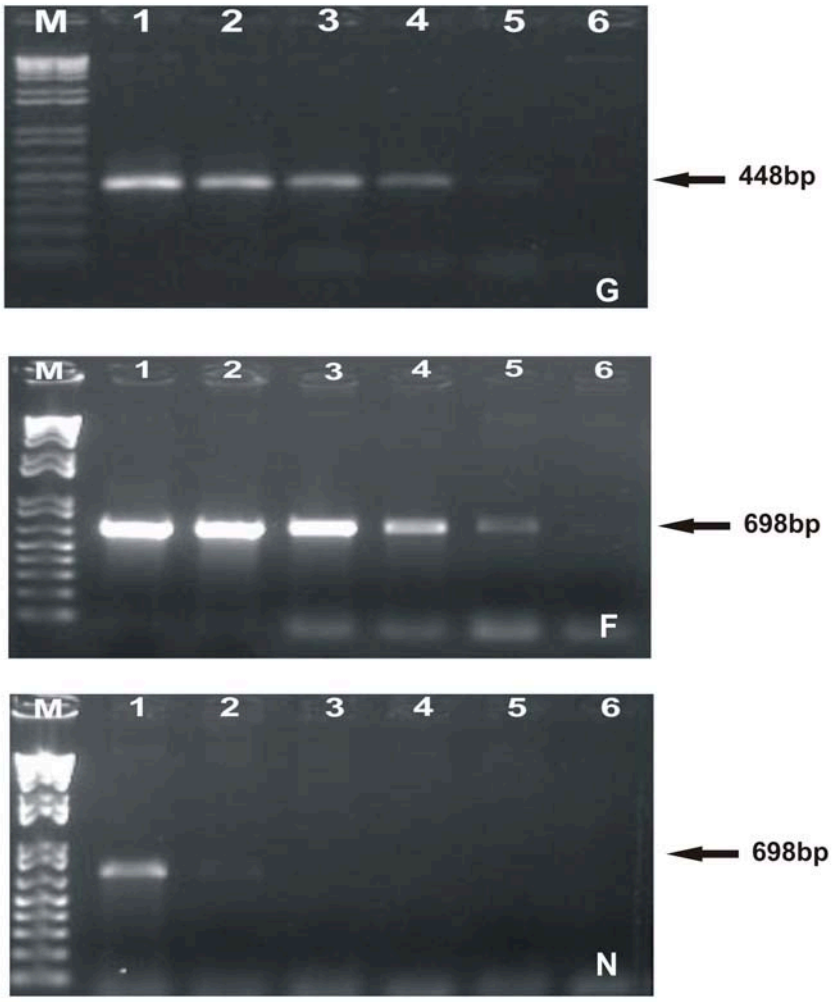
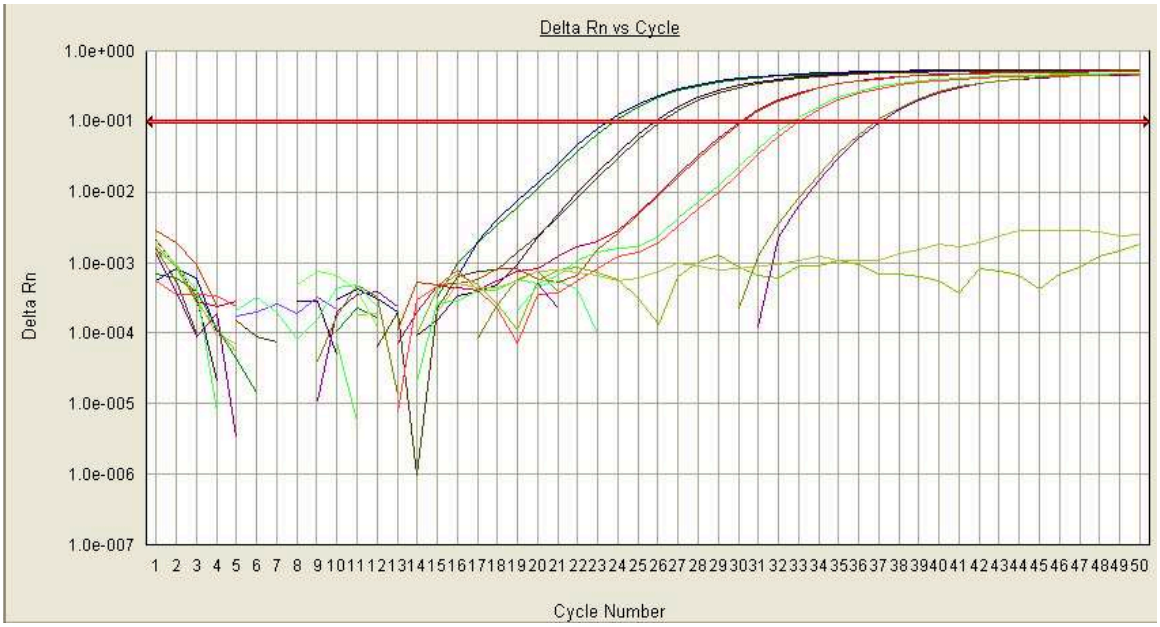


Figure 2- Detection limits of different conventional RT-PCR. The isolate SHSBR/669/03 was 10-fold serial diluted (10^1- fold to 10^6 fold) and the RT-PCR method was performed for the G, F and N genes detection. M: Leader 1kb plus; lines 1-6: 10^1 to 10^6- fold dilution.

F



N

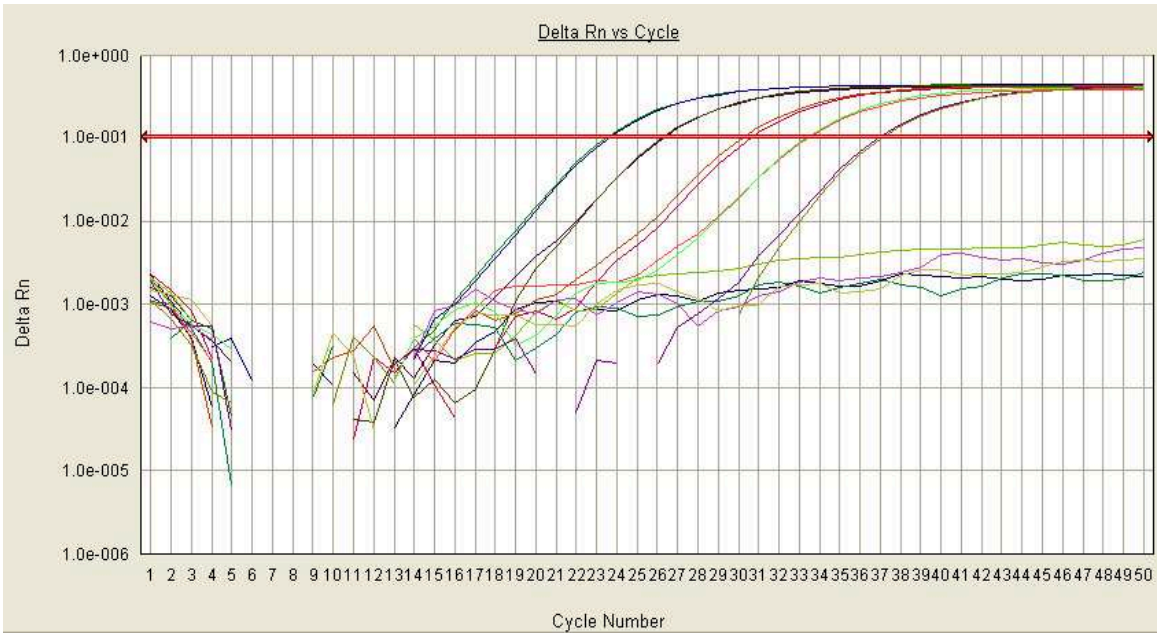


Figure 3. Detection limits of F- and N- based real time RT-PCR. Amplification plots of the SHSBR/669/03 10-fold serially diluted. Replicates from 10^1 to 10^5 dilutions were positive and 10^6 were negative.

ARTIGO III

INHIBITION OF AVIAN METAPNEUMOVIRUS (AMPV) REPLICATION BY RNA INTERFERENCE TARGETING NUCLEOPROTEIN GENE (N) IN CULTURED CELLS

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Short communication

Inhibition of avian metapneumovirus (AMPV) replication by RNA interference targeting nucleoprotein gene (N) in cultured cells

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Abstract

Avian metapneumovirus (AMPV) is the primary causative agent of severe rhinotracheitis in turkeys. It is associated with swollen head syndrome in chickens and is the source of significant economic losses to animal food production. In this study, we designed specific short interfering RNA (siRNA) targeting the AMPV nucleoprotein (N) and fusion (F) genes. Three days post-virus infection, virus titration, real time RT-PCR, and RT-PCR assays were performed to verify the effect of siRNA in AMPV replication. A marked decrease in virus titers from transfected CER cells treated with siRNA/N was observed. Also, the production of N, F, and G mRNAs in AMPV was decreased. Results indicate that N-specific siRNA can inhibit virus replication. In future studies, a combination of siRNAs targeting the RNA polymerase complex may be used as a tool to study AMPV replication and/or antiviral therapy.

Keywords: Avian metapneumovirus; RNA interference; Short interfering RNA; Inhibition; Real time PCR

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process initiated by double-stranded RNA (dsRNA). This phenomenon was first observed in the nematode Caenorhabditis elegans (Fire et al., 1998) and then in other organisms, such as plants, invertebrates (Drosophila), and vertebrates (Bitko et al., 2005; Buckingham, 2006; Ronemus et al., 2006). The RNAi pathway is triggered by a longer dsRNA which is cleaved in 21–25 nucleotides (nt) fragments (siRNAs) by RNAse III-like protein DICER. The siRNAs are subsequently incorporated into the RNA-induced silencing complex (RISC), which recognizes and cleaves the target messenger RNA (mRNA) (Elbashir et al., 2001a). Previous studies showed that many viruses can be inhibited by siRNA, such as foot and mouth disease virus (Chen et al., 2006; Grubman and de los Santos, 2005), poliovirus (Gitlin et al., 2005), SARS (Li et al., 2005; Wang et al., 2004), influenza virus (Ge et al., 2003, 2004a,b), human respiratory syncytial virus (Bitko and Barik, 2001; Bitko et al., 2005), and avian metapneumovirus (Munir et al., 2006).

AMPV belongs to the Paramyxoviridae family, Pneumovirinae subfamily, within the genus Metapneumovirus (Cook, 2000; Easton et al., 2004; Njenga et al., 2003). It was first reported in the late 1970s in South Africa (Buys and du Preez, 1980) and subsequently in France and in the UK (Giraud et al., 1986; McDougall and Cook, 1986). There are four subtypes of AMPV (A–D) throughout the world (Bäyon-Auboyer et al., 2000; Dar et al., 2001; Juhasz and Easton, 1994; Toquin et al., 2000). In Brazil, only AMPV subtype A (AMPV/A) was detected so far (D'Arce et al., 2005; Dani et al., 1999).

Metapneumoviruses contain a non-segmented, negative-sense RNA with an approximately 13,000-nt long genome. The AMPV genome consists of eight viral genes arranged as follows: '3–N–P–M–F–M2–SH–G–L–5'. Its RNA genome, in association with nucleoprotein (N), large protein (L), and phosphoprotein (P) are often referred to as RNA polymerase complex (Easton et al., 2004). The N protein intimately wrapped with the viral RNA genome forms a nucleoprotein complex (N-RNA) and becomes resistant to RNases. N-RNA serves also as biological template, because RNA polymerase complex does not recognize pure deproteinized genomic RNA (Barik, 2004). RNA polymerase complex is responsible for the synthesis of all viral RNA, including mRNA, replicative intermediates, and the progeny

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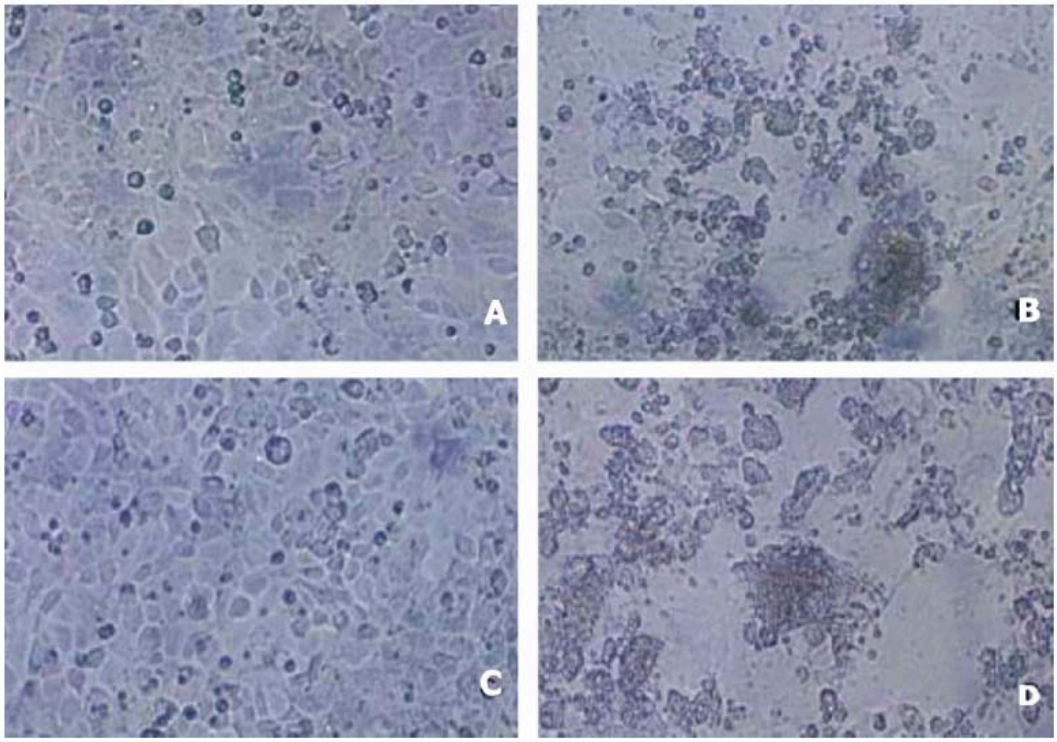

Fig. 1. CPE in transfected cells after 3 days (200×). CER cells were transfected with siRNAs at 75 nM. The transfected cells were infected after 6 h with AMPV subtype A: (A) negative control (without virus and siRNAs); (B) siRNA/GFP (control group); (C) siRNA/N; (D) siRNA/F.

RNA genomes (Easton et al., 2004). AMPV has two main surface glycoproteins in its membrane: the attachment (G) protein and the fusion (F) protein, which is responsible for fusion of viral and cell membranes (Walsh and Hruska, 1983). In the present study, we tested an siRNA sequence targeting the AMPV N and F genes to evaluate its inhibition effect in AMPV/A.

Based on previous research (Elbashir et al., 2001b), we designed 21-nt siRNA sequences with a G/C content of 30–70% and 2-nt 3′ overhangs. A leader sequence region of 5′ subgenomic AMPV N mRNA (GenBank accession number DQ175638) was targeted by siRNA/N [sense r(CCGGCGUGCCUCAAGGGUAUU)dTdT] and a leader sequence region of 5′ subgenomic AMPV F mRNA (GenBank accession number DQ175632) was targeted by siRNA/F [sense r(UGCCCUCCGGAACACAAAUGA)dTdT]. A negative control siRNA/GFP [sense, r(GACGGGAACUACAAGACACGU)dTdT] was designed, targeting the green fluorescent protein (GFP). The selected siRNA sequences were submitted to BLAST analysis, ensuring that only the intended gene was targeted. All RNA oligonucleotides were synthesized by IDT (Coralville, Iowa). CER (Chicken related embryo) cells line were cultured in Eagle's minimal essential medium (E-MEM) with 10% fetal bovine serum. CER cells were plated in 24-well plates (2×10^5 cells/well) for 1 day and transfected for 30 min with a total amount of 25, 50, 75, and 100 nM per well of siRNA duplexes (siRNA/N, siRNA/F and siRNA/GFP), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfected cells were infected after 6 h with AMPV/A (SHSBR/662/03 strain) at M.O.I. of 0.01 and examined 3 days later. Virus cytopathic effect (CPE) could be observed after 3 days incubation. Cells transfected with siRNA/N showed a significant decrease of virus

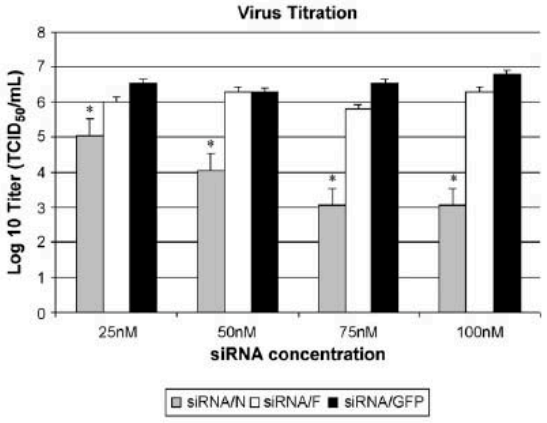

Fig. 2. Transfected cells monolayer were infected with AMPV after 6 h post-transfection. Supernatants were collected 3 days after infection and assayed for virus titration. $^*p < 0.05$ when compared to the control group.

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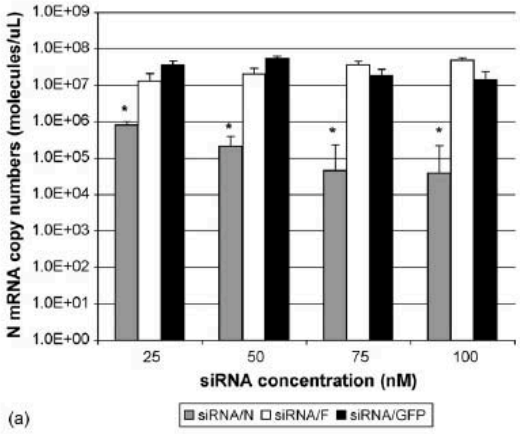
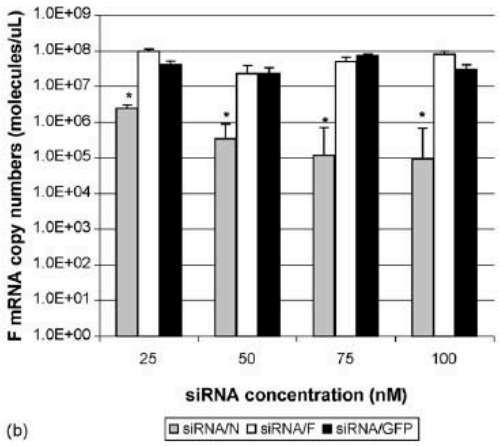
CPE, whereas those transfected with other siRNAs showed syncytium formation, rounding of refringent cells and many detached cells at all concentration (Fig. 1). Culture supernatants were assayed by virus titration, real time RT-PCR, and RT-PCR to investigate AMPV inhibition by siRNA.

siRNA-transfected and virus-infected cultures were recovered and titrated by $TCID_{50}$ assay (Reed and Muench, 1938). Culture supernatants transfected with siRNA/N at 25, 50, 75, and 100 nM concentrations, presented reduction of AMPV titers by 96.8, 99.4, 99.9 and 99.9%, respectively, when compared to the siRNA/F and siRNA/GFP treated samples (Fig. 2).

Real time RT-PCR was employed to determine N mRNA and F mRNA reduction. RNA was isolated from infected cells using Trizol (Invitrogen, Carlsbad, CA), and first-strand cDNA was made using Superscript III Rnase H-reverse transcriptase kit (Invitrogen, Carlsbad, CA). Real time PCR amplification was performed using Quantitec Probe PCR kit (Qiagen, Hilden, Germany), with final concentrations of 900 nM for each primer, and 300 nM for the Taqman



(a)



(b)

Fig. 3. Attenuation of AMPV mRNA copy numbers by siRNAs in N mRNA (a) and F mRNA (b). $^{*}p < 0.05$ when compared to the control group.

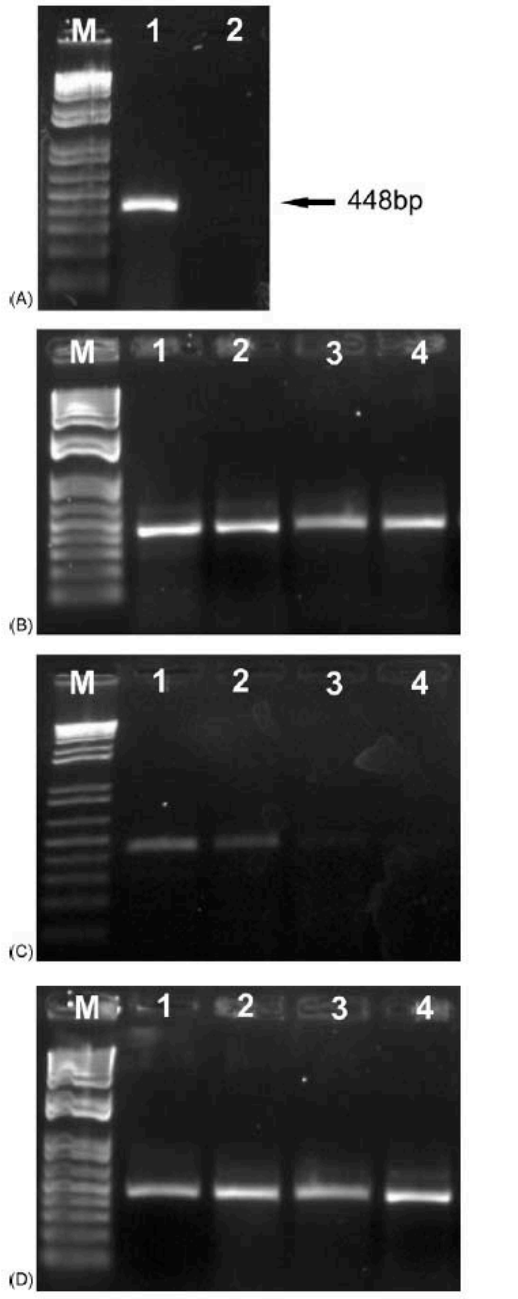


Fig. 4. Effect of different siRNAs on G mRNA production (448 bp) in the culture supernatants from infected cells, as indicated: (A), 1 positive and 2 negative control; (B) siRNA/GFP; (C) siRNA/N; (D) siRNA/F; M, leader 1 kb plus; numbers 1–4, 25, 50, 75, 100 nM, respectively.

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probe, in a total reaction volume of 25 μL, containing 1 μL of cDNA. Primers for N mRNA were: AMPVN+494 (5′-CAAAAGCCGTCTGCCTTGGAT-3′), AMPVN-567 (5′-GAGGCCAACTTGGTGAAAATG-3′), and the Taqman probe AMPVN+516FAMTAMRA (5′-CTCCCGTTATTCTATTATG-CATTGGTGCCC-3′). Primers for F mRNA were: AMP-VF+3643 (5′-TGCCAACTTCATCAGGACAGA-3′), AMPVF-3721 (5′-TCAATATACCAAACCCCTTCCTTCT-3′), and the Taqman probe AMPVF+3667FAMTAMRA (5′-AGTTT-GATGTTGAACAATCGTGCCATGGT-3′). All samples were run in duplicate and carried out on an ABI PRISM 7500 real time PCR cycler (Applied Biosystems, Foster City, CA). For absolute quantification, a standard curve was created based on spectrophotometric determination of PCR products of each gene (Bustin, 2000; Whelan et al., 2003). Copy number was calculated using the formula: molecules/μL = [g/μL DNA/(PCR product length in base pairs × 660) × 6.022×10^{23}]. siRNA/N was compared with the control using Student's *t*-test with a level of significance of $p < 0.05$.

Real time RT-PCR results presented reduction of AMPV N mRNA by 97.8, 99.6, 99.7 and, 99.7%, when transfected with siRNA/N at 25, 50, 75, and 100 nM concentrations, respectively, as compared to siRNA/F and siRNA/GFP treated samples (Fig. 3a). Similar results were obtained with RT-PCR targeting the F protein on samples treated with siRNA/N (Fig. 3b). The absence of PCR inhibitors was confirmed by detection of the internal control (beta-actin gene) in all samples (data not shown).

To confirm that siRNAs can inhibit virus replication, 1 μL of cDNA was applied for RT-PCR by using Taq Polymerase Recombinant (Invitrogen, Carlsbad, CA) with AMPV G gene-specific primers (Bäyon-Auboyer et al., 2000). The RT-PCR conditions were: 94 °C, 3 min; 25 cycles of (94 °C, 30 s, 54 °C, 30 s, 72 °C, 1 min); 72 °C, 10 min. A marked reduction in G mRNA copies was observed only in the cells transfected with siRNA/N (Fig. 4).

The present study reports an siRNA sequence targeting the N gene which was able to inhibit the AMPV production in vitro. We observed a decrease of infectious particles production as well as a reduction of mRNA production by targeting N mRNA with siRNA. The F mRNA and G mRNA were also inhibited by siRNA/N, confirming the essential role of N protein in AMPV replication. Munir et al. (2006) successfully described AMPV subtype C inhibition targeting other component of RNA polymerase complex, the phosphoprotein. In future studies, a combination of siRNAs targeting the RNA polymerase complex should be further explored as a tool to study AMPV infections or as an antiviral therapy.

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4. Considerações Finais

4.1. A BETA-ACTINA DAS CÉLULAS CER É SEMELHANTE A DAS CÉLULAS BHK_{21}

As células CER são frequentemente utilizadas para isolar diversos vírus animais. Estas células eram classificadas como híbridas de fibroblastos de galinhas e de células BHK_{21}, sem que sua origem tenha sido certificada. Sua caracterização é fundamental para que os resultados baseados no seu uso sejam validados, principalmente para estudos moleculares. No presente estudo, a avaliação da beta-actina das células CER foi realizada. A beta-actina das células BHK_{21} e CER foram detectadas utilizando oligonucleotídeos hamster-específicos. Além disso, pela análise filogenética as células CER e BHK_{21} apresentaram uma identidade genética próxima às células BHK_{21}. Tais resultados sugerem que as células CER devem ser re-classificadas como uma linhagem de células mamíferas.

4.2. ESTUDO COMPARATIVO DO RT-PCR CONVENCIONAL E DO RRT-PCR PARA A DETECÇÃO DO AMPV/A

O diagnóstico do AMPV baseado em sinais clínicos não é confiável, uma vez que o AMPV pode causar diferentes manifestações da doença e também apresentar variações genéticas. Por esse motivo, técnicas seguras de diagnóstico são importantes. No presente estudo, as técnicas RT-PCR convencional (para a detecção dos genes N e F) e RRT-PCR (para a detecção dos genes N e F) foram testadas e detectaram com sucesso os isolados AMPV/A. O RT-PCR convencional (para a detecção do gene G) foi capaz de detectar tanto o AMPV/A quanto o AMPV/B. O RT-PCR convencional (para a

detecção do gene F) e o RRT-PCR (para a detecção dos genes F e N) apresentaram maior sensibilidade que os outros testes. Além disso, o RRT-PCR gera resultados rápidos, sensíveis e com múltiplas amostras testadas ao mesmo tempo, tornando-se uma ferramenta alternativa para o isolamento viral. Até o momento, no Brasil, apenas o AMPV/A foi detectado. Por isso, o constante monitoramento das aves deve ser realizado para que outros subtipos circulantes possam ser detectados.

4.3. INIBIÇÃO DA REPLICAÇÃO DO AMPV ATRAVÉS DA INTERFERÊNCIA POR RNA PARA O GENE DA NUCLEOPROTEÍNA EM CULTIVOS CELULARES.

A interferência por RNA é considerada uma tecnologia terapêutica promissora contra doenças virais. Neste estudo, foram testadas moléculas de siRNA contra os genes da nucleoproteína e da fusão do AMPV. Todavia, não foi observado nenhuma alteração no efeito citopático, título viral ou na produção de mRNA após a utilização do siRNA/F. Não foi possível, portanto, constatar se o silenciamento da proteína F produziria algum efeito na replicação viral. Em estudos futuros, devem ser testados outros siRNAs alternativos contra o gene F. O presente estudo promoveu a inibição do AMPV em cultivos celulares através da utilização de moléculas de siRNA para o gene N viral. Em estudos futuros, a associação de siRNAs contra complexo da RNA polimerase deve ser avaliada como uma eficiente ferramenta para evitar o escape viral na terapia antiviral.

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