UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

Márcia Bianchi dos Santos

"ESTUDOS EXPERIMENTAIS COM ISOLADOS DO METAPNEUMOVIRUS AVIÁRIO (aMPV) SUBTIPOS A E B EM FRANGOS DE CORTE"

Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Genética e Biologia Molecular na área de Microbiologia

Orientadora: Profa. Dra. Clarice Weis Arns
Co-orientador: Prof. Dr. Fernando Rosado Spilki

Campinas
2010

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Sa59e

Santos, Márcia Mercês Aparecida Bianchi dos
Estudos experimentais isolados do metapneumovirus aviário (aMPV) subtipos A e B em frangos de corte / Márcia Mercês Aparecida Bianchi dos Santos. – Campinas, SP: [s.n.], 2010.

Orientadora: Clarice Weis Arns.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Metapneumovirus aviário. 2. Atenuação. 3. Propagação *in vitro*. 4. Vacinas. I. Arns, Clarice Weis, 1956-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(rcdt/ib)

Título em inglês: Experimental studies with avian metapneumovirus (aMPV) subtypes A and B isolates in broiler chickens.
Palavras-chave em inglês: Avian metapneumovirus; Protection; Atenuação; *In vitro* propagation; Vaccines.
Área de concentração: Microbiologia.
Titulação: Doutora em Genética e Biologia Molecular.
Banca examinadora: Clarice Weis Arns, Liana Verinaud; Isabela Cristina Simoni, Marcelo Vasconcelos Meireles, Helio José Montassier.
Data da defesa: 30/04/2010.
Programa de Pós-Graduação: Genética e Biologia Molecular.

Campinas, 30 de março de 2010

Banca Examinadora

Profa Dra Clarice Weis Arns (Orientadora)
(Titular)

Assinatura

Profa. Dra. Iiana Verinaud
(Titular)

Assinatura

Dra. Isabela Cristina Simoni
(Titular)

Assinatura

Prof. Dr. Marcelo Vascocelos Meireles
(Titular)

Assinatura

Prof. Dr. Helio José Montassier
(Titular)

Assinatura

Prof. Dr. Marcelo Brocchi
(Suplente)

Assinatura

Prof. Dr. Fabio Trindade Maranhão Costa
(Suplente)

Assinatura

Profa. Dra. Alda Maria Backx Noronha Madeira
(Suplente)

Assinatura

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada **Estudos experimentais com isolados do metapneumovirus aviário (aMPV) subtipos A e B em frangos de corte.**

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

(X) tem autorização da(s) seguinte(s) Comissão(ões) de Bioética ou Biossegurança*:
Comissão de Ética no Uso de Animais CEUA, sob Protocolo(s) nº1716-2.

** Caso a Comissão seja externa à UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno: Márcia M. A. Bianchi dos Santos

Orientador: Profa. Dra. Clarice Weis Arns

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Nome: Profa. Dra. ANA MARIA A. GUARALDO
Função: Presidente

Trabalho executado no Laboratório de Virologia
Animal do Instituto de Biologia da Universidade
Estadual de Campinas

Agradecimentos

À minha mãe, meu braço direito e minha maior incentivadora. Obrigada pelo exemplo de vida e por me fornecer suportes psicológico e financeiro (claro!) indispensáveis para que eu chagasse até aqui.

À Profa. Dra. Clarice Arns, pela liberdade e confiança. Obrigada pela orientação, pelos valiosos conselhos e oportunidades, e principalmente pela generosidade (você foi uma mãe!). Agradeço também ao Prof. Dr. Marcelo Meireles por ter me orientado durante toda a minha graduação e principalmente por ser tão querido e ter despertado meu interesse pela pesquisa (você foi um pai!).

À minha linda família! Agradeço o companheirismo dos meus irmãozinhos, as pessoas mais doces deste mundo. Obrigada Su, minha madrastinha querida, pelas conversas e pela força e coragem que têm me servido de exemplo. Obrigada meu pai, meu herói...sinto tantas saudades! Você ficaria orgulhoso de mim!

Ao Mark, meu Branquelinho. Espero que um dia você aprenda o português para ler isto: Você é a mais doce e inesperada surpresa da minha vida! Obrigada pelo amor e apoio durante o nosso primeiro inverno juntos e pela paciência com o meu inglês e alemão. Finalmente, obrigada pelas correções dos papers...outros virão (hehehe)!

À galera do LVA...são tantos! Obrigada à Dra. Helena Lage e ao Prof. Dr. Fernando Spilki (acredite, eles eram apenas Helen e Fer!) pelo apoio indispensável durante meu doutorado. Devo um agradecimento especial ao Matheus (o fofinho) porque esta tese não sairia sem a ajuda dele dentro e fora do LVA, obrigada por me aguentar! Obrigada Lu Kohn, Maria Ângela, Judite, Daniel, Paulinho (do meu coração), Gi e Luzinha (querida companheira) pelos momentos

incríveis que passamos juntos no LVA. Agradeço também aos melhores funcionários da UNICAMP, Paulinha e Geneci, por toda a ajuda e risadas.

Aos meus amigos queridos de Campinas...amigos para sempre! Jacy, Erico, Jana, Gu e Diogo. Aos meus primos, que são meus amigos desde que eu nasci: Lu, Aninha e Carol. Eu a-do-ro vocês!
Ao Prof. Dr. Hafez, pela oportunidade de me fazer realizar um sonho. Agradeço também a todos do Institut für Geflügelkrankheiten de Berlim, especialmente à Dra. Dörte Lüschow e ao Valter por me receberem tão bem.

Ao pessoal do GEMOCA, obrigada a todos pela disponibilidade (e pelo 7500, claro!), principalmente à Elaine e Profa. Laura Ward.

À Fundação de Amparo à Pesquisa do Estado de São Paulo e à Funcamp, pelo apoio financeiro.

ÍNDICE

1. RESUMO

O Metapneumovirus aviário (aMPV) pertence à família Paramyxoviridae, subfamília Pneumovirinae, gênero *Metapneumovirus.* O vírus, relatado pela primeira vez no Brasil em 1995, é o agente etiológico da Rinotraqueíte em perus (TRT) e está associado também à Síndrome da Cabeça Inchada (SHS) em frangos e poedeiras comerciais.

O presente estudo foi dividido em três partes. Na primeira foi avaliada a suscetibilidades de oito sistemas celulares para a propagação de amostras virais do aMPV subtipos A e B. As células chicken embryo related (CER), Vero e baby hamster kidney cells (BHK-21) demonstraram ser as mais apropriadas para a multiplicação de ambos os subtipos. Além disso, cultivo de anel de traquéia (TOC) e cultivo primário de embrião de galinha (CEF) foram permissíveis à infecção por aMPV após terem sido isolados e propagados em CER. As curvas da cinética viral foram realizadas nas três linhagens celulares e ambos os subtipos tiveram títulos mais altos em CER durante as primeiras 30 horas após a infecção. Não foram observadas diferenças significativas entre os títulos obtidos em células CER e Vero, demonstrando que as células CER são tão adequadas à propagação do aMPV quanto as células Vero.

A segunda parte do trabalho consistiu em analisar a virulência de uma amostra de aMPV subtipo B após sofrer passagens seriadas em células CER. Cinco variantes provenientes das passagens em CER foram inoculadas em galinhas e a excreção viral foi analisada. Os resultados obtidos com as amostras de traquéia demonstram que a virulência do aMPV diminui gradualmente enquanto o título viral aumenta com o número de passagens. Em contrapartida, nas amostras de seio nasal foi observado aumento da carga viral, demonstrando que não houve diminuição no *fitness* viral para este órgão. As seqüências do gene G das amostras utilizadas para desafio foram obtidas, porém este gene parece não ser afetado pela propagação em células CER.

Na terceira e última parte deste estudo, foi avaliada a proteção viral conferida por uma vacina comercial contra isolados brasileiros do aMPV subtipos A e B em

frangos de corte. Para tanto, uma amostra de cada subtipo foi avaliada quanto à sua virulência. O isolado do subtipo B foi detectado em um período mais longo e em maiores quantidades quando comparado ao subtipo A. Os resultados da analise da proteção demonstram que algumas aves imunizadas receberam proteção viral completa contra o vírus virulento heterólogo. Porém, a mesma vacina forneceu proteção viral parcial quando as aves foram desafiadas com o vírus virulento homologo ao vacinal.

ABSTRACT

"EXPERIMENTAL STUDIES WITH ISOLATED AVIAN METAPNEUMOVIRUS (aMPV) SUBTYPES A AND B IN BROILER CHICKENS"

Avian metapneumovirus (aMPV) belongs to the Paramyxoviridae family, Pneumovirinae subfamily, within the genus *Metapneumovirus*. The virus, first described in Brazil in 1995, is responsible for an acute rhinotracheitis in turkeys (TRT) and is associated with swollen head syndrome in broiler chickens and layer hens.

The present study is divided in three parts. In the first part, eight cell culture systems were evaluate for the propagation of aMPV subtypes A and B. The chicken embryo related (CER) cells, Vero and baby hamster kidney cells (BHK-21) cells were shown to be the most appropriate for propagation of both subtypes of aMPV. In addition, propagation of aMPV in chicken embryo fibroblasts (CEF) and tracheal organ culture (TOC) remained efficient after the primary isolation and several passages of viruses in the CER cell line. The growth curves were created using CER, Vero and BHK-21 cell lines. Compared with growth, both yielded higher titres in CER cells during the first 30 hours after infection, but no significant difference was observed in the results obtained from CER and Vero cells. This data show that CER cell are adequate for aMPV propagation, giving similar results to Vero cells.

The second part of this study was conducted to analyze the virulence of an aMPV subtype B strain after serial passage in CER cells. To accomplish this, chickens were infected with 5 different variants derived from serial passage and the amount of viral shedding was determined. The results of tracheal samples showed that the virulence decreases gradually with passage, while *in vitro* viral titre increases. However, an increase in viral shedding was observed in sinusal samples, demonstrating no decrease in fitness for this organ. The G gene sequences of challenge samples were analyzed, however this gene appears to not be affected when aMPV is propagated in CER cells.

Finally, the last part of this study aimed to examine a commercially available vaccine in broiler chickens in terms of it ability to provide virological protection against aMPV subtypes A and B. To accomplish this, the virulence of each virulent strain was analyzed. The results demonstrate that the subtype B virulent strain could be observed longer and in larger quantities compared to subtype A. A complete heterologous virological protection was provided by the subtype B vaccine; however, a lack of complete homologous virological protection was observed when chickens were challenged with the homologous subtype B strain.

2.0 INTRODUÇÃO GERAL

2.1 aMPV

O aMPV (do inglês avian metapneumovirus), relatado pela primeira vez no fim dos anos de 1970 (Buys & du Pereez, 1980) e no Brasil em 1995 (Arns & Hafez) , é o agente etiológico da Rinotraqueíte em perus (TRT) e está associado também à Síndrome da Cabeça Inchada (SHS) em frangos e poedeiras comerciais (Gough & Jones, 2008). O aMPV pertence à família *Paramyxoviridae,* subfamília *Pneumovirinae,* gênero *Metapneumovirus* (Bayon-Auboyer *et al*., 2000; Munir & Kaur *et al*., 2006) e é causador de uma doença aguda e altamente contagiosa do trato respiratório em aves comerciais e populações de aves silvestres na maior parte do mundo (Jones, 2009). Inicialmente, somente dois subtipos eram reconhecidos, A e B, diferenciados pela análise da sequência de nucleotídeos de uma das glicoproteínas de superfície, a proteína G (Juhasz &Easton, 1994). Em 1996 um terceiro subtipo bastante diverso dos dois primeiros, nomeado subtipo C, foi identificado nos EUA em perus (Seal, 1998). Finalmente um quarto subtipo, chamado subtipo D, mais próximo dos subtipos A e B foi descrito na França (Bayon-Auboyer *et al.,* 2000), onde também foi identificada uma variante do subtipo C em patos (Toquin *et al.,* 2006).

2.2 Genoma viral

O aMPV, que era classificado no gênero *Pneumovirus*, foi recentemente designado como um Metapneumovírus aviário dentro do gênero *Metapneumovírus* (Collins *et al*., 2001). Estes vírus apresentam o genoma RNA, fita simples, negativa e contém oito genes organizados de forma diferente dos 10 genes dos pneumovirus (Easton *et al*., 2004). No genoma dos pneumovírus, o fragmento genômico F-M2 situa-se entre os genes G e L, enquanto que, nos metapneumovírus, esse mesmo fragmento apresenta-se em translocação e se localiza entre os genes M e SH (Collins,*et al*., 2001; Randhawa, *et al*., 1997; Pringle, 1998). Os metapneumovírus são vírus envelopados, com um genoma RNA de sentido negativo, com aproximadamente 13 Kb em extensão (Ling, *et al*., 1992; Fenner, Gibbs *et al*., 1993).

O genoma do aMPV é constituído por oito genes virais dispostos na seguinte ordem: Nucleocapsídeo - Fosfoproteína- Matriz - Fusão- Segunda Matriz- Pequena hidrofóbica - glicoproteína- grande polimerase (3´-M-P-M-F-M2-SH-G-L-5.), flanqueado pelas sequências *leader* e *trailer* nas posições 3' e 5', respectivamente (Easton *et al.*, 2004).

O RNA genômico está associado com as proteínas N, L e P, que formam o complexo da RNA-polimerase. Este complexo é necessário para a replicação e transcrição viral (Barik, 2004a; Easton *et al.,* 2004). A proteína L é o principal componente deste complexo e se liga na porção 3´ das seqüências "*leaders*" e 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 *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 helicoidal para o genoma viral. Ela é sintetizada em grandes quantidades, uma vez sintetizadas, estas se ligam aos RNAs genômicos virais recentemente transcritos. No fim do ciclo de vida viral intracelular, os genomas de fita negativa encapsidados junto com outras proteínas estruturais, são empacotados dentro de partículas virais (Easton *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 *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 *et al.*, 1987), e a proteína F, essencial para a fusão da membrana viral e celular (Walsh & Hruska, 1983). A proteína F é sintetizada primeiramente em F0, que se torna ativa por uma clivagem em F1 e F2 assim que a proteína chega ao complexo de Golgi (Collins *et al.*, 1984; González-Reyes *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 & Collins, 1989).

2.3 Variabilidade

O aMPV é classificado em quatro subtipos A, B, C e D baseado na análise de sequências de nucleotídeos e aminoácidos. Inicialmente, acreditava-se que havia apenas um sorotipo de aMPV, contendo dois subtipos A e B diferenciados pela análise da seqüência de nucleotídeos da glicoproteína ou utilizando anticorpos monoclonais (Collins *et al.*, 1993, Juhasz & Easton, 1994). Após quase 20 anos do primeiro isolamento de aMPV, o subtipo C foi descrito em perus nos Estados Unidos pelo seqüenciamento dos genes das proteínas M (Seal, 1998), F (Seal *et al.*, 2000; Tarpey *et al.*, 2001), N e P (Dar *et al.*, 2001). Finalmente um subtipo D, geneticamente distinto dos subtipos A, B e C, foi descrito na França (Bayon-Auboyer *et al.,* 2000), onde também foi identificada uma variante do subtipo C em patos (Toquin *et al.*, 2006). No Brasil, as amostras de aMPV isoladas e caracterizadas até 2005 são descritas como subtipo A (Dani *et al.*, 1999; D'Arce *et al.,* 2005) e apresentaram identidade de 95,8 a 99% com as sequências de nucleotídeos de outras amostras disponíveis no GenBank para este mesmo subtipo (Ferreira, 2007). Em 2007 isolados brasileiros caracterizados como subtipo B foram descritos em aves comerciais pela primeira vez, apresentando identidade de 95,1-96,1% entre as sequencias de aminoácidos das amostras brasileiras e outras do subtipo B dispoíveis no GenBank (Chacón *et al.,* 2007).

A Proteína G, responsável pela adesão celular e o principal determinante antigênico do aMPV, tem até 99% de identidade entre as sequencias de nucleotídeos dentro de um mesmo subtipo. Em contraste, quando subtipos diferentes são comparados, as sequencias de nucleotídeos têm identidade de 33.2- 38% e a de aminoácidos de 56-61% (Bayon-Auboyer *et al.*, 2000). Um estudo filogenético dos subtipos A, B e C revela que os subtipos A e B têm uma relação mais estreita entre si do que a relação que ambos os subtipos têm com o subtipo C. A seqüência de aminoácidos da proteína F do subtipo C tem

aproximadamente 78% de identidade com as seqüências dos subtipos A e B, identidade esta inferior àquela encontrada entre os subtipos A e B (83-89%, Njenga, 2003). A análise filogenética das seqüências de aminoácidos das proteínas G, SH e L do subtipo C demonstra que este subtipo está mais próximo de isolados do metapneumovírus humano (hMPV) do que dos outros subtipos de aMPV (Toquin *et al*., 2003).

2.4 Histórico e Distribuição geográfica

No fim dos anos de 1970 uma infecção aparentemente nova e bastante grave foi descrita pela primeira vez na África do Sul (Buys & Du Preez, 1980). A doença, caracterizada por sintomas clínicos respiratórios, foi nomeada Rinotraqueíte dos perus (TRT) e provocou um efeito devastador na indústria Sul Africana de perus sem aparente recuperação (Cook, 2009).

Alguns anos mais tarde, uma doença com sinais clínicos similares foi relatada na França (Giraud *et al.,* 1986) e logo após na Inglaterra onde o agente causador foi isolado (McDougall & Cook, 1986; Wyeth *et al.,* 1986) e caracterizado como um pneumovirus (Cavanagh & Barrett, 1988). Inicialmente denominado TRT, o vírus passou a ser conhecido mais tarde como APV e mais recentemente tornou-se o aMPV.

Pouco tempo depois dos relatos na França e Inglaterra, o aMPV foi descrito em aves comerciais de outras partes da Europa como Alemanha, Holanda e Espanha (Hafez, 2000; Diaz De Espada & Perona, 1984; Goren, 1985) e em Israel (Perelman, *et al*., 1988). Na América do Sul foi descrita sorologia positiva para o aMPV no Chile e América Central (Jones, 1996; Toro *et al*., 1998), no Zimbabwe em avestruzes (Cadman *et al.,* 1994) e no mar Báltico em gaivotas (Heffels-Redmann *et al*., 1998). No Brasil o aMPV foi isolado pela primeira vez a partir de material proveniente de matrizes antes da introdução da vacina no país (Arns & Hafez, 1995). Em um estudo epidemiológico realizado no estado de São Paulo entre os anos de 2007 e 2009 com 168 amostras de traquéia e seios nasais provenientes de galinhas comerciais foram encontradas 38 amostras (22,61%) positivas para o Vírus da Bronquite Infecciosa (IBV), 5 (2,97%) para aMPV subtipo

A e 11 (6,54%) para aMPV subtipo B, sendo encontrados também 7 (4,16%) casos de co-infecção de aMPV e IBV, o que comprova a associação já descrita entre esses vírus (Martini, 2009; Morley & Thomson, 1984). Os últimos relatos da doença provocada pelos subtipos A e B são de 2008 na China e Nigéria em frangos e perus (Owoade *et al.*, 2008). O aMPV subtipo A identificado nestes países é essencialmente idêntico aos encontrados no Brasil e Reino Unido, dando margem à hipótese destes vírus terem uma origem comum. Nos EUA, as primeiras confirmações de surtos por estes vírus em perus ocorreram no Estado do Colorado (Cook *et al.,* 1999), e logo depois em Minnesota (Goyal *et al.,* 2000). Este último Estado é o único exemplo de erradicação da doença, onde nenhum caso clinico ou isolamento do vírus é feito há mais de cinco anos (Jones, 2009). Atualmente, Austrália e Canadá são provavelmente as duas únicas regiões do mundo onde a doença nunca foi identificada (Cook, 2009).

A disseminação do aMPV tem sido motivo de grande discussão. Como o vírus alcançou a Europa e outras partes do mundo ainda mais distantes da África do Sul não se sabe. Uma hipótese tem sido a transmissão a longa distância por aves migratórias (Jones, 1996), embora não haja indícios de infecção em aves migratórias européias. O mais questionável é o fato de que o subtipo C, encontrado nos EUA, e os subtipos A e B, na América do Sul e Central, não são encontrados simultaneamente nestas regiões apesar do trajeto migratório de aves aquáticas selvagens entre o norte e sul. Foram encontradas evidencias de infecção em aves selvagens nos EUA (Shin *et al.,* 2002; Turpin *et al.,* 2008), porém não se sabe se estas aves são realmente capazes de transmitir a doença para aves domésticas. No Brasil, o aMPV subtipos A e B foram detectados em aves exóticas, sinantrópicas e selvagens. Todas as amostras têm similaridade de até 100% com as amostras vacinais dos subtipos A e B utilizadas no Brasil (Felippe *et al.,* in prep).

2.5 Sinais Clínicos

O primeiro relato da doença causada pelo aMPV, caracterizada por sinais clínicos respiratórios graves, foi nomeada TRT. A replicação do vírus ocorre nos

tecidos nasais e sinusais, na traquéia e pulmões, porém ela é mais limitada ao trato respiratório superior, onde as partículas virais podem ser detectadas mais facilmente até dez dias após a infecção (Cook, 2000; Van De Zande *et al.*, 1999).

Em perus jovens a enfermidade é caracterizada por espirros, corrimento nasal, estertores traqueais, edema dos seios infraorbital, nasal e muitas vezes frontal, com descarga ocular. O corrimento nasal pode se tornar mucopurulento se houver infecção bacteriana secundária. Algumas variações são observadas na descrição dos sinais clínicos, isto é atribuído a condições precárias de manejo ou à presença de outros agentes infecciosos que frequentemente estão associados ao aMPV na TRT provocando o agravamento do quadro clínico, além do aumento da morbidade e da mortalidade (Cook *et al.*, 1991; Gough, 2008).

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 & Jones, 1993). O aparecimento dos sinais clínicos é rápido e a infecção pode disseminar em 24 horas (Stuart, 1989).

Em poedeiras e matrizes de perus, a infecção pelo AMPV provoca queda de mais de 70% na produção dos ovos associado a má qualidade da casca do ovo e peritonite (Jones *et al.,* 1988; Stuart, 1989; Cook *et al.*, 1996). Tosse, envolvendo comprometimento do trato respiratório superior, pode levar a prolapso de útero em matrizes. Quando a doença é diagnosticada, a morbidade em aves de todas as idades é normalmente de 100%. A mortalidade varia 0,4% a 50%, particularmente em aves jovens susceptíveis. Em infecções pouco complicadas, a recuperação ocorre normalmente em 10 a 14 dias (Gough, 2008). Em poedeiras, a queda na produção de ovos pode exceder os 70% acompanhada de má qualidade da casca do ovo e peritonite (Jones *et al*., 1988).

Apesar ser sabido que o aMPV é capaz de infectar galinhas e induzir resposta sorológica específica (Wyeth *et al*., 1987), alguns autores ainda relatam dificuldade em estabelecer uma firme conexão entre o vírus e a enfermidade em galinhas (Cook, 2000). O aMPV já foi isolado de frangos e poedeiras em todas as idades (Picault *et al.*, 1987; Buys *et al.*, 1989b) e infecções experimentais têm sido

realizadas nestas aves (Jones *et al.*, 1987; Majó *et al.*, 1995; Catelli *et al.*, 1998). Isolados dos subtipos A e B do aMPV podem provocar lesão em trato respiratório, acompanhando de inchaço dos sinus infraorbitários em frangos de corte, sugerindo o papel do aMPV como patógeno primário da doença (Aung et al, 2008). Por isso, o aMPV também é freqüentemente associado à SHS em galinhas, além de estar certamente associado a outros agentes (Cook, 2000), como o IBV (Morley & Thomson, 1984) e a bactéria *Escherichia coli* (Droual & Woolcock, 1994).

A SHS em galinhas é caracterizada pelos seguintes sinais clínicos: 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). Normalmente menos de 4% do lote é afetado, embora sinais respiratórios generalizados sejam observados. A mortalidade raramente excede 2% e em matrizes a produção de ovos de matrizes é freqüentemente afetada. Em poedeiras comerciais a infecção por aMPV também interfere na qualidade dos ovos (Cook, 2000; Gough, 2008). Em frangos de corte 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 inoculações experimentais (Catelli *et al.*, 1998).

2.6 Diagnóstico

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, já que a enfermidade é multifatorial (Cook, 2000).

B) ISOLAMENTO VIRAL

O aMPV pode ser isolado do trato respiratório superior e pulmões de aves infectadas, porém a fonte mais rica de vírus é sem dúvidas a secreção nasal ou o raspado sinusal. É importante coletar as amostras o mais rápido possível após a infecção já que na maioria dos casos os vírus permanecem nos cornetos nasais e sinus infraorbitários por apenas seis a sete dias (Jones, 1996). O isolamento viral de amostras de aves com sinais clínicos graves raramente é bem sucedido; o agravamento do quadro clínico normalmente é decorrente de infecção bacteriana secundária em aves predispostas por infecção viral primária. Isto provavelmente explica as falhas na tentativa de isolamento do vírus em amostras de galinhas com SHS (Gough, 2008). Se os sinais clínicos forem muito evidentes recomenda-se que as aves sem sintomatologia clínica presentes no mesmo lote das aves doentes sejam selecionadas (Cook, 2000).

A metodologia comumente utilizada para o isolamento primário de aMPV em amostras de campo inclui inoculação em ovos embrionados (Naylor & Jones, 1994; Panigrahy *et al.*, 2000; Cook & Ellis, 1990) ou em cultivo de anel de traquéia (TOCs) (Mcdougall & Cook, 1986; Wyeth *et al.*, 1986). O isolamento direto do aMPV em cultivo de células é raro, embora cultivos primários de células de embrião de galinha (CEF; Picault *et al.*, 1987) tenham sido utilizados com sucesso para o isolamento do subtipo C (Goyal *et al.*, 2000) e cultivos de linhagem de células CER para os subtipos A e B (Hafez & Weiland 1990; Arns & Hafez, 1995).

Os TOCs foram utilizados por muitos anos para o isolamento desses vírus. Este tipo de cultivo pode ser preparado a partir de embriões de galinhas ou perus pouco antes do nascimento ou pintos de um a dois dias de vida. Após a inoculação do material suspeito, a cultura pode ser mantida por várias semanas para a observação de ciliostase (Gough, 2008). 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 *et al.*, 1991). Entretanto, o subtipo C não provoca ciliostase (Cook *et al.*, 1999), sendo considerado impróprio o uso de TOCs para o isolamento de aMPV nos EUA.

Os embriões de galinha ou peru com 6 a 8 dias de idade podem ser utilizados para o isolamento do aMPV. A amostra é inoculada no saco da gema e após 8 dias o líquido alantóide e a membrana do saco da gema são coletados para nova passagem. Normalmente são necessárias algumas passagens para a observação de hemorragias e mortalidade do embrião (Cook, 2000). Este é um método caro e laborioso, porém capaz de detectar todos os subtipos do aMPV.

O cultivo de células não é comumente utilizado para o isolamento primário do aMPV. Entretanto, uma vez isolados, podem ser inoculados em cultivos de linhagens celulares para a propagação viral e aumento do título. As células Chicken Embryo-Related (CER) são utilizadas com sucesso para a propagação dos subtipos A e B (Hafez & Weiland 1990; Arns & Hafez, 1995). O subtipo C pode crescer em diferentes cultivos celulares como “baby grivet monkey kidney” (BGM), kidney (MA-104), Quail Tumor (QT) 35, BHK-21, cultivo primário de células de cornetos nasais e rins de perus ou cultivo primário de células de rins de galinha (Kong *et al.*, 2006, Patnayak *et al.*, 2005; Tiwari *et al.*, 2006). As células Vero são permissíveis para a propagação tanto dos subtipos A e B quanto do subtipo C (Goyal *et al.,* 2000; Cook, 2000).

O material inoculado em cultivos celulares pode apresentar efeito citopático (ECP) caracterizado por áreas dispersas com arredondamento celular e formação de sincício após poucas passagens (Buys *et al.*, 1989a; Cook *et al.*, 1999). A identificação do AMPV pode ser confirmada por microscopia eletrônica (Giraud *et al.*, 1986; Mcdougall & Cook, 1986; Buys *et al.*, 1989a) ou através de métodos imuno-químicos (Baxter-Jones *et al.*, 1986; Jones *et al.*, 1988; Catelli *et al.*, 1998).

C) DETECÇÃO VIRAL

A RT-PCR (transcrição reversa-reação em cadeia da polimerase) tem sido amplamente utilizada para o diagnóstico das infecções por AMPV. Ela oferece resultados mais rápidos que o isolamento viral (Mase *et al.*, 1996; Naylor *et al.*, 1997; Bäyon-Auboyer *et al.*, 1999; Dani *et al.*, 1999). Além disso, ela é importante para a caracterização de isolados virais e para realização de estudos moleculares e epidemiológicos (Jing *et al.*, 1993). O significado de um resultado positivo pela

RT-PCR para a detecção de uma infecção ativa ainda deve ser estabelecido, pois a técnica detecta o RNA viral e não o aMPV viável (Cook, 2000).

A RT-PCR convencional é muito conveniente para estudos epidemiológicos em grande escala, e não fornece qualquer indicação da quantidade de RNA viral presente na amostra, a não ser quando utilizada uma técnica semi quantitativa baseada na diluição do RNA ou cDNA (Cavanagh *et al.,* 1999). A RRT-PCR (real-time transcrição reversa-reação em cadeia da polimerase) também passa a ser utilizada como método rápido de detecção, promovendo, além disso, a quantificação do RNA viral. A RRT-PCR pode fazer distinção entre RNAs mensageiros com seqüências muito parecidas e requer menor quantidade de moldes de RNA do que outros métodos de análise de expressão gênica (Guionie *et al.,* 2007).

D) SOROLOGIA

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

2.7 Controle

Boas práticas de manejo e biosseguridade são importantes para ajudar a prevenir a infecção pelo aMPV e minimizar seus efeitos. Porém rapidamente tornou-se claro que estas medidas por si só não controlam a infecção pelo vírus indicando que uma intervenção urgente para controlar a doença em aves

comerciais era necessária. A imunidade maternal não confere proteção contra o vírus, nem interfere no sucesso da vacinação (Naylor *et al.,* 1997). A erradicação da doença normalmente não é possível, provavelmente devido ao tamanho e complexidade da indústria avícola na maioria das áreas afetadas. Apesar disso, existe um exemplo de erradicação da doença no Estado de Minessota-EUA, onde nenhum caso clínico ou isolamento do vírus é feito há mais de quatro anos (Jones *et al.,* 2009). Curiosamente, enquanto na Europa considerava-se essencial vacinar 100% do lote, nos EUA resultados satisfatórios foram obtidos em lotes onde apenas duas aves em um grupo de 1000 receberam a vacina (Gulati *et al.,* 2001). Além disso, em Minessota apenas as áreas ou granjas afetadas pela doença foram vacinadas e a administração da vacina foi cancelada assim que os surtos foram controlados. Aparentemente, em regiões onde a densidade nos lotes é baixa, a infecção por aMPV pode ser erradicada com a aplicação da vacina seguida de monitoramento cuidadoso e estrita atenção à bioseguridade (Cook, 2009; Jones, 2009).

2.8 Proteção vacinal

O principal método de controle da infecção pelo aMPV é a utilização de vacinas vivas atenuadas através de passagens seriadas em culturas de células ou de vacinas inativadas. Estas vacinas, se administradas cuidadosa e corretamente, demonstraram conferir excelente proteção de aves comerciais contra o aMPV (Cook, 2009). Uma única vacinação pode oferecer proteção aos perus durante toda a vida. Entretanto, pode ocorrer re-infecção 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, as vacinas vivas contra o aMPV e o Vírus da bronquite infecciosa (IB) devem ser administradas em momentos diferentes, devido a ocorrência de interferência na proteção contra ambas (Cook *et al.,* 2001), embora a vacinação simultânea contra aMPV e a doença de Newcastle não prejudique a eficácia de nenhuma das duas (Ganapathy *et al.,* 2005).

Após a administração da vacina viva, a vacina inativada oferece uma redução dos efeitos da infecção pelo aMPV durante o período de postura. Desta forma, o programa completo de vacinação composto por uma dose de vacina viva seguida de uma vacina inativada é necessário para uma proteção completa de aves adultas (Cook, Orthel *et al.*, 1996).

No Brasil estão disponíveis vacinas dos subtipos A e B do aMPV. As vacinas contra os subtipos A e B têm sido utilizadas com sucesso na maioria dos países afetados pela doença e oferecem boa proteção cruzada entre os dois subtipos (Cook *et al.*, 1995; Eterradossi *et al.*, 1995; Toquin *et al.*, 1996; Van de Zande *et al.*, 2000). Estas vacinas também oferecem proteção contra o isolado Colorado, subtipo C, porém não vice-e-versa (Cook *et al.*, 1999). Apesar disso, vários relatos sugerem que uma maior atenção deve ser dada ao desenvolvimento e método de administração das vacinas contra aMPV. Por exemplo, em perus, algumas semanas após a vacinação contra o aMPV, observou-se a ocorrência de doença respiratória diagnosticada como TRT. Segundo Catelli *et al.* (2006) este fenômeno acontece devido à recirculação do vírus vacinal entre as aves que não receberam ou receberam uma dose inadequada da vacina, levando à reversão de sua virulência. Um estudo epidemiológico longitudinal feito em Israel revela que tanto amostras vacinais quanto amostras de campo do aMPV dos subtipos A e B podem ser detectados simultaneamente em perus (Banet-Noach *et al.*, 2009). No Brasil (Villarreal *et al.*, 2009) e Itália (Cecchinato *et al.*, 2009) evidencias a campo sugerem que as vacinas existentes no mercado podem não oferecer proteção completa contra novas amostras circulantes do vírus. E finalmente, na Itália o desafio de aves vacinadas com uma amostra viral recente demonstrou que a proteção é inferior à conferida para uma amostra viral antiga (isolado de 1987). Os autores sugerem que tenha havido mudanças em regiões antigênicas fundamentais do vírus permitindo que este se replique e cause doença em animais vacinados (Catelli *et al.,* 2010).

As falhas no desempenho vacinal podem ser atribuídas a uma série de fatores, incluindo: Atenuação exacerbada da amostra vacinal (a baixa replicação implica em incapacidade de induzir resposta imune protetora), atenuação

insuficiente (com possível reversão da virulência do vírus vacinal), administração vacinal inadequada (ocorrência de falha na proteção ou reação severa) e em alguns casos divergência genética entre as amostras vacinal e de campo (Cook, 2009; Catelli *et al.,* 2010).

De fato, apesar de a vacinação prevenir a doença e suas conseqüências econômicas, não garante que a circulação viral seja suprimida (mesmo que em pequenas quantidades). O vírus respiratório sincicial bovino (BRSV), vírus de mesma família do aMPV e gênero próximo, é capaz de se replicar em animais com níveis detectáveis de anticorpos específicos (Beem, 1987; Van der Poel *et al.*, 1993). Além disso, a imunização prévia de animais contra o BRSV através de infecção ou vacinação não elimina completamente a circulação viral (Piazza, F. M., 1993; Schrijver, R. S. *et al.*, 1998). Dadas as altas taxas de mutação dos vírus RNA (Domingo & Holland**,** 1997) não seria surpresa se uma imunização estimulada, que previna a doença clínica, mas não elimine a circulação viral, induzisse a seleção de novas variantes (Larsen *et al.*, 2000; Valarcher *et al.*, 2000; Schat *et al.*, 2007; Mckinley *et al.*, 2008).

Embora vacinas vivas e atenuadas sejam eficazes quando administradas com precisão, esforços têm sido feitos para formular novas vacinas que não revertam sua virulência. O gene da proteína F do aMPV expresso num vetor de poxvírus (Qingzong *et al.,* 1994) e uma vacina de DNA que expressa o gene F ou N (Kapczynski & Sellers, 2003), foram testadas experimentalmente e demonstraram proteção parcial. Outro estudo envolvendo sistema de reversão genética para produzir um clone infeccioso de aMPV parece ser um ramo promissor no desenvolvimento de novas vacinas (Naylor *et al.,* 2004; Ling *et al.,* 2008). E finalmente a vacinação *in ovo* tem demonstrado ser um maneira pratica e efetiva de administração vacinal para a proteção de perus e galinhas contra a infecção pelo aMPV (Worthington *et al.,* 2003; Tarpey & Huggins, 2007).

Em síntese, existe um pequeno número de subtipos de aMPV, A, B, C e D. O subtipo D é raro (somente um relato na França), o C parece ser importante somente nos EUA, porém cada vez menos. Os subtipos A e B são os mais disseminados e importantes. As vacinas em circulação são eficazes se

administradas corretamente, mas problemas relacionados à reversão de virulência podem surgir e há evidencias de possível evolução do vírus. Neste contexto o desenvolvimento de novas vacinas mais seguras está sendo estudado.

3.0 Objetivos

3.1 Objetivos Gerais

Estudos experimentais com isolados do aMPV subtipos A e B em frangos de corte.

3.2 Objetivos específicos

- Determinação das linhagens celulares mais adequadas à propagação do aMPV subtipos A e B por meio de passagens seriadas do vírus em diferentes cultivos celulares e análise das curvas de crescimento;
- Investigação de modelo experimental para desafio de frangos de corte com o aMPV por avaliação da excreção viral.
- Investigação da proteção conferida por uma vacina do subtipo B contra isolados do aMPV subtipos A e B em frangos de corte pelas técnicas de RT-PCR em tempo real e titulação viral;

4.0 ARTIGOS

Artigo I

Propagation of Avian Metapneumovirus Subtypes A and B Using Chicken Embryo Related and Other Cell Systems

Lia Treptow Coswig, Márcia Bianchi dos Santos, Hafez Mohamed Hafez, Helena Lage Ferreira, and Clarice Weis Arns

Journal of Virologycal Methods, In press

Journal of Virological Methods xxx (2010) xxx–xxx

Propagation of avian metapneumovirus subtypes A and B using chicken embryo related and other cell systems

Lia Treptow Coswig[a], Márcia Bianchi dos Santos[b,*], Hafez Mohamed Hafez[c], Helena Lage Ferreira[d], Clarice Weis Arns[b]

[a] Ministério da Agricultura, Pecuária e Abastecimento, Av. General San Martin, 1000 Recife, PE 50630-060, Brazil
[b] Laboratory of Virology, Institute of Biology, State University of Campinas – UNICAMP, Campinas, SP, Brazil
[c] Institute of Poultry Diseases, Faculty of Veterinary Medicine of the "Freie Universität Berlin", Königsweg 63, Berlin 14163, Germany
[d] Avian Virology and Immunology, CODA-CERVA-VAR, Groeselenberg 99, B-1180 Uccle, Brussels, Belgium

Article history:
Received 20 August 2009
Received in revised form 15 February 2010
Accepted 18 February 2010
Available online xxx

Keywords:
Avian metapneumovirus
Cell propagation
CER

A B S T R A C T

Primary isolation of avian metapneumovirus (aMPV) is carried out using tracheal organ culture (TOC) or chicken embryonated eggs with subsequent adaptation in chicken embryo fibroblasts (CEF) or Vero cultures. This study was conducted to evaluate six different cell lines and two avian culture systems for the propagation of aMPV subtypes A and B. The chicken embryo related (CER) cells were used successfully for primary isolation. In addition to Vero and baby hamster kidney (BHK-21) cells, CER cells were also shown to be the most appropriate for propagation of aMPV considering high titres. Propagation of A and B subtypes in CEF and TOC remained efficient after the primary isolation and several passages of viruses in the CER cell line. The growth curves were created using CER, Vero and BHK-21 cell lines. Compared with growth, both yielded higher titres in CER cells during the first 30 h after infection, but no significant difference was observed in the results obtained from CER and Vero cells. This data show that CER cells are adequate for aMPV subtypes A and B propagation, giving similar results to Vero cells.

1. Introduction

Avian metapneumovirus (aMPV) is classified as a member of the *Metapneumovirus* genus within the *paramyxoviridae* family of viruses (Pringle, 1998). The virus causes upper respiratory tract infection in turkeys and chickens of all ages and is present in a wide range of countries (Jones, 1996; Cook, 2000). aMPV has been classified into four subtypes A, B, C and D based on antigenic and genetic characterization (Toquin et al., 2000; Dar et al., 2001; Alvarez et al., 2003). The virus was first described in South Africa in 1978 (Buys and Du Preez, 1980) and was reported thereafter in Europe (Hafez et al., 2000), the United States (Cook et al., 1999), the Middle East, Asia and Africa (Njenga and Seal, 2003; Owoade et al., 2008), Brazil (Arns and Hafez, 1992; D'Arce et al., 2005) and other parts of the world (Buys et al., 1989a,b; Cook, 2002). aMPV infections can be detected by serology and molecular methods; however they do not provide a sample of live virus. If such a sample is required, virus isolation is to be performed (Goyal et al., 2000; Shin et al., 2000). The methods of choice for primary isolation of aMPV are either the use of embryonated eggs (Naylor and Jones, 1994; Panigrahy et al., 2000) or chicken tracheal organ cultures (TOCs) (McDougall and Cook, 1986). Once the virus has been isolated in one of these two systems, it can be adapted to growth in cell cultures (Grant and Wilding, 1987; Buys et al., 1989a; Williams et al., 1991). Primary isolation of subtype C aMPV was achieved by seven blind passages of the field sample in chicken embryo fibroblasts (CEF) (Goyal et al., 2000) followed by adaptation of the isolate in Vero cells. The chick embryo related (CER) cell line, classified as a mixture of CEF and baby hamster kidney (BHK21) cells, was described for the first time in Japan as being capable of supporting rabies virus replication (Smith et al., 1977). Thereafter CER cells were reported as a successful method for aMPV preliminary isolation as well as for the propagation of aMPV from turkey flocks in Germany and chicken flocks in Brazil (Hafez and Weiland, 1990; Arns and Hafez, 1992). CER cells have also been used to propagate infectious bursal disease virus, infectious bronchitis virus and bovine respiratory syncytial virus (Cardoso et al., 2000; Ferreira et al., 2003; Spilki et al., 2005). To produce large quantities of aMPV strains for characterization and vaccination purposes, it is important that the virus grows to high titers *in vitro* in cell culture. In this study, aMPV subtypes A and B isolation are compared to a number

* Corresponding author at: Laboratório de Virologia, Departamento de Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), P.O. Box 6109, CEP 13081-970, Campinas, SP, Brazil.
Tel.: +55 19 3521 6258; fax: +55 19 3521 6276.
E-mail addresses: lia.coswig@agricultura.gov.br (L.T. Coswig), marciamerces@yahoo.com.br (M.B.d. Santos), hafez.mohamed@vetmed.fu-berlin.de (H.M. Hafez), Helena.ferreira@var.fgov.be (H.L. Ferreira), arns@unicamp.br (C.W. Arns).

0166-0934/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.jviromet.2010.02.018

Table 1
Cell culture systems.

Name of cell	Cell type	Species of origen	Source
CER	Chick embryo related	Chicken/Hamster	F.U. Berlin, Berlin, Germany
Vero	kidney	African green monkey	ATCC CCL-81
BHK-21	Baby hamster kidney	Hamster	ATCC CCL-10
HEP-2	Laryngeal carcinoma	Human	ATCC–CCL 23
MDBK	Bovine kidney	Bovine	ATCC CCL-22
ED	Equine dermis	Equine	ATCC-CRL-6288
CEF	Chicken embryo fibroblast	Chicken	Primary culture

of avian cell substrates to determine those most suitable for virus propagation.

2. Materials and methods

2.1. Cells

The basis of this study was CER cells which were described firstly by Smith et al. (1977). BHK-21 (baby hamster kidney), Vero (African green monkey kidney cells), MDBK (Madin–Darby bovine kidney cells), HEp-2 (human laryngeal carcinoma), ED (equine dermis), and a primary culture of chicken embryo fibroblasts (Laboratory of Virology, Institute of Biology, State University of Campinas, Brazil) were also used as shown in Table 1. The cells were grown in flasks of 25 cm^2 at 37 °C, using an initial concentration of 1.5×10^5 cells/mL in Eagle's minimal essential medium (E-MEM) free of antibiotics and supplemented with 10% fetal calf serum (FCS). For virus production, monolayers were grown to 85% of the cell confluence and the cells were then inoculated with 100 $TCID_{50}$ (50% tissue culture infective dose) of each viral isolate. Adsorption was allowed for 1 h at 37 °C. Subsequently, the medium was replaced and the flasks were incubated at 37 °C. The cells were monitored daily for the development of the viral cytopathic effect (CPE) using an inverted microscope (Axiovert 100, Carl Zeiss, Oberkochen, Germany). Cells and supernatants containing viruses were harvested following the development of the CPE or 5 days after inoculation if there was no appreciable CPE.

2.2. Viruses and virus titration

Two strains of aMPV were used for these experiments.

The first was a Brazilian strain designated as SHS-BR-121 (Arns and Hafez, 1995; Dani and Arns, 1999), which was isolated from broiler after 11 passages in CER and confirmed to belong to subtype A using PCR. Second, a German strain designated as STG-SHS-1439, isolated in 1990 from a broiler breeder flock with typical signs of SHS, in CER cells after 7 passages (Hafez, 1993) and confirmed to be subtype B using PCR (Hafez et al., 2000).

The strains were propagated in different cells and after 5 passages in each cell line, aliquots were titrated in the same cells as well as in CER cells following standard procedures. Titres were calculated by the Reed and Muench method (1938) and expressed as the log 10 tissue culture infective dose per mL ($TCID_{50}$/mL).

2.3. Permissibility of TOC and CEF to both subtypes A and B aMPV primarily isolated in CER cells

Once isolated in CER cells, the viruses were adapted to grow in TOC or CEF cultures. The aMPV subtypes A and B samples were passaged in TOC, examined daily for ciliary activity (Wilding et al., 1986) and in CEF cultures (Panigrahy et al., 2000) examined for appearance of the CPE. The presence of virus was confirmed by indirect immunofluorescent assay (IFA) according to Baxter-Jones and Grant (1986) method.

2.4. Analysis of replication kinetics of subtypes A and B aMPV in CER, BHK-21 and Vero cell lines

Growth curves were assessed following infection of preformed CER, BHK-21 or Vero cell monolayers. The infection was achieved within each viral isolate and the adsorption was allowed for 1 h at 37 °C. Subsequently, the medium was replaced and the flasks were incubated at 37 °C for different time intervals (0, 6, 12, 18, 24, 30, 36, 42, 48, 52, 58 and 60 h post-infection, p.i.). After incubation, the supernatants were harvested and assayed for the presence of the virus. All experiments were performed in triplicates. Infectious virus titres were calculated by the Reed and Muench method (1938) and expressed as the log 10 tissue culture infective dose per mL ($TCID_{50}$/mL).

2.5. Statistical analysis

Statistical analysis was performed using the unpaired *T*-test (GraphPad Prism 5.0 software). Results are given as mean values (±SE) and *p* values less than 0.05 were considered relevant.

3. Results

3.1. Susceptibility of different cells to aMPV subtypes A and B

Viruses grown in different cells were titrated in the same cells as well as in CER cells. The titres obtained are present in Table 2. CER, BHK-21, Vero, MDBK and HEp-2 were susceptible to infection with subtypes A and B aMPV, in contrast to ED cells, which did not produce a detectable CPE after 5 passages. The difference between titres obtained in CER and homologous cells for the subtype A strain was not statistically significant. For the subtype B strain there was a statistically significant difference ($p \leq 0.03$) between titres obtained in BHK-21, Vero and CER cells.

3.2. Permissibility of TOC and CEF to both aMPV subtypes A and B primarily isolated in CER cells

Both culture systems are permissible to infection with aMPV subtypes A and B after primary isolation and several passages in CER cells. In TOCs, the subtypes A and B took 3 passages and up to 7 days to cause ciliostasis. In CEF cells, free cells were observed

Table 2
Titres of infectious virus recovered after 5 passages of aMPV in different cell lines.

Cell lines	log 10 TCID50/mL SHS-BR-121 Homologous cells	CER cells	STG-SHS-1439 Homologous cells	CER cells
CER	–[a]	7.5	–[a]	6.0
Vero	6.0	6.5	5.5	5.8
BHK-21	5.5	5.8	5.0	5.5
HEP-2	4.5	5.0	4.1	4.9
MDBK	1.5	1.8	1.3	1.5
ED	–[a]	–[a]	–[a]	–[a]

[a] Significant minor at $p < 0.05$.

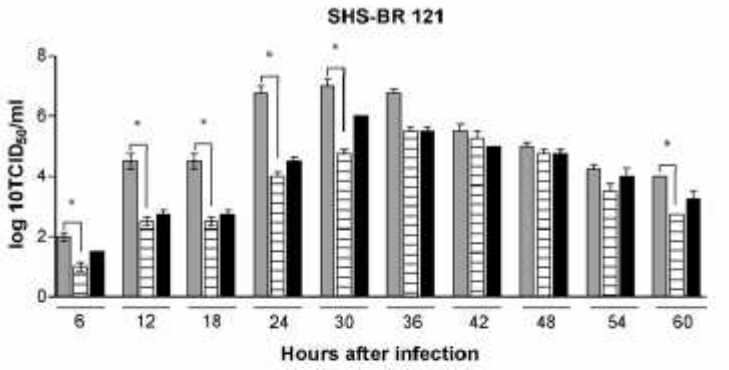


Fig. 1. Comparison of subtype A aMPV strain growth characteristics in CER, BHK-21 and Vero cell lines. Data consists of mean titre of three infected wells with the respective standard deviation bars, at different time intervals (hours after infection).

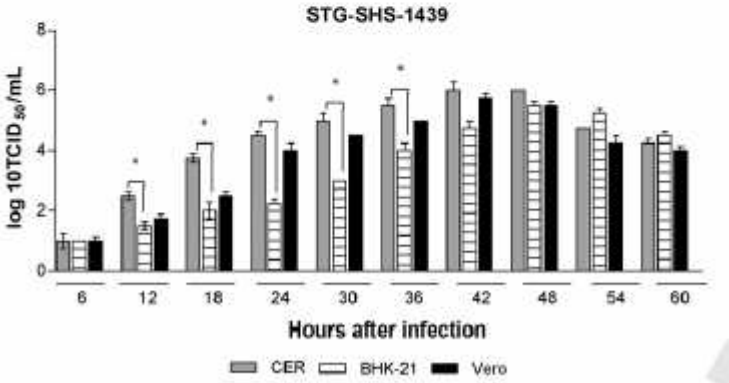


Fig. 2. Comparison of subtype B aMPV strain growth characteristics in CER, BHK-21 and Vero cell lines. Data consists of mean titre of three infected wells with the respective standard deviation bars, at different time intervals (hours after infection).

floating on the surface of the cultures with both viruses within 2 passages. Complete CPE was observed on the third passage within 24 h for CEF cells inoculated with subtype A and within 48 h with subtype B. Using IFA analysis, the viruses were detected during the first passage.

3.3. Analysis of replication kinetics of aMPV subtypes A and B in CER, BHK-21 and Vero cell lines

Growth curves of subtype A revealed statistically significant differences in titres obtained with CER and BHK-21 cells ($p < 0.05$) after 6, 12, 18, 24, 30 and 60 h of infection (Fig. 1A). The same difference was observed for subtype B after 12, 18, 24, 30 and 36 h of infection (Figs. 1 and 2). Both viruses grew to higher titres in CER than in BHK-21 cells. CER and Vero cells revealed no relevant differences considering titres for both viruses.

4. Discussion

Primary isolation of aMPV from field samples is carried out using chicken or turkey TOC or in chicken embryonated eggs (McDougall and Cook, 1986; Naylor and Jones, 1994; Panigrahy et al., 2000). Once the virus has been isolated in one of these two systems, it can be adapted easily to grow in CEF, chicken embryo liver (CEL) or Vero cultures (Grant and Wilding, 1987; Buys et al., 1989a; Williams et al., 1991). After primary isolation, aMPV subtype C has been reported to grow in other cell systems such as baby grivet monkey kidney (BGM), kidney (MA-104), quail tumor (QT) 35, BHK-21 and both primary turkey turbinate and kidney cells or chicken kidney primary cells (Kong et al., 2006; Patnayak and Goyal, 2005; Tiwari et al., 2006). Primary isolation using cell cultures is generally rare, although it has been reported for aMPV in CEF cells and QT-35 cell substrates in the US (Goyal et al., 2000).

The purpose of this study was to find suitable cell substrates for the propagation of aMPV subtypes A and B to achieve high titres. Both subtypes, isolated primarily in CER cells, yielded higher titres in CER, Vero and BHK-21 cells than in MDBK and HEp-2 cells. No detectable infectious virus was produced in ED cells. When subtype A and subtype B strains propagated in different cells were titrated in the same cells, the titres were lower than those observed by titration in CER cells, probably because the viruses were originally isolated in CER cells. Nevertheless, only the difference ($p \leq 0.03$) obtained from titres of BHK-21, Vero and CER cells for the subtype B strain was of statistically significant relevance. These findings are in agreement with results of Patnayak and Goyal (2005) who reported that vaccine strains adapted to grow in Vero cells produced higher titres in this cell than in other cells.

Considering that cell culture passage leads quickly to attenuation of the virus (Williams et al., 1991) and the most characteristics of CER cells are acquired from the parental hamster cells (Urmanova and Tsareva, 1996a,b,c), the multiplication of both strains was assayed using two different avian culture systems such as CEF and TOC culture systems. Only subtle differences in the number of passages necessary for CEP or ciliostasis appearance could be observed and it was concluded that the primary isolation using the CER cell line does not interfere with the permissibility of CEF and TOC to infection with subtypes A or B strains. Other avian primary cells has been reported previously to be permissive for aMPV subtype C production when the virus was adapted for propagation in Vero cells (Kong et al., 2006). The CER cells, which is susceptible to viral infection with a wide spectrum of different pathogens like the rabies virus and other avian and mammal viruses (El Karamany et al., 1979; Sinibaldi et al., 1990; Simoni et al., 1999; Cardoso et al., 2000, 2004; Spilki et al., 2005), has also been reported as a successful method for aMPV preliminary isolation and propagation (Hafez and Weiland, 1990; Arns and Hafez, 1995), but does not appear to have been used widely (Cook, 2002). Such cell culture may easily be maintained and has been used successfully for the primary isolation and propagation of aMPV subtypes A and B (D'Arce et al., 2005); this may also be an alternative for detection of other avian

The growth curves of the subtypes A and B strains were produced using the cell cultures that yielded higher titres in the first experiment described here, i.e. CER, Vero and BHK-21 cells. The biggest statistically significant differences among titres obtained from CER and BHK-21 cells were observed during the first 30 h after infection for both viruses, with higher titres in the CER cells. Despite the original virus adaptation in CER cells, no significant difference was observed in the results obtained from CER and Vero cells up to 60 h after infection, confirming that these cells are equally permissive to aMPV infection.

The overall results of this study indicate that CER cells can be used for primary isolation of aMPV. In addition to Vero cells, BHK-21 and CER cells can also be used to propagate subtypes A and B aMPV to high titres. Future research should be done to determine whether Vero and BHK-21 cells could be used for primary isolation of aMPV from field samples.

References

Alvarez, R., Lwamba, H.M., Kapczynski, D.R., Njenga, M.K., Seal, B.S., 2003. Nucleotide and predicted amino acid sequence-based analysis of the avian metapneumovirus type C cell attachment glycoprotein gene: phylogenetic analysis and molecular epidemiology of U.S. pneumoviruses. J Clin Microbiol 41 (4), 1730–1735.

Arns, C.W., Hafez, H.M., 1992. Swollen head syndrome in poultry flocks in Brazil. In: Proceedings of the 41st Western Poultry Disease Conference, pp. 81–84.

Arns, C.W., Hafez, H.M., 1995. Isolation and identification of Avian Pneumovirus from broiler breeder flocks in Brazil. In: Jensen, M.M. (Ed.), Proceedings of the 44th Western Poultry Diseases Conference, pp. 124–125.
Baxter-Jones, C., Wilding, G.P., Grant, M., 1986. Immunofluorescence as a potential diagnostic method for turkey rhinotracheitis. Vet Rec 119 (24), 600–601.
Buys, S.B., Du Preez, J.H., 1980. A preliminary rport on the isolation of a virus causing sinusitis in turkeys in South Africa and attemps to attenuatethe virus. Turkeys 28, 36–46.
Buys, S.B., Preez, Du, J.H., Els, H.J., 1989. The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. Onderstepoort J Vet Res 56 (2), 87–98.
Buys, S.B., Du, P., Els, J.H., 1989b. Swollen head syndrome in chickens: a preliminary report on the isolation of a possible aetiological agent. J S Afr Vet Assoc 60 (4), 221–222.
Cardoso, T.C., Rahal, P., Pilz, D., Teixeira, M.C.B., Arns, C.W., 2000. Replication of classical infectious bursal disease virus in the chicken embryo related cell line. Avian Pathol. 29, 213–217.
Cardoso, T.C., Silva, L.H., Albas, A., Ferreira, H.L., Perri, S.H., 2004. Rabies neutralizing antibody detection by indirect immunperoxidase serum neutralization assay performed on chicken embryo related cell line. Mem. Inst. Oswaldo Cruz 99, 531–534.
Cook, J.K., 2000. Avian pneumovirus infections of turkeys and chickens. Vet J 160 (2), 118–125.
Cook, J.K.A., Huggins, M.B., Orbell, S.J., et al., 1999. Preliminary antigenic characterization of an avian Pneumovirus isolated from a commercial turkeys in Colorado, USA. Avian Pathol 28, 607–617.
Cook, J.K.C.D., 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathology 31, 117–132.
Dani, M.A., Durigon, E.L., Arns, C.W., 1999. Molecular characterization of Brazilian avian pneumovirus isolates: comparison between immunochemiluminescent Southern blot and nested PCR. J Virol Methods 79 (2), 237–241.
Dar, A., Tune, K., Munir, S., Panigrahy, B., Goyal, S.M., Kapur, V., 2001. PCRbased detection of an emerging avian pneumovirus in US turkey flocks. J Vet Diagn Invest 13 (3), 201–205.
D'Arce, R.C., Coswig, L.T., Almeida, R.S., Trevisol, I.M., Monteiro, M.C., Rossini, L.I., Di Fabio, J., Hafez, H.M., Arns, C.W., 2005. Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. Avian Pathol 34, 133–136.
El Karamany, R.M., Imam, I.Z., Farid, A.H., Saber, M.S., 1979. Susceptibility of CER cell line to Rift Valley Fever virus. J. Egypt Public Health Assoc. 54, 105–114.
Ferreira, H.L., Pilz, D., Mesquita, L.G., Cardoso, T., 2003. Infectious bronchitis virus replication in the chicken embryo related cell line. Avian Pathol. 32, 413–417.
Goyal, S.M., Chiang, S.-J., Dar, A.M., Nagaraja, K.V., Shaw, D.P., Halvorson, D.A., Kapur, V., 2000. Isolation of avian pneumovirus from an outbreak of respiratory illness in Minnesota turkeys. J Vet Diagn Invest 12 (2), 166–168.
Grant, M.B.-J.C., Wilding, G.P., 1987. An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. Vet Rec 120 (12), 279–280.
Hafez, H.M., 1993. The role of pneumovirus in the swollen head syndrome of chickens. Review fuer Archiv Gefluegelkunde 57, 181–185.
Hafez, H.M., Weiland, F., 1990. Isolation of turkey rinotracheitis virus from turkeys. Tierarztliche Umschau 45, 103–111.
Hafez, H.M.H.M., Prusas, C., Naylor, C.J., Cavanagh, D., 2000. Presence of avian pneumovirus type A in continental Europe during the 1980s. J Vet Med B Infect Dis Vet Public Health 47 (8), 629–633.
Jones, R.C., 1996. Avian pneumovirus infection: Questions still unanswered. Avian Pathol 25 (4), 639–648.
Kong, B.-W., Foster, L.K., Foster, D.N., 2006. Comparison of avian cell substrates for propagating subtype C avian metapneumovirus. Virus Res 116, 58–68.
McDougall, J.S., Cook, J.K., 1986. Turkey rhinotracheitis: preliminary investigations. Vet Rec 118 (8), 206–207.
Naylor, C.J., Jones, R.C., 1994. Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. Vaccine 12 (13), 1225–1230.
Njenga, M.K.L., Han, M., Seal, B.S., 2003. Metapneumoviruses in birds and humans. Virus Res 91 (2), 163–169.
Owoade, A.A., Ducatez, M.F., Hubschen, J.M., Sausy, A., Chen, H., Guan, Y., Muller, C.P., 2008. Avian metapneumovirus subtype A in China and subtypes A and B in Nigeria. Avian Dis 52 (3), 502–506.
Panigrahy, B., Senne, D.A., Pedersen, J.C., Gidlewski, T., Edson, R.K., 2000. Experimental and serologic observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. Avian Dis 44 (1), 17–22.
Patnayak, D.P.T.A., Goyal, S.M., 2005. Growth of vaccine strains of avian pneumovirus in different cell lines. Avian Pathol 34 (2), 123–126.
Pringle, C.R., 1998. Virus taxonomy—San Diego 1998. Arch Virol 143 (7), 1449–1459.
Reed, J., Muench, H., 1938. A simple method for estimating fifty percent endpoints. American Journal Hygiene 27, 493.
Shin, H.J., McComb, B., Back, A., Shaw, D.P., Halvorson, D.A., Nagaraja, K.V., 2000. Susceptibility of broiler chicks to infection by avian pneumovirus of turkey origin. Avian Dis 44 (4), 797–802.
Simoni, I.C., Fernandes, M.J.B., Custódio, R.M., Madeira, A.M.B.N., Arns, C.W., 1999. Susceptibility of cell lines to avian viruses. Rev. Microbiol 30 (4), 373–376.
Sinibaldi, L., Goldoni, P., Pietropaolo, V., Viti, D., Orsi, N., 1990. Extraction and purification of gangliosides from CER cells, a cell line suitable for rabies virus replication. Microbiologica 13 (4), 339–342.
Smith, A.L., Tigno, G.H., Mifune, K., 1977. Isolation and assay of rabies serogroup viruses in CER cells. Intervirology 8, 92–99.
Spilki, F.R., Almedia, R.S., Campalans, J., Arns, C.W., 2005. Susceptibility of different cell lines to infection with bovine respiratory syncytial virus. J Virol Methods 131 (2), 130–133.
Tiwari, A., Patnayak, D.P., Chander, Y., Goyal, S.M., 2006. Permissibility of different cell types for the growth of avian metapneumovirus. J Virol Methods 138 (1–2), 80–84.
Toquin, D., Bäyon-Auboyer, M.H., Senne, D.A., Eterradossi, N., 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis 44 (4), 977–982.
Urmanova, M.A., Tsareva, A.A., 1996a. The cytogenetic study of established Syrian hamster cell lines. I. A comparative analysis of the karyotypes of the BHK-21 (C-13) sublines. Tsitologiia 38, 630–638.
Urmanova, M.A., Tsareva, A.A., 1996b. The cytogenetic study of established Syrian hamster cell lines. II. A comparative analysis of the karyotypes of cell lines HaK, CER and BHK-21 (C-13). Tsitologiia 38, 639–645.
Urmanova, M.A., Tsareva, A.A., 1996c. The cytogenetic study of established Syrian hamster cell lines. III. An analysis of the NOR-marker chromosomes in Syrian hamster cell cultures by using differential chromosome Ag? G restaining. Tsitologiia 38, 646–649.
Wilding, G.P., Baxter-Jones, C., Grant, M., 1986. Ciliostatic agent found in rhinotracheitis. Vet Rec 118 (26), 735.
Williams, R.A., Savage, C.E., Jones, R.C., 1991. Development of a live attenuated vaccine against turkey rinotracheitis. Avian Pathol 20, 45–55.

Artigo II

Experimental analysis of Avian Metapneumovirus Subtype B after serial passage in CER cells

Márcia Bianchi dos Santos, Matheus Cavalheiro Martini, Helena Lage Ferreira, Fernando Rosado Spilki, Hafez Mohamed Hafez and Clarice Weis Arns

Experimental analysis of Avian Metapneumovirus Subtype B after serial passage in CER cells

Márcia Bianchi dos Santos[a], Matheus Cavalheiro Martini[a], Helena Lage Ferreira[b], Hafez Mohamed Hafez[c], Fernando Rosado Spilki[d] and Clarice Weis Arns [a]*

[a] Laboratory of Virology, Institute of Biology - State University of Campinas, Campinas, SP, Brazil

[b] Veterinary and Agrochemical Research Centre, Brussels, Belgium

[c] Institute of Poultry Diseases, Faculty of Veterinary Medicine of the "Freie Universität Berlin", Berlin, Germany

[d] Feevale University, Novo Hamburgo, Brazil

[a] Corresponding author:
Laboratório de Virologia - Departamento de Microbiologia e Imunologia
Instituto de Biologia-Universidade Estadual de Campinas (UNICAMP),
P.O. Box: 6109 - CEP: 13081-970 - Campinas-SP-Brasil.
e-mail: arns@unicamp.br
e-mail: marciamerces@yahoo.com.br
e-mail: crmv2007@hotmail.com

[b] Avian Virology and Immunology, CODA-CERVA-VAR,
Groeselenberg 99, B-1180 Uccle, Brussels, Belgium
e-mail: helena.ferreira@var.fgov.be

[c] Institute of Poultry Diseases,
Faculty of Veterinary Medicine - "Freie Universität Berlin"
Königsweg 63, 14163 Berlin – Germany
e-mail: hafez.mohamed@vetmed.fu-berlin.de

[d] Instituto de Ciências da Saúde, Universidade Feevale
Novo Hamburgo, RS, Brasil.
e-mail: fernandors@feevale.br

Abstract

Respiratory disease caused by avian metapneumovirus (aMPV) has a strong negative impact on the economy of the poultry industry in many countries. Since aMPV infection cannot be properly treated, preventive control measures including live attenuated vaccines have been developed against subtypes A, B and C by serial passage of the virulent virus in cell cultures, and experimental trials have been done with these vaccines. The objective of this study was to analyze the infectivity of an aMPV subtype B strain after serial passage in CER cells. To accomplish this, chickens were infected with 5 different passages and the amount of viral shedding determined. The results of tracheal samples showed that the viral infectivity decreases gradually with passage, while *in vitro* replication rate increase. The G gene sequences of virus passages derived from serial passage were analyzed, however no amino acid changes were found.

1. Introduction

The Avian metapneumovirus (aMPV), classified as a member of the m*etapneumovirus* genus within the p*aramyxoviridae* family of viruses (Pringle 1998), is responsible for an acute rhinotracheitis in turkeys (TRT) and swollen head syndrome (SHS) in chickens. In turkey and chicken breeder flocks, aMPV can also affect the reproductive tract resulting in loss of egg production and quality (Jirjis et al., 2002; Gough and Jones, 2008).

First described in 1970 in South Africa, aMPV have been, based on nucleotide sequence variability, classified into four subtypes A through to D (Juhasz and Easton, 1994; Toquin et al., 1999; Bayon-Auboyer et al., 2000). Since the early 1990s, the great majority of aMPV detected have been of subtypes A and B (Juhasz & Easton, 1994; Naylor *et al.*, 1997; Toquin *et al.*, 2000; D'arce et.al., 2005; Njenga et al., 2003, Owoade et al., 2008; Banet-Noach, et al., 2009). Subtype C was first identified in the United States (Seal et al., 1998) and subsequently the subtype D was described in France (Bäyon-Auboyer *et al.*, 1999). The gene G of aMPV is the major antigenic variant for determining the subtypes of aMPV (Lwamba et al., 2005). Since G protein is responsible for viral attachment to the cell membrane and subsequent entry as was demonstrated for respiratory syncytial virus (RSV) which is in same subfamily of aMPV (Levine et al,1987), it may has a key function in the virus life cycle and may be an important determinant of virulence.

Primary isolation of aMPV from field samples is carried out using chicken or turkey TOC or in chicken embryonated eggs (McDougall and Cook 1986; Naylor and Jones 1994; Panigrahy 2000). Once the virus has been isolated in one of these two systems, it can be adapted easily to growth in CEF, chicken embryo liver (CEL), chick embryo related (CER), baby hamster kidney (BHK-21) or Vero cultures (Grant and Wilding, 1987; Buys et al., 1989a; Williams et al., 1991; Coswig et al., 2010). Live attenuated vaccines have been developed against subtypes A, B and C by serial passage of the virulent virus in cell cultures, and experimental trials must be done with these vaccines (Williams et al., 1991; Cook et al., 1995; Patnayak et al., 2002, Ganapathy and Jones, 2007; Catelli et. al., 2010). However,

in contrast to disease in turkey, uncomplicated infection in chicken is frequently so mild that it may go unnoticed unless perhaps SHS develops (Jones, 1996). Moreover, experimental infections are typically less severe then those observed under field conditions (Catelli and Cook, 1998), making it difficult to develop a consistent challenge model for aMPV in chickens.

This study was conducted to determine the influence of serial passage in CER cells on the virulence of an aMPV strain, defined as viral shedding of challenged susceptible chickens.

2. Material and methods

2.1 Virus and Serial Passage of the Virus in Cell Cultures. An aMPV strain named STG-SHS-1439, isolated from a broiler breeder flock with typical signs of SHS (Hafez, 1993) and confirmed to be subtype B using PCR (Hafez and Hess., 2000) was utilised for these experiments. Serial passage of the virus was carried out in CER cells. The cells were grown at 37^{o}C under an atmosphere of 5% CO_2 in eagle's minimal essential medium (E-MEM) free of antibiotics and supplemented with 10% fetal calf serum (FCS). For serial passage, inoculated cells were disrupted by three freeze and thaw cycles and 1 mL of supernatant was inoculated onto freshly prepared CER cell monolayers. After incubation for 1 hour at 37^{o}C and 5% CO2, the medium was replaced and the flasks were incubated at the same conditions. The monolayers were monitored daily for the development of the viral cytopathic effect (CPE) and virus suspension after 48 hours of incubation was defined as one passage. The procedure was repeated 5 times. The virus passage representing each challenge passage were named 3P, 4P, 5P, 6P and 7P to be used in the *in vivo* experiment. Infectious virus titres were calculated by the Reed and Muench method (1938) and expressed as the log10 tissue culture infective dose per mL ($TCID_{50}$/mL).

2.2 Experimental design. At two weeks of age, five groups of five birds were inoculated by oculonasal route with 0.20 ml of each aMPV virus passage. A non-challenged control group was included in the experiment. At 5 days post infection

(d.p.i.) all birds were euthanized, by dislocation of cervical vertebrae, for the tracheal and sinusal sample collection. The sinusal swab and tissue scraped from trachea were collected individually and suspended in 1 ml of E-MEM containing enrofloxacin (1mg/ml) and stored at -80°C until used.

2.3 Analysis of viral shedding by real-time RT-PCR and virus isolation. RNA was extracted from each sample using QIAamp viral RNA extraction kit (Qiagen) followed by the generation of cDNA with Superscript III reverse transcriptase enzyme (Invitrogen, Brazil). The real-time RT-PCR (qRT-PCR) for aMPV subtype B was developed in an Applied Biosystems 7500 real time PCR cycler (Applied Biosystems, Foster City, USA). The primers and TaqMan probe for the F (fusion) gene were based on the sequence of the prototype British isolate UK/8/94 (GenBank accession number Y14294.1). The sequences were as follows: forward primer, 5′-CAGCAACAAAGTCGGGATCA-3′; reverse primer, 5′-GTGTTGTCTATAGTTATTGTGTCTGCCTCATT-3′; and TaqMan probe, 5′-FAM-CAACTCAACAAAGGATGCACACACATACCC-TAMRA-3′. Each 25 µL reaction volume included 1 µL of cDNA and 24 µL of Quantitec Probe PCR kit (Qiagen, Hilden, Germany) with final concentrations of 500 nM of each primer and 200 nM of the Taqman probe. The F gene results were compared with a G gene-specific primer and probe set designed for detection of aMPV subtype B previously described (Gionie et al, 2007). Ultra-pure water was used as the negative template control (NTC) and PCR products for F and G gene containing the target sequence was used as DNA standard (Bustin and Muller, 2000). 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. Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. In order to convert threshold cycles in copy numbers, an external standard curve was created using serial dilutions (from 10^2 to 10^8 copies) of known copy numbers of G and F gene of aMPV. Copy number was calculated using the

following formula: molecules/μL = g/μL DNA/(PCR product length in base pairs x 660) x 6.022x1023].

The correlation coefficients between the two qRP-PCR methods were calculated by a computer program in Excel software (the “correl” function returns the correlation coefficient for two data sets).

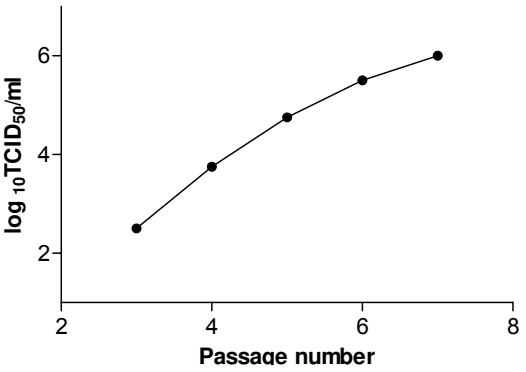
Individual samples were further used to inoculate CER cells. When viral cytopathic effect (CPE) was obtained, the original samples were titrated in CER cells following standard procedures. Titres were calculated by the Reed and Muench method (1938) and expressed as the $\log_{10}$ tissue culture infective dose per 50 µl ($TCID_{50}$/50 µl).

2.4 Sequence analysis of G gene. PCR amplification was performed with a G gene-specific primer sets designed for detection of aMPV subtype B previously described (Guionie et al, 2007). The PCR products with nucleotide sequence size of 1249 bp were gel-purified using a Gel extraction Kit (Qiagen), ligated into the pGEM-T vector and transformed into JM109 competent cells (Promega). The plasmid was extracted with QIAprep Spin Miniprep kit (Qiagen) and sequenced with the T7 forward and SP6 reverse primers.

3. Results

3.1 Serial Passage. Fig. 1 shows the replication curve of the aMPV strain based on viral titre of each passage in CER cells. After 3 cell culture passages, the virus showed typical signs of adaptation, as reflected by increases in virus titre and proportion of infected cells. The CPE consisted of round refringent cells with subsequent detachment from the monolayer and clusters of small syncytia.

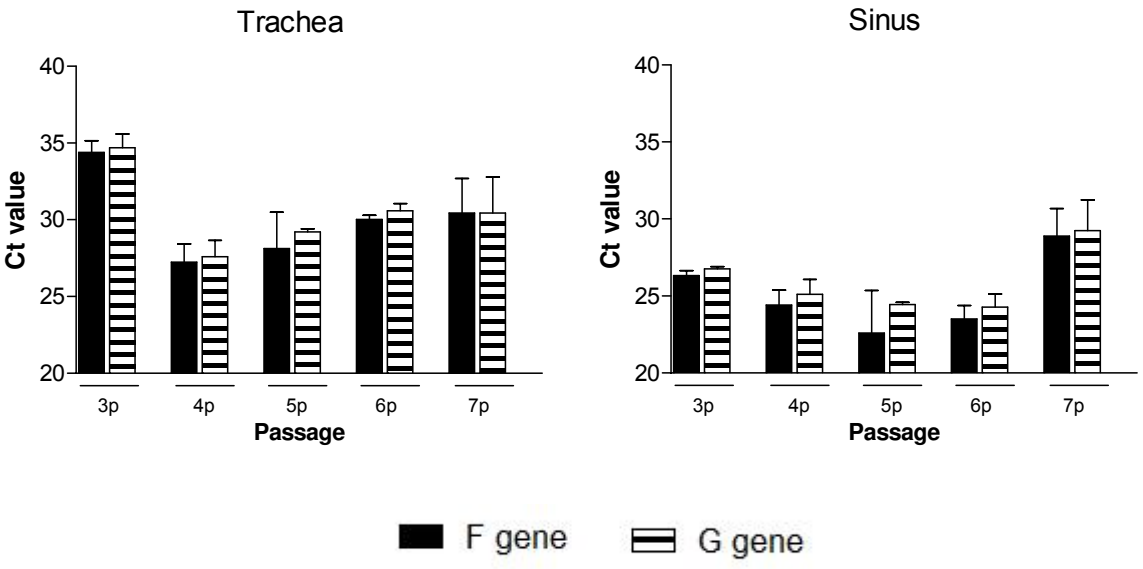
Figure 1. Mean aMPV titre on each passage numbers in CER cells. These same passages were used to challenge the birds.



3.2 Analysis of viral shedding. Viral RNA was detected by both F and G-based qRT-PCR methods in sinusal swab and tissue scraped from trachea from all birds in all five challenged groups (fig. 2). The Ct number obtained from sinus samples declined as higher was the passage level inoculated in chickens, following the growth of viral titre with the serial passages in CER cells, except for the 7P. However, when testing tracheal samples, the Ct number increased as higher was the passage level inoculated in chickens, except for the 3P. The amount of viral shedding detected in trachea shows the gradual decrease in infectivity of the virus with serial passage, while *in vitro* replication rate increase. The lowest Ct number was obtained in sinusal samples of group challenged with 5P (Ct: 31.43) and in tracheal samples of group challenged with 4P (Ct: 27.92). No virus was detected from the control group.

There was general agreement (correlation= 0.98 to 0.99) between the two qRT-PCR methods used.

Figure 2. Mean amount of viral shedding (in Ct number) from groups challenged with each virulent passage number determined using F and G-based RRT-RT-PCR.



Infectious virus was recovered from the trachea and sinus of all the birds in all challenged groups. The highest virus titre was obtained in group challenged with P4 for tracheal samples (2.2 $\log_{10}$ $TCID_{50}$/50 µl) and P5 for sinusal samples (3.1 $\log_{10}$ $TCID_{50}$/50 µl). No virus was isolated from the control group.

Table 1. Mean titres of virus in sinus or trachea from chickens challenged with different passages of virulent aMPV.

Passage	Vírus Titre (log10 TCID50 /mL)	
	Trachea	Sinus
3p	2.1	1.7
4p	2.2	2.9
5p	1.7	3.1
6p	1.9	2.8
7p	1.6	3.0

3.3 Analysis of G gene. Three sequences were obtained from serial passages of the virus in CER cells, the P1, P5 and P10 representing the first, fifth and tenth passages. Only one nucleotide change in the gene that did not result in amino acid substitution was detected. The nucleotide and deduced nucleotide similarity is of 99%.

4. Discussion

In agreement with observations made by others authors (Williams et al., 1991; Cook and Ellis, 1990; Kong et al., 2008), serial passage of the aMPV subtype B strain in CER cells resulted in decrease of viral infectivity. In spite of increasing viral titre with CER passage level, the results of qRT-PCR for tracheal samples showed that the viral fitness in chickens decreased with the passage level. However, investigating sinusal samples the Ct number did not increase as fast as those observed in tracheal samples and a signal of decrease in fitness appeared in the last passage only. A high correlation was observed between the two qRT-PCR methods used in this study indicating that F-based qRT-PCR is as sensitive as the previously reported G-based qRT-PCR (Gionie et al., 2007). When using cell based viral titration, a decrease in viral titres was also observed in the tracheal samples collected from birds challenged with the three last passages, however no signal of decrease in viral titre was observed in sinusal samples. In fact, it is remarkable that, as observed on aMPV isolation and titration assays from samples recovered during the bird experiments, the levels on infectious titres excreted at the upper respiratory tract of birds increases after serial CER passages, while tracheal virus shedding decreases. This may be an effect of selective adaption to a certain group of receptors *in vitro*. The qRT-PCR and viral titration data also reveal that the best source of aMPV for virus detection and isolation are the nasal secretion. Consistent with Domingo and Holland (1997) observations, these finds show that aMPV, as many other RNA viruses, uses well known evolutionary strategies to ensure its multiplication and stability in cell culture. It can also be supported by a previous report which showed that the aMPV subtype C virulence was enhanced by *in vivo* passages (Tiwari et al. 2006), unlike *in vitro* passage.

It is well established that viruses utilize co-receptors and alternative receptors for their entry into cells, and this is an important determinant of virulence (Domingo and Holland, 1997). The G protein gene of aMPV is responsible for cell attachment (Levine et al., 1987) and it is the major antigenic variant for determining the subtypes of this virus (Juhasz and Easton, 1994; Lwamba et al., 2005). Deletions in this gene were detected in early passage when a subtype C strain of aMPV was propagated in a mammalian cell line (Kong et al., 2008). Taking into account the key function of G gene for the viral life cycle, three sequences of aMPV derived from serial passage were obtained in order to investigate possible RNA modifications. Surprisingly, only one nucleotide alteration resulting in no amino acid substitution was detected. It appeared that G gene was not affected when aMPV was propagated in CER cells, but other alternative glycoproteins could play more functional roles such as the F protein. A previous study showed that although an aMPV subtype A vaccine strain attenuated in Vero cells have retained the complete genome without any deletions, two amino acid substitutions were detected in F gene and none in the SH-G genes (Catelli et al., 2006). Moreover, recombinant mutants of aMPV subtype A demonstrated that the SH and G proteins were dispensable for virus propagation in cell culture (Naylor et al., 2004), however none of naturally deleted mutant genes have been reported to date.

The results of this study indicate that serial passage of the aMPV subtype B strain in CER cells quickly leads to decrease of viral infectivity with no mutation in G gene. Knowledge of the types of hosts and cell types in which viral replication may lead to decreases in viral fitness in the authentic host may help in the design of live-attenuated vaccines. Furthermore this kind of study can help to improve on a challenge model in chickens for aMPV subtype B.

5. References

Banet-Noach C., Simanov L., Laham-Karam N., Perk S., Bacharach E. 2009. Longitudinal survey of avian metapneumoviruses in poultry in Israel: infiltration of field strains into vaccinated flocks. Avian Diseases 53(2),184–9.

Bäyon-Auboyer, M. H., Jestin V. 1999. Comparison of F-, G- and N-based RTPCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Archives of Virology, v.144, n.6, p.1091- 09.

Bäyon-Auboyer, M. H., Arnauld, C., 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. Journal of General Virology, v.81, n.Pt 11, Nov, p.2723-2733.

Bustin, S.A. & Mueller, R. 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond), 109, 365-379.

Catelli, E., Cook, J. K. A. 1998. The use of virus isolation, histopathology and immunoperoxidase techniques to study the dissemination of a chicken isolate of avian neumovirus in chickens. Avian Pathology, v.27, p.632.640.

Catelli E., Cecchinato M., Savage C. E., Jones R. C., Naylor C. J. 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine 24, 6476–82.

Catelli E., Lupini C., Cecchinato M., Ricchizzi E., Brown P., Naylor C. J. 2010. Field avian metapneumovirus evolution avoiding vaccine induced immunity. Vaccine. 28(4), 916-21.

Cook, J. K. 2000. Avian Pneumovirus Infections of Turkeys and Chickens. The Veterinary Journal, v.160, Aug, p.118-125.

Cook J. K., Ellis M. M. 1990. Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ cultures.Avian Pathol. Jan;19(1):181-5.

D'arce, R. C., Coswig, L. T., Almeida, R. S., Trevisol, I. M., Monteiro, M. C., Rossini, L. I., Di Fabio, J., Hafez, H. M. & Arns, C. W. 2005. Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. Avian Pathology 34, 133-136.

Domingo E., Holland J. J. 1997. RNA virus mutations and fitness for survival. Annu Rev Microbiol.;51:151-78. Review.

Ganapathy K. and Jones R.C. 2007. Vaccination of chicks with live attenuated subtype B avian metapneumovirus vaccines: protection against challenge and immune responses can be unrelated to vaccine dose. Avian Dis. Sep;51(3):733-7.

Gionie, O., Toquin, D., Sellal, E., Bouley, S., Zwingelstein, F., Allée, C., Bougeard, S., Lemiere, S., Eterradossi, N., 2007. Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. J. Virol. Meth. V. 139, p. 150-158.

Gough, R. E. and Jones, R. C. 2008. Avian metapneumovirus. In 'Diseases of Poultry', 12th Edition. Eds. Y M Saif et al.. Blackwell Publishing, Ames Iowa. Pp 101-110.

Hafez, H. M. 1993. The role of pneumovirus in swollen head syndrome of chickens: review. Archiv für Geflügelkunde, v.57, p.181-185.

Hafez, H. M., Hess M. 2000. Presence of avian pneumovirus type A in continental Europe during the 1980s. Journal of Veterinary Medicine Series B, infectious Diseases and Veterinary Public Health v.47, n.8, Oct, p.629-33.

Jirjis F. F., Noll S. L., Halvorson D. A., Nagaraja K. V., Shaw D. P. 2002. Pathogenesis of avian pneumovirus infection in turkeys. Vet Pathol. 39(3):300-10.

Jones, R. C. 1996. Avian pneumovirus infection: questions still unanswered. . Avian Pathology v.25, p.639-648.

Juhasz, K. and A. J. Easton. 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. Journal of General Virology, v.75 (p 11), Nov, p.2873-80.

Kong, B-W., Foster, L. K., Foster, D. N. 2008. Species-specific deletion of the viral attachment glycoprotein of avian metapneumovirus Virus Research 132 114–121.

Levine, S., Klaiber-Franco, R., Paradiso, P. R. 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J. Gen. Virol. 68 (Pt 9), 2521–2524.

Lwamba, H. C., Alvarez, R., Wise, M. G., Yu, Q., Halvorson, D., Njenga, M. K., Seal, B.S. 2005. Comparison of the full-length genome sequence of avian metapneumovirus subtype C with other paramyxoviruses. Virus Res. 107, 83–92.

Naylor C., Shaw K., Britton P., Cavanagh D. 1997. Appearance of type B avian pneumovirus in Great Britain. Avian Pathology 26, 327–38.

Naylor, C.J., Brown, P.A., Edworthy, N., Ling, R., Jones, R.C., Savage, C.E., Easton, A.J. 2004. Development of a reverse-genetics system for avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. J. Gen. Virol. 85, 3219–3227.

Njenga, M. K., Lwamba H. M. 2003. Metapneumoviruses in birds and humans. Virus Research, v.91, n.2, Feb, p.163-9.

Owoade A. A., Ducatez M. F., Hübschen J. M., Sausy A., Chen H., Guan Y., Muller C.P. 2008. Avian metapneumovirus subtype A in China and subtypes A and B in Nigeria. Avian Dis. Sep;52(3):502-6.

Patnayak, D. P., Sheikh, A. M., Gulati, B. R, & Goyal, S. M., 2002. Experimental and field evaluation of a live vaccine against avian pneumovirus. Avian Pathology, 31, 377-382.

Reed, J.I.; Muench, H. 1938. A simple method for estimating fifth percent endpoints. American Journal Hygiene, v.27, p.493-497.

Seal, B. S. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Research, v.58, n.1-2, Nov, p.45-52. 1998.

Toquin, D., Bayon-Auboyer, M. H., Eterradossi, N., Morin, H., Jestin, V., 1999. Isolation of a pneumovirus from a Musckovy duck. Vet. Rec. 145, 680.

Toquin, D., Bayon-Auboyer, M. H., Senne, D. A., Eterradossi, N., 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis. 44, 977–982.

Tiwari, A. P., D. P., Chander, Y., Goyal, S. M. 2006. Permissibility of different cell types for the growth of avian metapneumovirus. J Virol Methods 138(1-2): 80-4.

Williams, R. A., Savage, C. E., Jones, R. C. 1991. Development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathology, 20, 45-55.

Artigo III

Protection study of avian metapneumovirus subtypes A and B in Brazil

Márcia Bianchi dos Santos[a], Matheus Cavalheiro Martini[a], Helena Lage Ferreira[b], Paulo Anselmo Fellipe[a], Fernando Rosado Spilki[a] and Clarice Weis Arns [a*]

Protection study of Brazilian avian metapneumovirus subtypes A and B

Márcia Bianchi dos Santos[a], Matheus Cavalheiro Martini[a], Helena Lage Ferreira[b], Luciana Helena Antoniassi da Silva[a], Paulo Anselmo Fellipe[a], Fernando Rosado Spilki[c] and Clarice Weis Arns [a]*

[a] Laboratory of Virology, Institute of Biology - State University of Campinas, Campinas, SP, Brazil

[b] Veterinary and Agrochemical Research Centre, Brussels, Belgium

[c] Feevale University, Novo Hamburgo, Brazil

[a] Corresponding author:
Laboratório de Virologia - Departamento de Microbiologia e Imunologia
Instituto de Biologia-Universidade Estadual de Campinas (UNICAMP),
P.O. Box: 6109 - CEP: 13081-970
Campinas-SP-BRASIL.
Telephone: +55-19-3521- 6258, Fax: +55-19-3521- 6276.
e-mail: arns@unicamp.br
e-mail: marciamerces@yahoo.com.br

[b] Avian Virology and Immunology, CODA-CERVA-VAR,
Groeselenberg 99, B-1180 Uccle, Brussels, Belgium
e-mail: helena.ferreira@var.fgov.be

[c] Instituto de Ciências da Saúde, Centro Universitário Feevale
Novo Hamburgo, RS, Brasil.
e-mail: fernandors@feevale.br

Abstract

Avian metapneumovirus (aMPV) is a respiratory pathogen associated with the swollen head syndrome (SHS) in chickens and includes four subtypes, which are differentially distributed worldwide. In Brazil, subtypes A and B are circulating in the same area and in the last years the majority of the detected aMPV belongs to subtype B. This study was conducted to compare the virulence of recently isolated aMPV subtypes A and B from Brazilian chicken flocks and analyze the virological protection afforded to chickens inoculated with an established subtype B vaccine. The results demonstrate that the subtype B virulent strain could be observed longer and in larger quantities using real time RT-PCR compared to a parallel group challenged with a subtype A. A complete heterologous virological protection was provided by an established subtype B vaccine; however, a lack of complete homologous virological protection was observed when chickens were challenged with a virulent subtype B of aMPV.

1. Introduction

The avian metapneumovirus (aMPV) is classified as a member of the *Metapneumovirus* genus within the Paramyxoviridae family (Pringle, 1998). This virus is able to replicate in the respiratory tract, especially the upper tissues, resulting in an acute respiratory disease in turkeys and chickens with significant economic losses, especially if the viral infection is associated with secondary pathogens (Jirjis et al., 2002; Gough & Jones, 2008). In broiler chickens, aMPV is, among other agents, involved in the swollen head syndrome (SHS) (Cook, 2000).

Based on molecular analysis of the genome, aMPV can be classified into four subtypes, A, B, C and D, (Juhasz & Easton, 1994; Toquin et al., 2000; Dar et al., 2001; Alvarez et al., 2003) and is present in many countries. Subtypes A and B are more widespread in the world, whereas subtype C is only found in the United States and subtype D was once reported in France (Cook, 2000; Njenga et al., 2003). In Brazil, where a high density of poultry farms exists, studies on diseased chicken and turkey flocks prove a high prevalence of aMPV subtypes A and B (D'Arce et al., 2005; Chacon et al., 2007). Recent surveys indicate that in Brazil the majority of the detected aMPV in the last years belongs to subtype B. Furthermore, they reveal the circulation of at least two subpopulations of this subtype in Brazil (Chacon et al., 2009; Villarreal et al., 2009).

Once aMPV infection was diagnosed in Europe, live vaccines became available (Cook et al., 1989ab; Williams et al., 1991ab) improoving the welfare and reducing economic effects of aMPV infections in turkey flocks (Cook, 2009). In Brazil, after the first detection of aMPV in 1995, mass vaccination in commercial flocks was established. However, disease in vaccinated birds has still been found in regions with high poultry density (Cecchinato et al., 2009; Chacon et al., 2009; Banet-Noach et al., 2009; Catelli et al., 2010). Despite the evidence of excellent cross protection between the A and B subtypes (Cook et al., 1995; Eterradossi et al., 1995), in some cases disease may have been caused by infection with the subtype not included in the vaccine (Banet-Noach et al., 2005, 2009; Cook, 2009). In other cases it could have been caused by genetic variations between the field and vaccine strains (Banet-Noach et al., 2009; Catelli et al., 2010) or by poor vaccine

administration leading to reversion to virulence (Catelli et al., 2006; Ricchizzi et al., 2009).

The first aim of this study was to examine a commercially available subtype B vaccine in broiler chickens in terms of its ability to provide virological protection against aMPV subtypes A and B. To accomplish this, two recently isolated subtypes A and B aMPV strains from Brazilian chicken flocks were analyzed.

2. Materials and methods

2.1 Collection of field samples and aMPV subtyping by nested RT-PCR

From December 2006 to December 2008, sinusal swab and tissue scraped from trachea were collected from respiratory diseased chickens of vaccinated and non-vaccinated flocks against aMPV. The field samples were suspended in 1 mL of E-MEM containing 1 mg/mL enrofloxacin and, after shaking, centrifuged for 5 minutes at 3500 g to sediment the cellular debris. The supernatants were harvested and stored at -80°C until used. A subtype specific RT-nested PCR, based on G gene sequence able to differentiate A and B subtypes, was used to detect and classify aMPV. RNA was extracted from pools of birds from the same flock and cDNA prepared, followed by nested PCR using the method described by Naylor et al. (1997).

2.2 Virus isolation

Virus isolation was performed on positive samples by RT-nested PCR. The samples were serially passaged in Chicken Embryo Related (CER) cells until the development of the viral cytopathic effect (CPE) and a new RT-nested PCR was used to confirm virus presence. The strains Chicken/A/BR/775/06 and Chicken/B/BR/877/08 were titrated in CER cells and end points were calculated by the method of Reed and Muench (1938). For experimental inoculation, both strains were used at a dose of 3,0 log10$TCID_{50}$/50µL per bird.

2.3 Vaccine

The chickens were inoculated with a commercial aMPV subtype B vaccine, at a dose of 2,5 log10$TCID_{50}$/50µL, by the oculonasal route at the dose recommended by the manufacturer.

2.4 Experimental design

2.4.1 Analysis of challenge viruses: experimental pilot study

At two weeks of age, two groups of 25 birds were divided and inoculated by the oculonasal route with 0.20 ml of subtype A or B strains. A non-challenged control group was included in the experiment. At 3, 5, 7, 10 and 14 d.p.i. five birds from each group were euthanized followed by dislocation of cervical vertebrae. Sinusal swab and tissue scraped from trachea were collected individually and suspended in 1 ml of E-MEM containing 1 mg/mL enrofloxacin. After shaking, the samples were centrifuged for 5 minutes at 3500 g to sediment the cellular debris. The supernatants were harvested and stored at -80°C until used.

2.4.2 Protection study

Fifty-one birds were used in total. Two groups were equally divided in 21 birds and vaccine was applied to the one-day-old broiler chickens in one of the groups. At 21 days of age 18 birds of each group were subdivided in two groups and challenged with 0.20 ml of virulent subtypes A or B strains by the oculonasal route. Samples of the three remaining birds of each group (vaccinated and non-vaccinated), before the virological exposure, were collected to determine whether vaccine virus could be detected 21 days after vaccination, these birds were then euthanized. A non-challenged control group was included in the experiment. At 3, 5 and 7 d.p.i. three birds from each group were euthanized. The sinusal and tissue tracheal samples were collected individually for virological examination and processed as the first experiment.

2.5 Virus titration

Individual samples of the experimental pilot study were further used to inoculate CER cells. When viral cytopathic effect (CPE) was obtained, the original samples were titrated in CER cells following standard procedures. Titres were calculated by the Reed and Muench method (1938) and expressed as the log_{10} tissue culture infective dose per 50 µl ($TCID_{50}$/50 µl).

2.6 Analysis of viral shedding by real-time RT-PCR and virus titration

The sinusal swab and tissue scraped from trachea were collected individually from both *in vivo* experiments. RNA was extracted from each sample and cDNA prepared, followed by real time RT-PCR based on amplification of the F (fusion) protein gene of aMPV subtype A (Ferreira et al., 2009) or B (Santos et al., in prep). Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. In order to convert threshold cycles in copy numbers, an external standard curve was created using serial dilutions of known copy numbers of F gene of aMPV. The samples were further analyzed by virus titration in CER cells. Serial 10-fold dilutions were inoculated on CER 96-well plates following standard procedures. Titres were calculated by the Reed and Muench method (1938) and expressed as the log_{10} tissue culture infective dose per 50 µL ($TCID_{50}$/50 µL).

2.7 Serum neutralization test

At the initial inoculation of the vaccine and at weekly intervals up to tree weeks, blood samples were collected from ten birds. The sera were assayed for the presence of antibodies by the serum neutralization (SN) test.

3. Results

3.1 aMPV subtyping

From the 101 analyzed biological samples, 20 were aMPV-positive. Nine samples belonged to subtype A and 11 were characterized as subtype B. In seven cases,

an aMPV and Infectious bronchitis virus (IBV) co-infection was detected. In the last years of this survey, the majority of the detected aMPVs belonged to subtype B.

3.2 Analysis of challenge viruses: experimental pilot study

3.2.1 Analysis of viral shedding

In the pilot study, chickens in groups infected with aMPV subtype A or B showed different viral shedding; subtype B virulent strain could be observed longer and in larger quantities using real time RT-PCR compared to subtype A strain. Subtype A RNA was detected at 3, 5 and 7 d.p.i. while subtype B virus was detected at all five time points. The highest RNA detection for subtype A was obtained in sinus at 5 d.p.i. (Ct: 33.21) and for subtype B at 3 d.p.i. (Ct: 27.18). When mean Ct number of sinusal and tracheal samples were compared, those at 3, 5, and 7 d.p.i. in the subtype B challenged group were significantly higher than those in the subtype A challenged group. Fig. 1 shows the recovery of virus from the birds infected with virulent aMPV subtype A and B.

3.2.2 Virus titration

Infectious virus was recovered from the trachea and sinus at all d.p.i. for subtype B challenged group. The highest virus titre was obtained in tracheal samples at 7d.p.i. (1.0 $\log_{10}$ $TCID_{50}$/50 µl) and in sinusal samples at 3 and 5 d.p.i. (1.4 $\log_{10}$ $TCID_{50}$/50 µl). Subtype A viruses were detected only in sinusal samples at 5 d.p.i. with titre mean of 0.6 $\log_{10}$ $TCID_{50}$/50 µl. No virus was isolated from the control group.

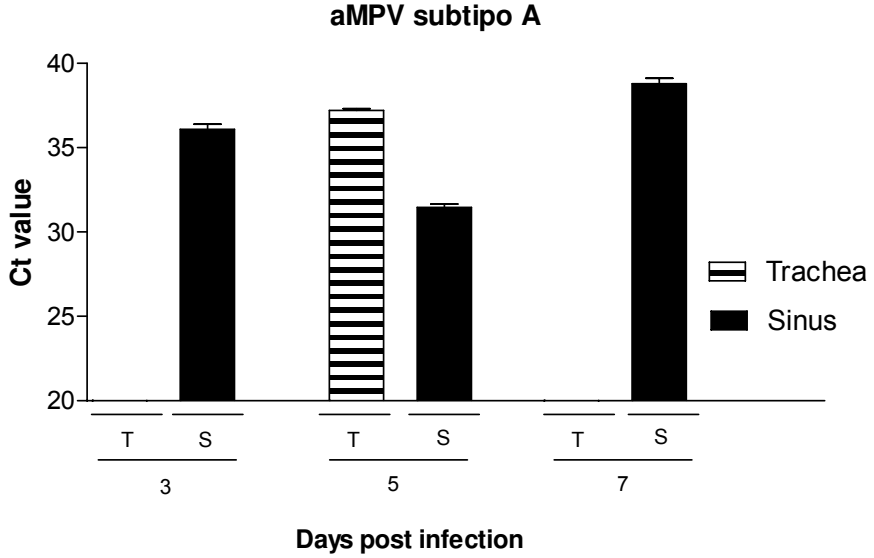
Table 1: Mean titres in trachea and sinus from chickens inoculated with virulent aMPV subtype B

Days post challenge	Virus titre (log10 TCID50/50µL) Trachea	Sinus
3	0,4*	1,4
5	0,5	1,4
7	1,0	0,4
10	0,6	0,4
14	0,4	0,4

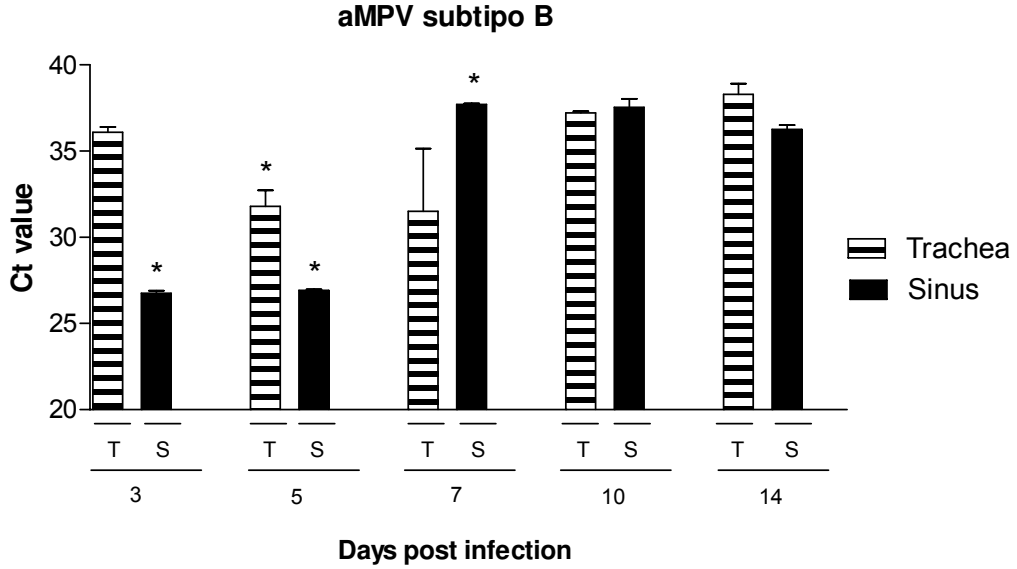
Titres are expressed in log10 TCID50/50µL
* Values are mean of five birds

Figure 1. RNA virus detection by real time RT-PCR from trachea and sinus of challenged birds with virulent subtype A or B aMPV at 3, 5, 7, 10 and 14 d.p.i. Asterisk (*) means Ct number of the subtype B challenged group (B) is significantly ($P<0.05$) higher than the corresponding value of the subtype A challenged group (A).

A)



B)

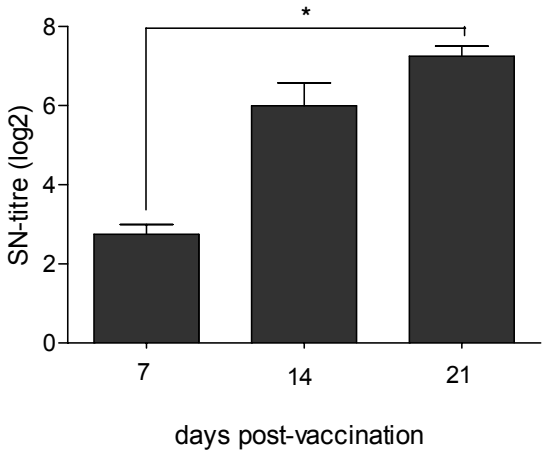


3.3 Protection study

3.3.1 Serological analyses

All pre-vaccination sera were negative for aMPV antibodies in the SN test. After initial inoculation with vaccine strain the titres of neutralizing antibodies reached their maximal level after three weeks. The maximum mean titre was 8 $\log_2$ (Fig. 1)

Figure 2. Mean serum neutralization titres of chickens after vaccination with subtype B aMPV. Asterisk (*) means SN titre are significantly (P<0.05) different.



3.3.2 Analysis of viral shedding

For the protection study, the mean viral detection by real-time RT-PCR or virus titration for the vaccinated and unvaccinated groups is reported graphically in fig. 5. Virus was detected by both real time RT-PCR and viral titration in all chickens which had not been vaccinated, but challenged at 21 days of age with virulent subtype B strain (group -/B), except for the tracheal samples collected at 7 d.p.i. in which no infectious virus was detected. In unvaccinated chickens challenged with subtype A strain (group -/A), viral RNA was detected at 3 and 5 d.p.i.; infectious virus was recovered from the trachea and sinus of all birds at 5 d.p.i. and in sinusal samples only at 3 d.p.i. The highest virus titre and Ct number was obtained in sinusal samples of group -/A at 3 d.p.i. (1,5 log10$TCID_{50}$/50µL; Ct: 31.43) and at 5 d.p.i. for group -/B (2,7 log10$TCID_{50}$/50µL; Ct: 27.92).

In chickens that had been vaccinated at one day of age and then challenged with homologous virus at 21 days of age (group B/B), viral RNA was detected in 4/9 birds after 3, 5 and 7 d.p.i. Only one bird in group B/B yielded infectious virus (1,2 and 1,5 log10$TCID_{50}$/50µL in sinusal and tracheal samples respectively). In Chickens previously vaccinated and then challenged with heterologous subtype A virus at 21 days old (group B/A), no virus was detected by both real time RT-PCR and viral titration. No virus was detected by real time RT-PCR or virus titration in vaccinated chickens not challenged and in chickens which had been neither vaccinated at one day old nor challenged. Table 1 shows the recovery of virus from the challenged birds which were initially vaccinated or not.

Figure 3. Results of protection study. Groups of 9 one day old chickens were vaccinated with commercial subtype B aMPV vaccine. After 21 days they were challenged with either homologous (group B/B) or heterologous virulent virus (group B/A). Unvaccinated chickens were challenged with one or the other virus (groups -/B and -/A). The figure shows the percentage of detected positive birds for aMPV by real time RT-PCR or virus titration in each group at the given time in d.p.i.

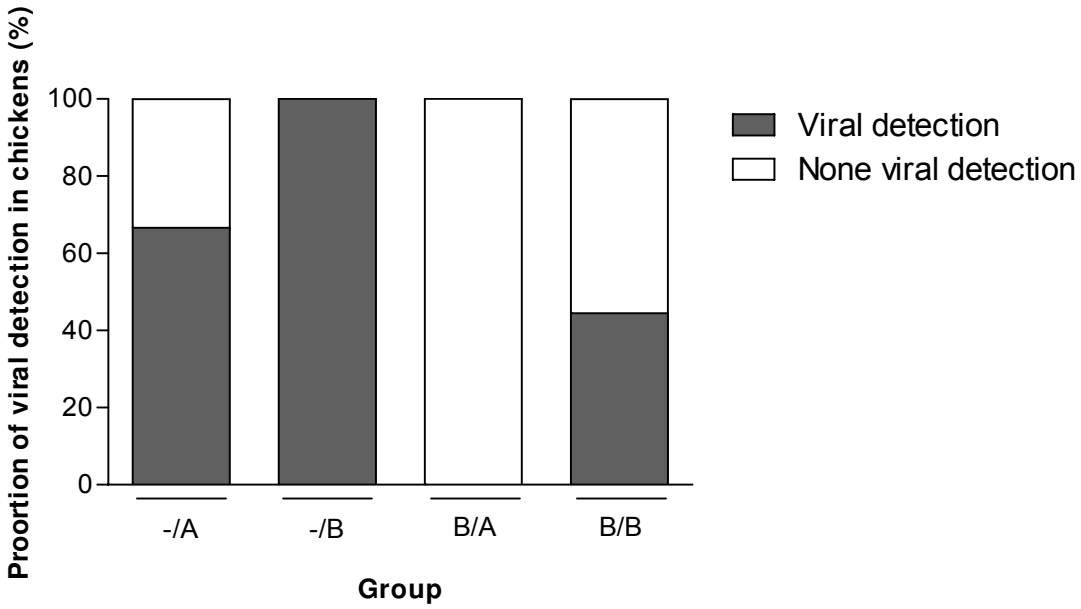


Table 2: Mean titres in trachea and sinus from chickens inoculated with virulent aMPV subtypes A and B

		Sinus					
vaccine	Challenge	3dpi		5dpi		7dpi	
		Titre	Ct	Titre	Ct	Titre	Ct
B	A	-	-	-	-	-	-
B	B	-/-/1.2**	-/-/31.41	-	-	-	-/-/>39
None	A	<1*	38.64	1.5	31.43	-	-
None	B	1.2	33.07	2.7	27.92	1.1	38.79
		Trachea					
vaccine	Challenge	3dpi		5dpi		7dpi	
		Titre	Ct	Titre	Ct	Titre	Ct
B	A	-	-	-	-	-	-
B	B	- /-/1.5	-/>39/29.45	-	-/-/>39	-	-/-/29.98
None	A	-	-/-/>39	1.2	36.77	-	-
None	B	1.4	31.91	1.7	30.67	-	32.56

Titres are expressed in log10 TCID50/50µL
* Values are means of tree birds, except when only some of the samples yielded virus, in which case all the values are shown (**)

4. Discussion

After the first detection of aMPV in Brazil (Arns and Hafez, 1995) massive vaccination was established in commercial flocks. However, recent studies show that aMPV subtypes A and B are still highly spread in Brazil affecting broilers, breeders, laying hens and turkeys (D'Arce et al., 2005; Chacon et al., 2007; Chacon et al., 2009). To analyze a commercial aMPV subtype B vaccine found in Brazil, a protection study was performed in order to examine the virological protection afforded to broiler chicken by inoculating them with the vaccine and Brazilian virulent aMPV subtypes A and B strains.

The results of this study demonstrate, under experimental conditions, a lack of complete homologous virological protection of immunized and subtype B challenged birds. Surprisingly, a complete heterologous virological protection was provided by the same subtype B vaccine, i.e., neither virus shedding nor viral RNA could be detected in tracheal or sinusal samples of immunized and subtype A challenged birds. Similar to the study of Naylor et al. (1997), heterologous clinical protection was observed after turkeys, inoculated initially with subtype B, were challenged with virulent subtype A strain. However, clear nasal exudate was observed in part of the birds inoculated initially with subtype B and challenged with virulent homologous strain, although no infectious virus was detected. In this study, full cross-protection did not occur when the birds were inoculated initially with subtype A. In contrast, Van de Zande (2000) showed that turkeys were afforded an homologous and heterologous clinical and virological protection for at least 11 weeks after they had been inoculated with either a virulent subtype A and B strains of aMPV; the cross-protection diminished after a given period.

Although the results lead to the conclusion that the heterologous protection was more efficient than homologous protection provided by the same subtype B vaccine, the virulence difference between the subtype A and B challenge strains should be taken into account. In the first part of this study, the virulence of both challenge viruses were analyzed and subtype B could be observed longer and in significantly larger quantities compared with subtype A virulent strain. Moreover, Catelli et al. (2010) suggest that protection levels able to resist virulent experimental challenge can be insufficient on farms where less ideal conditions prevail. Indeed protection between A and B subtypes in experimental conditions is generally good (Van de Zande et al., 2000) but in the field, a lack of heterologous protection has been seen (Naylor et al., 1997; Van de Zande et al., 1998; Catelli, 2006). Since these observations are based on the results of vaccine protection provided against a single subtype A or B challenge, it should be interpreted with caution.

In this survey, four out of nine broiler chickens immunized with the subtype B vaccine, then challenged with the virulent homologous strain, were found to receive

incomplete protection. However, infectious virus was recovered from one bird at 3 d.p.i. only. Lack of complete protection after homologous vaccination was also seen previously in two recent field studies carried out in Israel and Italy. In both studies the sequence analysis detected nucleotide differences between the vaccine and field strains in the SH and G genes, with predominance of non-synonymous amino acid alterations, suggesting that the field virus was able to overcome immunity induced by the vaccine (Banet-Noach et al., 2009; Catelli et al., 2010). Since the G protein is believed to be antigenically important, changes on it may provide an opportunity for the virus to potentially scape from previously established immunity, as already shown for BRSV and HRSV (Sullender et al., 1999; Valarcher et al., 2000). Indeed the reason for the failure of the commercial vaccine used in this survey to protect part of the birds from subtype B Brazilian strain may be the existence of a genetic divergence between the European vaccine and the Brazilian field strains. On the other hand, the lack of complete homologous protection may be a result of differential immune responses by individual birds, as the recovery of infectious virus was observed in one bird of B/B group only.

A field protection study carried out on naturaly infected Brazilian layer chickens showed an efficient protection. However, the subtype B lineage found on naturaly infected chickens was divergent from other lineages already described, suggesting that subpopulations of aMPV are present in Brazil. As a consequence of the amino acids mutations that are accumulating in Brazilian strains of aMPV subtype B, escape mutants could emerge for which the vaccines that worked before would provide insufficient protection (Villarreal et. al., 2009). Curiously, the fist results of this survey show that the majority of the detected aMPVs in Brazil in the last years belonged to subtype B.

This study confirms the distribution of aMPV subtypes A and B in Brazil and the contagious nature of these viruses. Continued surveillance is required to detect any changes in the subtypes A and B or introduction of a new subtype of aMPV. Moreover, it also reveals a lack of complete protection provided from a commercial European vaccine to Brazilian broiler chicken flocks. In such situation, viruses may

be free to replicate in vaccinates and shed to environment, allowing a large number of generations for mutations to occur.

5. References

ALVAREZ, R. L.; KAPCZYNSKI, D. R.; NJENGA, M. K.; SEAL, B. S. (2003). Nucleotide and predicted amino acid sequence-based analysis of the avian metapneumovirus type C cell attachment glycoprotein gene: phylogenetic analysis and molecular epidemiology of U.S. pneumoviruses. *J Clin Microbiol* **41**(4),1730-5.

ARNS, C. W. AND HAFEZ, H. M. (1995). Isolation and identification of Avian Pneumovirus from broiler breeder flocks in Brazil. In M.M. Jensen (Ed) Proceedings of the 44st Western Poultry Diseases Conference. pp 124-125.

BANET-NOACH C., SIMANOV L., PERK S. (2005). Characterization of Israeli avian metapneumovirus strains in turkeys and chickens. *Avian Pathology* **34**(3), 220–6.

BANET-NOACH C., SIMANOV L., LAHAM-KARAM N., PERK S., BACHARACH E. (2009). Longitudinal survey of avian metapneumoviruses in poultry in Israel: infiltration of field strains into vaccinated flocks. *Avian Diseases* **53**(2),184–9.

CATELLI E., CECCHINATO M., SAVAGE C. E., JONES R. C., NAYLOR C. J. (2006). Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. *Vaccine* **24**, 6476–82.

CATELLI E., LUPINI C., CECCHINATO M., RICCHIZZI E., BROWN P., NAYLOR C. J. (2010). Field avian metapneumovirus evolution avoiding vaccine induced immunity. *Vaccine*. **28**(4), 916-21.

CECCHINATO M., CATELLI E.,LUPINI C., RICHIZZI E., CLUBBE J., NAYLOR C. (2009) Evidence of AMPV attachment protein evolution coincident with mass live vaccine introduction in Italy. Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. Rauischholzhausen, Germany.

CECCHINATO, M., CATELLI, E., LUPINI, C., RIXXHIZZI, E., BROWN, P. & NAYLOR, C.J. (2008). Field avian metapneumovirus evolution avoiding vaccine induced immunity. Proceedings of the 7th International Symposium on turkey diseases, Berlin, Germany. pp 176-179

CHACÓN JL, BRANDÃO PE, BUIM M, VILLARREAL L, FERREIRA AJ. (2007) Detection by reverse transcriptase-polymerase chain reaction and molecular characterization of subtype B avian metapneumovirus isolated in Brazil. *Avian Pathology* **36**(5), 383-7.

CHACON J. L.; PEDROSO A. C.; TOQUIN D.; ETERRADOSSI N.; PATNAYAK D.; GOYAL S. AND FERREIRA A. J. P. (2009). Subtypes of avian metapneumovirus circulating in brazilian commercial flocks. Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. Rauischholzhausen, Germany.

COOK, J. K. A., HUGGINS, M. B., WOODS, M. A., ORBELL, S. J. & MOCKETT, A. P. A. (1995). Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. *Veterinary Record* **136***,* 392-393.

COOK J. K. A. (2009). Avian Metapneumovirus – Nearly 30 years of vaccination. In Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. Rauischholzhausen, Germany.

COOK, J. K. (2000). Avian pneumovirus infections of turkeys and chickens. *The Veterinary Journal* **160**(2), 118-25.

COOK J. K. A., ELLIS M. M., DOLBY C. A., HOLMES H. C., FINNEY P. M., HUGGINS M. B. (1989a). A live attenuated turkey Rhinotracheitis Virus Vaccine 1. *Avian Pathology* **18**, 511–22.

COOK J. K. A., HOLMES H. C., FINNEY P. M., ELLIS M. M., HUGGINS M. B., DOLBY C. A. (1989b). A live attenuated turkey Rhinotracheitis Virus Vaccine. 2. The use of the attenuated strains as an experimental vaccine. *Avian Pathology* **18,** 523–34.

COOK, J. K. (2000). Avian Pneumovirus Infections of Turkeys and Chickens. *The Veterinary Journal* **160,** 118-125..

DAR, A. M. T., K.; MUNIR, S.; PANIGRAHY, B.; GOYAL, S. M.; KAPUR, V. (2001). PCR-based detection of an emerging avian pneumovirus in US turkey flocks. *Journal of Veterinary Diagnostic Investigation* **13**, 201-5.

D'ARCE, R. C., COSWIG, L. T., ALMEIDA, R. S., TREVISOL, I. M., MONTEIRO, M. C., ROSSINI, L. I., DI FABIO, J., HAFEZ, H. M. & ARNS, C. W. (2005). Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. *Avian Pathology* **34**, 133-136.

ETERRADOSSI, N., TOQUIN, D., GUITTET, M. & BENNEJEAN, G. (1995). Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. *Journal of Veterinary Medicine* **42,** 175-186.

FERREIRA, H. L.; SPILKI, F. R. ; SANTOS, M. B.; ALMEIDA, R. S.; ARNS, C. W. (2009). Comparative evaluation of conventional RT-PCR and real-time RT-PCR (RRT-PCR) for detection of avian metapneumovirus subtype A. *Ciência Rural* **39**, 1445-1451,.

GOUGH, R. E., JONES, R. C. (2008). Avian metapneumovirus. In 'Diseases of Poultry', 12th Edition. Eds. Y M Saif et al.. Blackwell Publishing, Ames Iowa. pp 101-110.

JIRJIS F. F., NOLL S. L., HALVORSON D. A., NAGARAJA K. V., SHAW D. P. (2002). Pathogenesis of avian pneumovirus infection in turkeys. *Veterinary Pathology* **39**, 300-10.

JUHASZ, K. and EASTON A. J. (1994). Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. *Journal of General Virology*, **75**, p.2873-80.

NAYLOR C., SHAW K., BRITTON P., CAVANAGH D. (1997). Appearance of type B avian pneumovirus in Great Britain. *Avian Pathology* **26**, 327–38.

NJENGA, M. K.; LWAMBA, H. M.; SEAL, B. S. (2003). Metapneumoviruses in birds and humans. *Virus Research* **91**, 163-9.

PRINGLE, C. R. (1998). Virus taxonomy--San Diego 1998. *Arch Virol* **143**, 1449-59.

REED, J. AND MUENCH H. (1938). A simple method for estimating fifty percent endpoints. *American Journal Hygiene* **27**, 493.

RICCHIZZI E., CATELLI E., CECCHINATO M., LUPINI C., BROWN P. AND NAYLOR C. J. (2009). Turkey rhinotracheitis outbreak in 7 week old turkeys caused by a vaccine derived avian metapneumovirus. Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. Rauischholzhausen, Germany.

SANTOS, M. B.; MARTINI, M. C.; FERREIRA, H. L.; HAFEZ, H. M.; ARNS, C. W. (2010). Attenuation Analysis of Avian Metapneumovirus Subtype B by Real-Time RT-PCR. *In prep*

TOQUIN, D. B.-A., M. H.; SENNE, D. A.; ETERRADOSSI, N. (2000). Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. *Avian Diseases* **44**, 977-82.

VAN DE ZANDE S., NAUWYNCK H., CAVANAGH D., PENSAERT M. (1998). Infection and reinfection with Avian Pneumovirus subtype A and B on Belgian turkey farms and relation to respiratory problems. Journal of Veterinary Medicine **45**, 621–6.

VAN DE ZANDE S, NAUWYNCK H, NAYLOR CJ, PENSAERT M. (2000). Duration of crossprotection between subtypes A and B avian pneumovirus in turkeys. *Veterinary Record* **147**,132–4

VILLARREAL L. Y. B., SANDRI T. L., ASSAYAG L. J., RICHTZENHAIN L. J., MALO A., BRANDAO P. E. (2009). Field observations after natural infection of Brazilian layer chickens with a phylogenetically divergent lineage of subtype B AMPV. Proceedings of the VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. Rauischholzhausen, Germany.

WILLIAMS R. A., SAVAGE C. E., JONES R. C. (1991a). Development of a live attenuated vaccine against turkey rhinotracheitis. *Avian Pathology* **20**, 45–55.

WILLIAMS RA, SAVAGE CE, WORTHINGTON KJ, JONES RC. (1991b). Further studies on the development of a live attenuated vaccine against turkey rhinotracheitis. *Avian Pathology* **20**, 585–96.

5.0 CONCLUSÕES GERAIS

5.1 Cultivo do aMPV em diferentes sistemas celulares

O objetivo deste estudo foi encontrar cultivos celulares que permitissem a propagação em altos títulos do aMPV subtipos A e B. Ambos os subtipos, isolados primariamente em células CER, desenvolveram títulos maiores em CER, VERO e BHK-21. Quando os subtipos A e B, propagados nos diferentes cultivos celulares, foram titulados na célula homologa, os títulos foram menores do que os observados na titulação em CER. Considerando que passagens seriadas do aMPV em cultivo celular levam à rápida atenuação da amostra viral, a multiplicação de ambos os subtipos foi investigada em TOC e CEF. Apenas diferenças sutis quanto ao número de passagens necessárias para o aparecimento de ECP ou ciliostase foram observadas, levando à conclusão de que o isolamento primário em CER não interfere na permissibilidade de sistemas celulares aviários à infecção pelas amostras de subtipo A ou B. Além disso, as curvas de crescimento das amostras dos subtipos A e B foram analisadas nas células CER, VERO e BHK-21. Entre as células CER e BHK-21, diferenças estatisticamente significativa foram observados durante as primeiras 30 horas após a infecção para ambos os subtipos, com títulos mais altos nas células CER. Apesar de as amostras terem sido isoladas primariamente em CER, não houve diferença significativa entre os resultados obtidos em CER e Vero, sugerindo que estes cultivos celulares são igualmente permissíveis à infecção pelo aMPV.

5.2 Análise da virulência do aMPV após passagens seriadas

Este trabalho consistiu em analisar a virulência uma amostra de aMPV subtipo B após sofrer passagens seriadas em células CER. Para tanto, frangos de corte foram desafiados com cinco variáveis provenientes das passagens em CER e amostras de traquéia e seio nasal foram analisadas quanto à excreção viral. Os resultados obtidos por qRT-PCR nas amostras de traquéia demonstram que a virulência do aMPV diminui gradualmente enquanto o título viral aumenta com o número de passagens em células CER. Quando investigadas as amostras de seio nasal, o número de Ct não sofreu aumento tão rápido quanto ao observado nas

amostras de traquéia, ou seja, o sinal de atenuação apareceu somente nas aves desafiadas com a última passagem. Quando as amostras foram analisadas por titulação viral, também foi observada uma diminuição nos títulos das amostras de traquéia de acordo com o aumento do número da passagem, porém nenhum sinal de atenuação foi observado nas amostras de seio nasal. As seqüências do gene G das amostras utilizadas para desafio foram obtidas, porém este gene parece não ser afetado pela propagação em CER. Estes resultados sugerem que passagens seriadas do aMPV subtipo B em células CER podem levar a uma rápida diminuição no *fitness* viral, observada claramente quando o vírus se replica em traquéia. Em contrapartida, no seio nasal a replicação não se altera tão rapidamente, reforçando a idéia de que este é o local onde se encontra partículas viriais em maior abundância.

5.3 Estudo da proteção vacinal contra o aMPV subtipos A e B

Uma investigação molecular em lotes de galinhas com doença respiratória demonstra que aMPV subtipos A e B circulam no Brasil. O primeiro objetivo deste estudo foi analisar o nível de proteção viral conferido por uma vacina comercial do subtipo B contra isolados brasileiros dos subtipos A e B em frangos de corte. Para tanto, a virulência de ambos os subtipos foi analisada baseando-se na quantidade e duração da excreção viral. Os resultados desse estudo demonstram que algumas aves imunizadas receberam proteção viral parcial quando desafiadas com o vírus virulento homologo ao vacinal. O RNA viral ou a partícula infecciosa foram detectados em pelo menos um animal aos 3, 5 e 7 dpi. Curiosamente, a mesma vacina forneceu proteção viral completa contra o vírus virulento heterólogo. Embora tenha sido feita uma análise comparativa entre a proteção vacinal conferida contra os subtipos A e B do aMPV, deve-se levar em consideração a diferença de virulência entre os dois isolados. O isolado do subtipo B foi detectado em um período mais longo e em maiores quantidades quando comparado com o subtipo A. Além disso, níveis de proteção capazes de resistir à infecção pelo vírus em condições experimentais podem não ser suficientes a campo, onde as condições de manejo são outras. Talvez a falha na proteção

vacinal possa ser atribuída a variações genéticas entre a amostra vacinal européia e o isolado brasileiro, como conseqüência do acúmulo de mutações nas amostras do subtipo B que estão circulando no Brasil.

6.0 REFERÊNCIAS BIBLIOGRÁFICAS

1. Arns, C. W. e H. M. Hafez. Isolation and identification of Avian Pneumovirus from broiler breeder flocks in Brazil. *44st Western Poultry Diseases Conference Sacramento, California, USA.* 124-125 p. 1995.

2. Aung, Y. H., M. Liman, *et al.*, Reproducibility of swollen sinuses in broilers by experimental infection with avian metapneumovirus subtypes A and B of turkey origin and their comparative pathogenesis. *Avian Pathology*, 37: 65-74. 2008.

3. Banet-Noach C., L. Simanov*, et al.* Longitudinal survey of avian metapneumoviruses in poultry in Israel: infiltration of field strains into vaccinated flocks. *Avian Diseases,* 531: 84–9. 2009.

4. Barik, S. Control of nonsegmented negative-strand RNA virus replication by siRNA. *Virus Research,* 102: 27-35. 2004.

5. Baxter-Jones, C., G. P. Wilding*, et al.* Immunofluorescence as a potential diagnostic method for turkey rhinotracheitis*. Veterinary Record*, 119: 600-1. 1986.

6. Baxter-Jones, C., M. Grant*, et al.* A comparison of three methods for detecting antibodies to turkey rhinotracheitis virus. *Avian Pathology*, 18: 91.98. 1989.

7. Bäyon-Auboyer, M. H., V. Jestin*, et al.* Comparison of F-, G- and N-based RTPCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. *Archives of Virology*, 1441: 091-109. 1999.

8. Bäyon-Auboyer, M. H., C. Arnauld*, et al.* Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. *Journal of General Virology*, 81: 2723-2733. 2000.

9. Beem, M. Repeated infections with respiratory syncytial virus. *The Journal of Immunology, 98:1115*-1122. 1987.

10. Buys, S. B. e J. H. Du Preez. A preliminary report on the isolation of virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. *Turkeys*, 28: 36-46. 1980.

11. Buys, S. B., J. H. Du Preez*, et al.* The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. Onderstepoort. *Journal of Veterinary Research*, 56: 87-98. 1989a.

12. Buys, S. B. e J. H. Du Preez et al. Swollen head syndrome in chickens: a preliminary report on the isolation of a possible aetiological agent. *Journal of the South African Veterinary Association,* 60: 221-2. 1989b.

13. Cadman, H. F., P. J. Kelly*, et al.* A serosurvey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in ostriches (Struthio camelus) from Zimbabwe. *Avian Diseases*, 38: 621-5. 1994.

14. Catelli, E., J. K. A. Cook*, et al.* The use of virus isolation, histopathology and immunoperoxidase techniques to study the dissemination of a chicken isolate of avian neumovirus in chickens. *Avian Pathology*, 27: 632.640. 1998.

15. Catelli E., M. Cecchinato*, et al.* Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. *Vaccine,* 24: 6476–82. 2006.

16. Catelli E., Lupini C., *et al.* Field avian metapneumovirus evolution avoiding vaccine induced immunity. *Vaccine*, 28: 916-21. 2010.

17. Cavanagh D, Barrett T. Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis virus. *Virus Research,* 11:241-56. 1988.

18. Cavanagh, D. e P. Mawditt, *et al.* Longitudinal field studies of infectious bronchitis virus and pneumovirus in broilers using type-specific polymerase chain reactions. *Avian Pathology,* 28: 593-605. 1999.

19. Chacón, J. L., Brandão P. E., *et al.* Detection by reverse transcriptase-polymerase chain reaction and molecular characterization of subtype B avian metapneumovirus isolated in Brazil. *Avian Pathology,* 36: 383-7. 2007

20. Chettle, N. J. e P. J. Wyeth. Turkey rhinotracheitis: detection of antibodies using an ELISA test. *British Veterinary Journal*, 144: 282-7. 1988.

21. Cecchinato M., E. Catelli, *et al.* Evidence of AMPV attachment protein evolution coincident with mass live vaccine introduction in Italy. Proceedings of the *VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens.* Rauischholzhausen, Germany. 2009

22. Collins PL, Chanock RM, Murphy BR. Respiratory syncytial virus. In: Knipe DM, Howley thedn. Lippincott-Williams and Wilkns, Philadelphia PM, Editors Fields virology, 4, p1443:1486. 2001

23. Collins, M. S., R. E. Gough*, et al.* Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. *Avian Pathology*, 22: 469-479. 1993.

24. Collins, P. L., M. G. Hill*, et al.* Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. *Proceedings of National Academy of Sciences of the United States of America,* 93: 81-5. 1996.

25. Collins, P. L., Y. T. Huang*, et al.* Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. *Proceedings of the National Academy of Sciences of the United States of America*, 81: 7683-7. 1984.

26. Cook J. K., M. B. Huggins, *et al.* Infectious bronchitis virus vaccine interferes with the replication of avian pneumovirus vaccine in domestic fowl. *Avian Patholog*y, 30: 233-42. 2001.

27. Cook, J.K.A. & Ellis, M.M. Attenuation of turkey rhinotracheitis virus by alternative passage in embryonated chicken eggs and tracheal organ cultures. *Avian Pathology*, 19: 181-185. 1990.

28. Cook, J. K. A., M. M. Ellis*, et al.* The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. *Avian Pathology*, 201: 55-156. 1991.

29. Cook, J. K., M. B. Huggins*, et al.* Protection provided by a commercially available vaccine against different strains of turkey rhinotracheitis virus. *Veterinary Record*, 136: 392-3. 1995.

30. Cook, J. K. A., F. Orthel*, et al.* An experimental turkey rhinotracheitis (TRT) infection in breeding turkeys and the prevention of its clinical effects using liveattenuated and incativated TRT vaccines. *Avian Pathology*, 25: 231-243. 1996.

31. Cook, J. K. A., M. B. Huggins*, et al.* Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. *Avian Pathology*, 28: 607-617. 1999.

32. Cook, J. K. Avian Pneumovirus Infections of Turkeys and Chickens. *The Veterinary Journal*, 160: 118-125. 2000.

33. Cook J. K. A. Avian Metapneumovirus – Nearly 30 years of vaccination. In Proceedings of the *VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens.* Rauischholzhausen, Germany. 2009.

34. Dani, M. A., E. L. Durigon*, et al.* Molecular characterization of Brazilian avian pneumovirus isolates: comparison between immunochemiluminescent Southern blot and nested PCR. *Journal of Virological Methods*, 79: 237-41. 1999.

35. Dar, A. M., K. Tune*, et al.* PCR-based detection of an emerging avian pneumovirus in US turkey flocks. *Journal of Veterinary Diagnostic Investigation*, 13: 201-5. 2001.

36. D'arce, R. C., L. T. Coswig, *et al.* Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. *Avian Pathology,* 34: 133-136. 2005.

37. Diaz de Espada, E. e M. E. Perona. Etiología del sídrome de cabeza hinchada. *Revista de la Sección Española de la Asociación Mundial de Avicultura Científica*, 40: 36-42. 1984.

38. Domingo, E., and J. J. Holland. RNA virus mutations and fitness for survival. *Annual Reviews in Microbiology*, 51:151-178. 1997.

39. Droual, R. e P. R. Woolcock. Swollen head syndrome associated with E. coli and infectious bronchitis virus in the Central Valley of California. *Avian Pathology*, 23: 733-742. 1994.

40. Dupuy, L. C., S. Dobson*, et al.* Casein kinase 2-mediated phosphorylation of respiratory syncytial virus phosphoprotein P is essential for the transcription elongation activity of the viral polymerase; phosphorylation by casein kinase 1 occurs mainly at Ser (215) and is without effect. *Journal of Virology*, 73: 8384-92. 1999.

41. Easton, A. J., J. B. Domachowske*, et al.* Animal pneumoviruses: molecular genetics and pathogenesis. *Clinical Microbiology Reviews*, 17: 390-412. 2004.

42. Eterradossi, N., D. Toquin*, et al.* Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. *Zentralblatt für Veterinarmedizin, Reihe B,* 42: 175-86. 1995.

43. Fellipe, PA in prep

44. Fenner, F. J., E. P. J. Gibbs*, et al.* Paramyxoviridae. San Diego: *Academic Press.1993 (Veterinary Virology),* 1993.

45. Ferreira, HL. Estudo do efeito da interferência por rna (rnai) na replicação do metapneumovirus aviário (ampv) subtipo A (Doctoral thesis). Campinas (BR): Universidade Estadual de Campinas. 2007.

46. Ganapathy K, P. Cargill, *et al.* Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. *Avian Pathology,* 34: 297-302. 2005.

47. Gionie, O., D. Toquin, *et al.* Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. *Journal of Virological Methods,* 139: 150-158. 2007.

48. Giraud, P., G. Bennejean*, et al.* Turkey rhinotracheitis in France: preliminary investigations on a ciliostatic virus. *Veterinary Record*, 119: 606-7. 1986.

49. González-Reyes, L., M. B. Ruiz-Arguello*, et al.* Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. *Proceedings of the National Academy of Sciences of the United States of America*, 98: 9859-64. 2001.

50. Goren, E. A 'new disease' in chickens; diagnostic findings. *Tijdschrift voor Diergeneeskdunde,* 15: 1076-1077. 1985.

51. Gough, R. E. e Jones, R. C. Avian metapneumovirus. In 'Diseases of Poultry', 12th Edition. Eds. Y M Saif et al. Blackwell Publishing, Ames Iowa: 101-110. 2008.

52. Goyal, S. M., S-J. Chiang, *et al.* Isolation of avian pneumovirus from an outbreak of respiratory illness in Minnesota turkeys. *Journal of Veterinary Diagnostic Investigation, 12:* 166-168. 2000.

53. Grant, M., C. Baxter-Jones*, et al.* An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. *Veterinary Record*, 120: 79-80. 1987.

54. Gulati, B. R., D. P. Patnayak, *et al.* Protective efficacy of high-passage avian pneumovirus (APV/MN/turkey/1-a/97) in turkeys. *Avian Diseases, 45:* 593-597. 2001.

55. Hafez, H. M. Respiratory disease in turkeys: Serological surveillance for antibodies against Ornithobacterium rhinotracheal e (ORT) and turkey

rhinotracheitis (TRT). *1st International Symposium on Turkey Diseases*. Berlin, Germany: 138±145. 1988.

56. Hafez, H. M., M. Hess*, et al.* Presence of avian pneumovirus type A in continental Europe during the 1980s. Journal of Veterinary Medicine Series B, *Infectious Diseases and Veterinary Public Health*. 47: 629-33. 2000.

57. Hafez, H. M. The role of pneumovirus in swollen head syndrome of chickens: review. *Archiv für Geflügelkunde*, 571: 81-185. 1993.

58. Hafez, H. M. e F. Weiland. Isolation of turkey rhinotracheitis virus from turkeys. *Tierärztiliche Umschau*, 45: 103-111. 1990.

59. Heffels-Redmann, U., U. Neumann*, et al.* Serological evidence for susceptibility of sea gulls to avian pneumovirus (APV) infection. *International Symposium on Infectious Bronchitis and Pneumovirus Infections in Poultry*. Rauischholshausen Germany: 23-25. 1998.

60. Jing, L., J. K. A. Cook*, et al.* Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. *Avian Pathology.* .22: 771-783. 1993.

61. Jones, R. C., C. Baxter-Jones*, et al.* Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. *Veterinary Record,* 120: 301-302. 1987.

62. Jones, R. C., R. A. Williams, C *et al.* Experimental infection of laying turkeys with rhinotracheitis virus: Distribution of virus in the tissues and serological response. *Avian Pathology* 17:841-850. 1988.

63. Jones, R. C. Avian pneumovirus infection: questions still unanswered. *Avian Pathology,* 25: 639-648. 1996.

64. Jones R. C. Viral respiratory diseases (ILT, aMPV infections and IB): problems of vaccine control. *XVI world veterinary poultry association congress*, Marrakesh, Morocco, 2009, pp. 35-44. 2009.

65. Jones, R. C., R. A. Williams*, et al.* Experimental infection of laying turkeys with rhinotracheitis virus: distribution of virus in the tissues and serological response. *Avian Pathology*, 17: 841-850. 1988.

66. Juhasz, K. e A. J. Easton. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. *Journal of General Virology*, 75: 2873-80. 1994.

67. Kapczynski, D. R. e Sellers, H. S. Immunisation of turkeys with a DNA vaccine expressing either the F or N gene of avian metapneumovirus. *Avian Diseases, 47*:1376-1383. 2003.

68. Kong, B-W., L.K. Foster, *et al.* Comparison of avian cell substrates for propagating subtype C avian metapneumovirus. *Virus Res*earch, 116:58-68. 2006.

69. Larsen L.E., K. Tjørnehøj, et al. Extensive sequence divergence among bovine respiratory syncytial viruses isolated during recurrent outbreaks in closed herds. *Journal of Clinical Microbiology*, 38:4222-7. 2000.

70. Levine, S., R. Klaiber-Franco*, et al.* Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. *Journal of General Virology*, 68: 2521-4. 1987.

71. Ling, R., A. J. Easton*, et al.* Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. *Journal of General Virology*, 73: 1709-15. 1992.

72. Ling R., S. Sinkovic, *et al.* Deletion of the SH gene from avian metapneumovirus has a greater impact on virus production and immunogenicity in turkeys than deletion of the G gene or M2-2 open reading frame. *Journal of General Virology*. 89: 525-533. 2008.

73. Majó, N., G. M. Allan*, et al.* A sequential histopathologic and immunocytochemical study of chickens, turkey poults and broiler breeders

experimentally infected with turkey rhinotracheitis virus. *Avian Diseases*, 39: 887- 896. 1995.

74. Martini, M. C., L. H. A. Silva, *et al.* Infecção experimental de isolados de galinha e pomba do vírus da bronquite infecciosa com perfil filogenético diferente da vacinal. In: *XVII National Meeting of Virology*, 2009, Brasília. *Virus Reviews and Research*. Caxambu, MG. Sociedade Brasileira de Virologia, 2009.

75. Mase, M., S. Asahi*, et al.* Detection of turkey rhinotracheitis virus from chickens with swollen head syndrome by reverse transcriptase-polymerase chain reaction (RT-PCR). *Journal of Veterinary Medicine* B, 58: 359-61. 1996.

76. Mekkes, D. R. e J. J. De Wit. Comparison of three commercial ELISA kits for the detection of turkey rhinotracheitis virus antibodies. *Avian Pathology*, 27: 301- 305. 1998.

77. Mckinley E. T., D. A. Hilt, *et al.* Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. *Vaccine,* 26:1274-84. 2008.

78. Mcdougall, J. S. e J. K. Cook. Turkey rhinotracheitis: preliminary investigations. *Veterinary Record,* 118: 206-7. 1986.

79. Morley, A. J. e D. K. Thomson. Swollen head syndrome in broiler chickens. *Avian Diseases*, 28: 338-343. 1984.

80. Munir, S. e V. Kapur. Regulation of host cell transcriptional physiology by the avian pneumovirus provides key insights into host-pathogen interactions. *Journal of Virology*, 77: 4899-4910. 2003.

81. Munir, S., K. Kaur*, et al.* Avian metapneumovirus phosphoprotein targeted RNA interference silences the expression of viral proteins and inhibits virus replication*. Antiviral Research,* 69:46-51. 2006.

82. Naylor, C. J. e R. C. Jones. Turkey rhinotracheitis: a review. *Veterinary Bulletin,* 63: 439-449. 1993.

83. Naylor, C. J. e R. C. Jones. Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. *Vaccine,* 12: 1225-30. 1994.

84. Naylor, C. J., K. J. Worthington*, et al.* Failure of maternal antibodies to protect young turkey poults against challenge with turkey rhinotracheitis virus. *Avian Diseases*, 41: 968-71. 1997.

85. Naylor C. J., P. A. Brown *et al.* Development of a reverse-genetics system for avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. *Journal of General Virology*, 85: 3219-3227. 2004.

86. Njenga, M. K., H. M. Lwamba*, et al.* Metapneumoviruses in birds and humans. *Virus Research,* 91: 163-9. 2003.

87. O'brien, J. D. Swollen head syndrome in broiler breeders. *Veterinary Record,* 117: 619-620. 1985.

88. O'loan, C. J., G. Allan*, et al.* An improved ELISA and serum neutralisation test for the detection of turkey rhinotracheitis virus antibodies. *Journal of Virological Methods,* 25: 271-82. 1989.

89. Olmsted, R. A. e P. L. Collins. The 1A protein of respiratory syncytial virus is an integral membrane protein present as multiple, structurally distinct species. *Journal of Virology,* 63: 2019-29. 1989.

90. Owoade A. A., M. F. Ducatez, *et al.* Avian metapneumovirus subtype A in China and subtypes A and B in Nigeria. *Avian Diseases,* 52:502-6. 2008.

91. Panigrahy, B. S., J. C. Pedersen, *et al.* Experimental and serologic observations on avian pneumovirus (APV/turkey/Colorado/97) infection in turkeys. *Avian Diseases,* 44: 17-22. 2000.

92. Patnayak, D. P., Goyal, S. M. *et al.* Growth of vaccine strains of avian pneumovirus in different cell lines. *Avian Pathology,* 34: 123-6. 2005.

93. Perelman, B., M. Meroz*, et al.* Swollen Head Syndrome in broiler breeders in Israel. *Veterinary Record*, v.123: 444. 1988.

94. Piazza, F. M., S. A. Johnson, *et al.* Bovine respiratory syncytial virus protects cotton rats against human respiratory syncytial virus infection. *Journal of Virology,* 67:1503-1510. 1993.

95. Picault, J. P., P. Giraud*, et al.* Isolation of a TRTV-like virus from chickens with swollen head syndrome. *Veterinary Record*, 121: 135. 1987.

96. Pringle CR. Virus taxonomy - San Diego 1998. *Archives of Virology*, 143(7):1449-1459. 1998.

97. Qingzhong, Y., T. Barrett, *et al.* Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). *Vaccine, 12*: 569-573. 1994.

98. Randhawa, J. S., A. C. Marriott*, et al.* Rescue of synthetic minireplicons establishes the absence of the NS1 and NS2 genes from avian pneumovirus. *Journal of Virology*, 71: 9849-54. 1997.

99. Seal, B. S. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. *Virus Research,* 58: 45-52. 1998.

100. Seal, B. S., H. S. Sellers*, et al.* Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates. *Virus Research,* 66: 139-47. 2000.

101. Shin, H. J., K. V. Nagaraja KV, *et al.* Isolation of avian pneumovirus from mallard ducks that is genetically similar to viruses isolated from neighboring commercial turkeys. *Virus Research*, 26: 207-212. 2002.

102. Schat, K. A., E. Baranowski, *et al.* Animal vaccination and the evolution of viral pathogens. *Revue Scientifique Et Technique,* 26: 327-38. Review. 2007.

103. Schrijver, R. S., J. P. M. Langedijk, *et al.* A bovine respiratory syncytial virus strain with mutations in subgroup-specific antigenic domains of the G protein induces partial heterologous protection in cattle. *Veterinary Microbiology,* 63:159-175. 1998.

104. Stuart, J. C. Rhinotracheitis: turkey rhinotracheitis (TRT) in Great Britain. Recent Advances in Turkey science, *Poultry Science Symposium*. London: Butterworth, 217-224. 1989.

105. Tarpey, I., M. B. Huggins*, et al.* Cloning, expression and immunogenicity of the avian pneumovirus (Colorado isolate) F protein. *Avian Pathology*, 30: 471. 474. 2001.

106. Tarpey, I. e M. B. Huggins. Onset of immunity following *in ovo* delivery of avian metapneumovirus vaccines. *Veterinary Microbiology*, 20: 134-9. 2007.

107. Tiwari, A. P., Y. Chander, *et al.* Permissibility of different cell types for the growth of avian metapneumovirus. *Journal of Virological Methods,* 138: 80-4. 2006.

108. Toquin, D., C. De Boisseson*, et al.* Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. *Journal of General Virology*, 84: 2169-2178. 2003.

109. Toquin D, O. Guionie, *et al.* European and American subgroup C isolates of avian metapneumovirus belong to different genetic lineages. *Virus Genes*. 32: 97-103. 2006.

110. Toquin, D., N. Eterradossi*, et al.* Use of a related ELISA antigen for efficient RT serological testing following live vaccination. *Veterinary Record*, 139: 71-72. 1996.

111. Toro, H., H. Hidalgo*, et al.* Serologic evidence of pneumovirus in Chile. *Avian Diseases*, 42: 815-817. 1998.

112. Turpin E. A., D. E. Stallknecht, *et al.* Evidence of avian metapneumovirus subtype C infection of wild birds in Georgia, South Carolina, Arkansas and Ohio, USA. *Avian Pathology,* 37: 343-351. 2008.

113. Valarcher J. F., F. Schelcher, *et al.* Evolution of bovine respiratory syncytial virus. *Journal of Virology.* 74:10714-28. 2000.

114. Van De Zande, S., H. Nauwynck*, et al.* Comparative pathogenesis of a subtype A with a subtype B avian pneumovirus in turkeys. *Avian Pathology,* 28: 239-244. 1999.

115. Van De Zande*,* S., H. Nauwynck, *et al.* Duration of cross-protection between subtypes A and B avian pneumovirus in turkeys. *Veterinary Record,* 147: 132-134. 2000.

116. Van Der Poel, W. H. M., J. A. Kramps, *et al.* Dynamics of bovine respiratory syncytial virus infections, a longitudinal epidemiological study in dairy herds. *Archive of Virology,* 133:309-321. 1993.

117. Villarreal L. Y. B., Sandri T. L., *et al.* Field observations after natural infection of Brazilian layer chickens with a phylogenetically divergent lineage of subtype B AMPV. Proceedings of the *VI International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens.* Rauischholzhausen, Germany. 2009.

118. Walsh, E. E. e J. Hruska. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. *Journal of Virology*, 47: 171-7. 1983.

119. Worthington K. J., B. A., Sargent, *et al.* Immunity to avian pneumovirus infection in turkeys following in ovo vaccination with an attenuated vaccine. *Vaccine*. 28: 1355-1362. 2003.

120. Wyeth, P. J., R. E. Gough*, et al.* Preliminary observations on a virus associated with turkey rhinotracheitis. *Veterinary Record*, 119: 139. 1986.

121. Wyeth, P. I., N. I. Chettle*, et al.* Antibodies to TRT in chickens with swollen head syndrome. *Veterinary Record*, 120: 286-287. 1987.

Anexo I

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

Comparação entre as técnicas de RT-PCR convencional e RT-PCR em tempo real para a detecção do metapneumovírus aviários subtipo A

Helena Lage Ferreira[I] Fernando Rosado Spilki[II] Márcia Mercês Aparecida Bianchi dos Santos[III] Renata Servan de Almeida[IV] Clarice Weis Arns[III*]

ABSTRACT

Avian metapneumovirus (AMPV) belongs to Metapneumovirus genus of Paramyxoviridae family. Virus isolation, serology, and detection of genomic RNA are used as diagnostic methods for AMPV. The aim of the present study was to compare the detection of six subgroup A AMPV isolates (AMPV/A) viral RNA by using different conventional and real time RT-PCR methods. Two new RT-PCR tests and two real time RT-PCR tests, both detecting fusion (F) gene and nucleocapsid (N) gene were compared with an established test for the attachment (G) gene. All the RT-PCR tested assays were able to detect the AMPV/A. The lower detection limits were observed using the N-, F- based RRT-PCR and F-based conventional RT-PCR ($10^{0.3}$ to 10^{1} $TCID_{50}$ mL^{-1}). The present study suggests that the conventional F-based RT-PCR presented similar detection limit when compared to N- and F-based RRT-PCR and they can be successfully used for AMPV/A detection.

Key words: *avian metapneumovirus, G, F, N genes, real time RT-PCR, RT-PCR.*

RESUMO

O metapneumovírus aviário (AMPV) pertence ao gênero Metapneumovirus, família Paramyxoviridae. Isolamento viral, sorologia e detecção do RNA genômico são atualmente as técnicas utilizadas para o diagnóstico desse agente. O objetivo do presente estudo foi comparar a detecção de RNA viral de seis isolados de AMPV, subtipo A (AMPV/A), utilizando diferentes métodos de RT-PCR convencional e real time RT-PCR (RRT-PCR). Duas novas técnicas de RT-PCR convencional e duas técnicas de RRT-PCR, ambas para a detecção dos genes da nucleoproteína (N) e da proteína de fusão (F), foram comparadas com um RT-PCR previamente estabelecido para a detecção do AMPV (gene da glicoproteína -G). Todos esses métodos foram capazes de detectar os isolados AMPV/A. As técnicas RRT-PCR (genes F e N) mostraram os menores limites de detecção ($10^{0.3}$ to 10^{1} $TCID_{50}$ mL^{-1}). Os resultados sugerem que as técnicas RT-PCR convencional (gene F) e as técnicas de RRT-PCR (gene F e N) desenvolvidas no presente estudo podem ser utilizadas com sucesso para a detecção do AMPV/A. Além disso, o RRT-PCR gera resultados rápidos e sensíveis, o que o torna uma ferramenta alternativa para o isolamento viral.

Palavras-chave: *metapneumovírus aviário, genes G, F, N, real time RT-PCR, RT-PCR.*

INTRODUCTION

The avian metapneumovirus (AMPV), previously called avian pneumovirus (APV) or turkey rhinotracheitis virus (TRTV), is a member of the ***Paramyxoviridae*** family, ***Pneumovirinae*** subfamily, within the new genus ***Metapneumovirus*** (FAUQUET et al., 2005). It contains a non-segmented, negative-sense RNA genome of approximately 13,000nt length. The AMPV genome is composed by eight viral genes arranged in the following order: nucleocapsid–phosphoprotein–matrix–fusion–second matrix–small

hydrophobic–glycoprotein–large polymerase ('3–N–P–M–F–M2–SH–G–L–5') (GOUGH, 2003).

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. AMPV infection is also associated to egg drop in turkeys and ducks (GOUGH, 2003). The virus was first described causing clinical evident disease in South Africa. Nonetheless, major outbreaks of the disease were later reported in Europe, United States (US), United Kingdom, Middle East, Asia, and in other parts of the world (COOK & CAVANAGH, 2002). AMPV is also present in Brazilian flocks since at least 1992 (ARNS & HAFEZ, 1992).

Diagnosis of AMPV infection can be achieved by virus isolation in chicken or turkey tracheal tissue cultures (TOC). Alternatively, it can be obtained from cell cultures (D'ARCE et al., 2005; GIRAUD et al., 1986). Other methods allow the identification and characterization of AMPV, such as immunofluorescence staining or virus neutralization of the isolate with polyclonal or monoclonal antibodies (OTSUKI et al., 1996). Among serological methods, the ELISA (GIRAUD et al., 1986) is the most commonly used. However, serological results are delayed for at least 15 days needed for seroconversion. Molecular methods, such as reverse transcriptase- polymerase chain reaction (RT-PCR), allow the development of rapid, sensitive and specific detection of AMPV (BÄYON-AUBOYER et al., 1999; D'ARCE et al., 2005; DANI et al., 1999; GUIONIE et al., 2007; JUHASZ & EASTON, 1994). Different conventional RT-PCR were already developed by using primers defined either for the detection of all subgroups (BÄYON-AUBOYER et al., 1999; CECCHINATO et al., 2004), or for the specific identification of each of subgroups A–D (BÄYON-AUBOYER et al., 1999). In a recent study, sets of primers targeting attachment (G) gene and small hydrophobic (SH) gene were designed to identify the four AMPV subgroups by real time RT-PCR (RRT-PCR), which also provides the quantification of mRNAs (GUIONIE et al., 2007). Several RRT-PCR assays were also developed for detection of human metapneumovirus (hMPV) targeting fusion (F), nucleoprotein (N), phosphoprotein (P), and polymerase (L) genes (MAERTZDORF et al., 2004; PABBARAJU et al., 2007).

Different target genes can apparently alter the sensibility and specificity of virus detection by conventional (CECCHINATO et al., 2004) and RRT-PCR assays. Primers and probes targeting NS1, NP-1, and VP1 genes of Human bocavirus (HBoV) showed similar sensitivity and specificity in RRT-PCR assays (CHOI et al., 2008). On the other hand, nucleocapsid target genes were found to be consistently more sensitive than the polymerase targets of SARS coronavirus (SARS-CoV) in RRT-PCR tests (KEIGHTLEY et al., 2005). The aim of the present study was to compare the sensitivities and specificities of two newly defined conventional RT-PCR assays, two RRT-PCR tests detecting the F and N genes (FERREIRA et al., 2007), and an established test for the attachment (G) gene (BÄYON-AUBOYER et al., 1999) for detection of AMPV/A isolates.

MATERIALS AND METHODS

Virus strains: in this study, six Brazilian AMPV viruses were propagated in chicken embryo-related cell (CER) cultures. These viruses were isolated from trachea and nasal exudates in CER cells and they were named: chicken/A/BR/119/95, chicken/A/BR/121/95, SHSBR/662/03, SHSBR/668/03, SHSBR/669/03 and TRTBR/169, previously classified as AMPV/A (D'ARCE et al., 2005; DANI et al., 1999).

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 60ng 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

Conventional RT-PCR: two different pairs of AMPV-specific primers targeting the N, F genes were designed based on the conserved regions of the nucleotide sequences available for the F and N genes of AMPV/A to perform the conventional RT-PCR (Table 1). Also, AMPV-specific primers targeting the G gene previous described by BÄYON-AUBOYER et al. (1999) were used to compare the AMPV detection (Table 1). PCR reaction 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 $MgCl_2$, 0.2µM of each primer in a total reaction volume of 25µL containing 1µL of cDNA. Individual PCR amplification cycle of N or F genes was performed with an initial denaturation step at 94°C for 3min, followed by 35 cycles (94°C for 30s; 53°C for 30s; 72°C for 60s), and finally with an elongation step at 72°C for 7min. PCR reaction and amplification cycle of the G gene were performed as previously described (BÄYON-AUBOYER et al., 1999). PCR products (N gene– 698bp; F gene- 698bp; G

Table 1 - Primers and probes for each amplified AMPV/A gene by RT-PCR and RRT-PCR.

Molecular test	Gene	Primers or Taqman ®probes	Positions*	Sequence (5'- 3')	Ref.
RT-PCR	N	Nf	215-235	GCAAAACACACCGACTATGAG	this study
		Nr	892-912	TAGACCTCAGATACTTGCCTC	
Real time RT-PCR	N	AMPVN+494	494-514	CAAAAGCCGTCTGCCTTGGAT	(FERREIRA et al. 2007)
		AMPVN-567	547-567	GAGGCCAACTTGGTGAAAATG	
		AMPVN+516FAMTAMRA	516-545	CTCCCGTTATTCTATTATGCATTGGTGCCC	
RT-PCR	F	Ff	3178-3198	AGGGAGCTCAAAACAGTGTCA	this study
		Fr	3855-3875	CAGTACCACCCTTGATCTTCT	
Real time RT-PCR	F	AMPVF+3643	3643-3663	ATGCCAACTTCATCAGGACAGA	(FERREIRA et al. 2007)
		AMPVF-3721	3700-3721	TCAATATACCAAACCCCTTCCTTCT	
		AMPVF+3667FAMTAMRA	3367-3394	AGTTTGATGTTGAACAATCGTGCCATGGT	
RT-PCR	G	Ga1	5944-5964	CCGGGACAAGTATCYMKATGG	(BÄYON-AUBOYER et al. 1999)
		Gy	6390-6412	TCTCGCTGACAAATTGGTCCTGA	

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

gene- 448bp) were observed in 1% agarose gel electrophoresis, stained with ethidium bromide. Ultra-pure water was used as the negative template control (NTC).

Real time RT-PCR (RRT-PCR): Real-time PCR amplification (RRT-PCR) of N and F genes were performed as previously described (FERREIRA et al., 2007). Primers and Taqman® probes targeting the N and F mRNAs were used (Table 1). Briefly, the Quantitec Probe PCR kit (Qiagen, Hilden, Germany) was used with final concentrations of 900nM of each primer, and 300nM of the Taqman® probe in a total individual reaction volume of 25µL containing 1µL of cDNA (0.2 to 20ng). 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 15sec – 60°C 1min) 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 (Applied Biosystems, Foster City, USA). For absolute quantification, a PCR product containing the target sequence was used as DNA standard. The experiments were repeated three times on different days from the same cDNA stocks. Threshold cycle values (Ct) were used, as Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. In order to convert threshold cycles in copy numbers, 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^{-1} = (Xg μL^{-1} DNA/ [Length of PCR product in base pairs x 660]) x 6.022 x 10^{23}).

Detection Limit: In addition, 10-fold serial dilutions in DMEM of isolates chicken/A/BR/121/95 and SHSBR/669/03 were also extracted and used to evaluate the detection limit of each test. The titers from each isolate were performed in CER cells and calculated by the Reed-Muench method (REED & MUENCH, 1938) and expressed as median 50% tissue culture infectious dose ($TCID_{50}$) per mL of viral suspension.

Specificity: specificity tests were performed from stocks of other RNA viruses, including, infectious bronchitis virus (IBV) and respiratory syncytial virus (hRSV). One strain (STG SHS-1439, AMPV/B) from Germany was included in the analysis. Non-infected supernatants from CER cells were used as negative control.

RESULTS

Conventional RT-PCR: all the six isolates were detected using conventional G, F-, and N-based, RT-PCR (Figure 1A). 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).

RRT-PCR: the N- and F- based RRT-PCR assays were also able to detect all isolates (Table 2). A standard curve for N gene AMPV quantification was

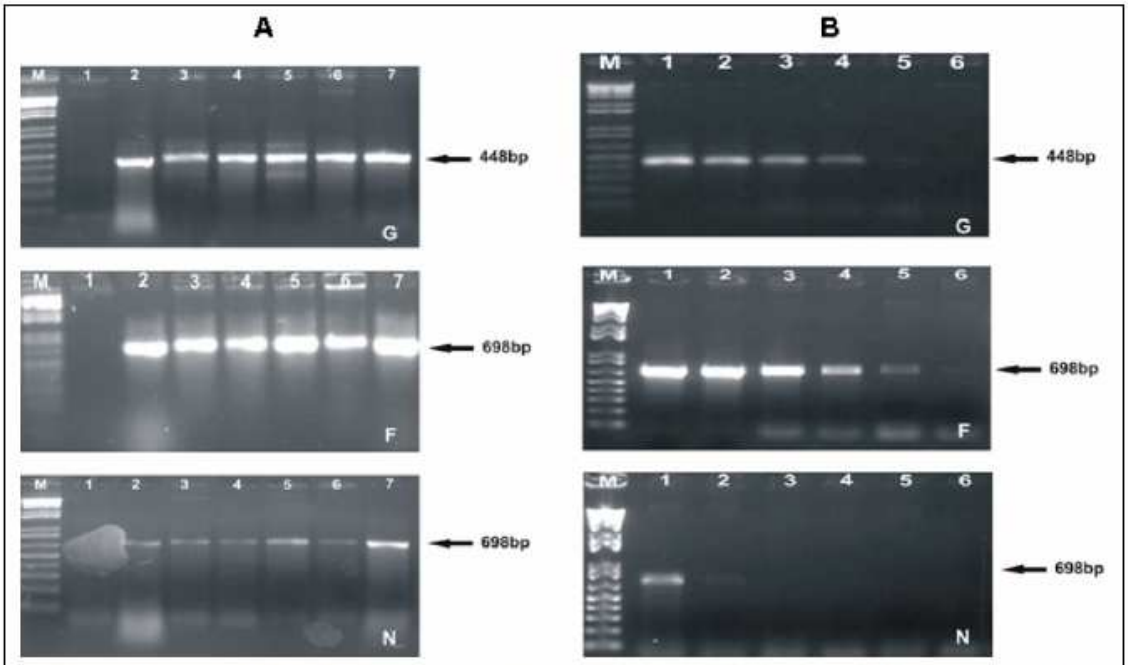


Figure 1 - A) Detection of AMPV/A six isolates by conventional RT-PCR for G, F and N gene. All RT-PCR products had the appropriate size on ethidium bromide stained agarose gels (G=448bp; F= 698bp and N=698bp). M: Leader 1kb plus; 1: negative control; 2: chicken/A/BR/119/95; 3: chicken/A/BR/121/95; 4: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

established using a PCR product containing a target sequence serially diluted from 8 x10^0 to 8 x10^{-7}. 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. For the N- based RRT-PCR, Ct values ranging from 18.39 ±0.434 to 23.70±0.199. The standard curve of F gene AMPV quantification was generated using F target sequence serially diluted from 10^0 to 10^{-8}. RRT-PCR efficiency was 99.95%, slope was -3.3229, a regression coefficient was 0.998116, with an intercept of 49.621. For the F-based RRT-PCR, the tested isolates showed Ct values ranging from 19.69±0.032 to 25.55±0.180.

Detection limit: in order to evaluate the detection limit, eight serial 10-fold dilutions in DMEM were prepared from two different isolates (chicken/A/BR/121/95 and SHSBR/669/03), and RNA was extracted (Table 3). The chicken/A/BR/121/95 titer ranges $10^{5.3}$–$10^{0.3}$ $TCID_{50}$ mL^{-1}, equivalent to $10^{4.3}$–$10^{-1.3}$ $TCID_{50}$ per reaction mix by using N- and F- based RRT-PCR, and F-based conventional RT-PCR. The SHSBR/669/03 titer ranges $10^{6.0}$–$10^{1.0}$ $TCID_{50}$ mL^{-1}, equivalent to $10^{5.0}$–$10^{0.0}$

Table 2 - Ct values and standard deviation of real time RT-PCR (F and N genes) in detecting the AMPV/A isolates.

Isolate	F gene			N gene		
	Ct value	Copy numbers	Std Deviation	Ct value	Copy numbers	Std Deviation
chicken/A/BR/119/95	24.02	1.89 x 10e7	±0.039	23.49	3.03 x 10e6	±0.217
chicken/A/BR/121/95	19.69	6.89 x 10e8	±0.032	18.39	1.40 x 10e8	±0.434
SHSBR/662/03	23.12	3.63 x 10e7	±0.083	22.49	4.67 x 10e6	±0.297
SHSBR/668/03	20.85	1.07 x 10e8	±0.225	21.89	5.49 x 10e6	±0.015
SHSBR/669/03	25.55	6.38 x 106	±0.180	23.70	3.23 x10e6	±0.199
TRTBR/169	20.05	3.46 x 10e8	±0.200	19.97	1.20 x 10e7	±0.298
Negative control	Undetermined	Undetermined		Undetermined	Undetermined	

Table 3 - Comparison of conventional RT-PCR (G, F, and N gene) and real time real time RT-PCR (F and N gene) assays and their detection limits in detecting serially diluted AMPV viral suspensions. Idem 1.

Viruses	$TCID_{50}$ mL^{-1}	G-based RT-PCR	F-based RT-PCR	F-based RRT-PCR	N-based RT-PCR	N-based RRT-PCR
		Length (448bp)	Length (698bp)	Ct^2 value	Length (698bp)	Ct value
chicken/A/BR/121/95	$10^{5.3}$	Positive	Positive	21.57 ±0.051	Positive	21.48 ±0.123
	$10^{4.3}$	Positive	Positive	25.15 ±0.082	Positive	25.02 ±0.075
	$10^{3.3}$	Positive	Positive	27.44 ±0.141	Negative	27.74 ±0.105
	$10^{2.3}$	Positive	Positive	31.59 ±0.165	Negative	32.04 ±0.273
	$10^{1.3}$	Positive	Positive	34.21 ±0.191	Negative	35.79 ±0.189
	$10^{0.3}$	Negative	Positive	38.12 ±0.397	Negative	38.39 ±0.315
	$10^{-1.3}$	Negative	Negative	Undetermined	Negative	Undetermined
	$10^{-2.3}$	Negative	Negative	Undetermined	Negative	Undetermined
SHSBR/669/03	10^{6}	Positive	Positive	19.47 ±0.086	Positive	19.39 ±0.126
	10^{5}	Positive	Positive	23.05 ±0.168	Positive	22.93 ±0.170
	10^{4}	Positive	Positive	26.77 ±0.154	Negative	27.18 ±0.153
	10^{3}	Positive	Positive	29.69 ±0.263	Negative	30.26 ±0.015
	10^{2}	Positive	Positive	32.65 ±0.082	Negative	33.17 ±0.436
	10^{1}	Negative	Positive	37.65 ±0.220	Negative	38.19 ±0.616
	10^{0}	Negative	Negative	Undetermined	Negative	Undetermined
	10^{-1}	Negative	Negative			

$TCID_{50}$ per reaction mix by using N- and F-based RRT-PCR, and F-based conventional RT-PCR. The N-based conventional RT-PCR presented detection limit of $10^{4.3}$ and $10^{5.0}$ $TCID_{50}$ mL^{-1} from chicken/A/BR/121/95 and SHSBR/669/03 isolates, respectively (Figure 1 B). The G-based conventional RT-PCR showed detection limit of two isolates ranging to $10^{1.3}$ to $10^{2.0}$ $TCID_{50}$ mL^{-1}. The best detection limits were obtained by using N-, F-based RRT-PCR and F-based conventional RT-PCR assays, which could detected detection limits ranging from $10^{0.3}$ to 10^{1} $TCID_{50}$ mL^{-1} of both isolates (Table 3). Our group was able to recover virus titers up to $10^{4.55}$ $TCID_{50}$ mL^{-1} at 5dpi from oral swabs, after experimental infection with $10^{5}TCID_{50}$ mL^{-1} AMPV/A and AMPV/B in chickens (unpublished data). This suggests that evaluated RT-PCR and RRT-PCR assays could be used for AMPV detection and quantification in experimental studies.

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, as no amplifications was detected for other RNA viruses. No specific band was visualized by N- and F- based conventional RT-PCR tests and Ct values were undetermined by N- and F-based RRT-PCR assays). The conventional RT-PCR for the G gene could detect AMPV/A and AMPV/B.

DISCUSSION

BÄYON-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 are in agreement with these authors because the G-based RT-PCR was able to detect the AMPV subtypes A and B. The conventional F-based RT-PCR and the RRT-PCR tested assays could specifically detect AMPV/A. BÄYON-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.

Interestingly, it is important to note that the detection limit of F-based conventional RT-PCR sustains comparison with RRT-PCR tested assays detection limits (detection of $10^{0.3}$.to 10^{1} $TCID_{50}$ mL^{-1}). This fact could be explained by the presence of a pyrimidine residue at their 3' end in primers AMPV-specific targeting the F gene. This parameter was suggested to increase the sensitivity in some PCR primers designed to detect an AMPV/A cloned F gene (CECCHINATO et al., 2004). The sensitivity of the N- and F-based RRT-PCR seemed to be lower than the recently reported G-based RRT-PCR for AMPV/A detection ($10^{-1.5}$ $TCID_{50}$ mL^{-1}; GUIONIE et al., 2007). Nonetheless, a previous study also described that the

N-based RT-PCR was more sensitive than other tests targeting different genes (MAERTZDORF et al., 2004). We could expect this due to 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 non-segmented negative-strand RNA viruses (BARIK, 1992). The N gene is the promoter closest gene, thus, the transcription process produces more N mRNA than G genes. Surprisingly, conventional N-based RT-PCR had the highest detection limit when compared with conventional F- and G- based RT-PCR assays for AMPV detection. The absence of a pyrimidine residue at their 3'in the primers AMPV-specific targeting the N gene can play on the sensitivity of conventional RT-PCR assays. On the other hand, the primers of tested RRT-PCR assays do not contain this parameter and no difference in the sensitivity was observed when compared N- and F- based RRT-PCR. The impact of pyrimidine residue at their 3' in the primers for RRT-PCR assays should be further investigated.

In addition, some positive signals can be detected due to non-specific amplification and/or probe disruption at the end of the amplification process in absence of target cDNA (LOISY et al., 2005). We considered thus that C values higher than 39 may indicate either a problematic sample, or RNA purification, or RRT-PCR reaction.

CONCLUSION

The present study shows that the conventional F-based RT-PCR presented similar sensitivity when compared to N- and F-based RRT-PCR and they can be successfully used for AMPV/A detection. Nonetheless, they should be used in association with conventional G-based RT-PCR for AMPV diagnosis, because it also detects N and D AMPV subgroups. The conventional F-based RT-PCR could also provide further nucleotide sequencing, which allows phylogenetic studies on the detected isolates. On the other hand, RRT-PCR assays can offer targeted mRNA detection, generating quantitative data. Although the RRT-PCR assays remains to be evaluated with field samples and it would be useful to virus shedding quantification in vaccine studies.

ACKNOWLEDGEMENTS

We are thankful to Geneci F. Davi and Paula S. Porto for their excellent technical assistance, Steven Van Borm and Tiago C. Pereira for providing sequence information for primer and probes synthesis, Prof Hafez M Hafez for providing the German strain (STG SHS1439). This work was supported by FAPESP grant number 03/14012-9.

REFERENCES

ARNS, C.W.; HAFEZ, M.H. Swollen head syndrome in poultry flocks in Brazil. In: WESTERN POULTRY DISEASE CONFERENCE, 41., 1992, Sacramento, USA. **Proceedings...** Davis, CA: Conference & Event Services, University of California, 1992. p.81-84.

BARIK, S. Transcription of human respiratory syncytial virus genome RNA in vitro: requirement of cellular factor(s). **Journal of Virology**, v.66, p.6813-6818, 1992.

BÄYON-AUBOYER, M.H. et al. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. **Archives of Virology**, v.144, n.6, p.1091-1109, 1999. Disponível em: <http://www.springerlink.com/content/vxcm8vulvfx3vmeh>. Doi: 10.1007/s007050050572.

CECCHINATO, M. et al. Design, validation, and absolute sensitivity of a novel test for the molecular detection of avian pneumovirus. **Journal of Veterinary Diagnostic Investigation**, v.16, n.6, p.582-585, 2004.

CHOI, J.H. et al. Development of real-time PCR assays for detection and quantification of human bocavirus. **Journal of Clinical Microbiology**, v.42, n.3, p.249-253, 2008. Disponível em: <http://www.journalofclinicalvirology.com/article/S1386-6532(08)00070-X>. Doi: 10.1016/j.jcv.2008.02.010.

COOK, J.K.; CAVANAGH, D. Detection and differentiation of avian pneumoviruses (metapneumoviruses). **Avian Pathology**, v.31, n.2, p.117-132, 2002.

D'ARCE, R.C. et al. Subtyping of new Brazilian avian metapneumovirus isolates from chickens and turkeys by reverse transcriptase-nested-polymerase chain reaction. **Avian**

DANI, M.A. et al. Molecular characterization of Brazilian avian pneumovirus isolates: comparison between immunochemiluminescent Southern blot and nested PCR. **Journal of Virological Methods**, v.79, n.2, p.237-241, 1999. Disponível em: <http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6T96-3WJDTS6-D-7&_cdi=5106&_user=687304&_orig=search&_coverDate=05%2F31%2F1999&_sk=999209997&view=c&wchp=dGLbVlW-zSkzS&md5=6b42016b9f823422152fecdd9a0d7060&ie=>. Doi: 10.1016/S0166-0934(99)00020-8.

FAUQUET, C.M. et al. **Virus taxonomy**: VIIIth Report of the International Committee on Taxonomy of Viruses. Amsterdam: Elsevier Academic, 2005. 1162p.

FERREIRA, H.L. et al. Inhibition of avian metapneumovirus (AMPV) replication by RNA interference targeting nucleoprotein gene (N) in cultured cells. **Antiviral Research**, v.74, n.1, p.77-81, 2007. Disponível em : <http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6T2H-4MR1GV1-1-1&_cdi=4919&_user=10&_orig=search&_coverDate=04%2F30%2F2007&_sk=999259998&view=c&wchp=dGLzVtb-zSkWb&md5=a1d63aa0ca8ae7b4fa06307793e8edc5&ie=>. Doi: 10.1016/j.antiviral.2006.12.002.

GIRAUD, P. et al. Turkey rhinotracheitis in France: preliminary investigations on a ciliostatic virus. **Veterinary Record**, v.119, n.24, p.606-607, 1986.

GOUGH, R.E. Avian pneumoviruses. In: SAIF, M. et al. **Diseases of poultry**. Ames: Iowa State, 2003. p.92-99.

GUIONIE, O. et al. Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. **Journal of Virological Methods**, v.139, n.2, p.150-158, 2007. Disponível em : <http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6T96-4MFJJ12-1-3&_cdi=5106&_user=10&_orig=search&_coverDate=02%2F28%2F2007&_sk=998609997&view=c&wchp=dGLzVtz-zSkWz&md5=70a2de1148e26bf618e402c54144d25e&ie=>. Doi: 10.1016/j.jviromet.2006.09.022.

JUHASZ, K.; EASTON, A.J. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. **Journal of General Virology**, v.75 (Pt 11), p.2873-2880, 1994. Disponível em : <http://vir.sgmjournals.org/cgi/reprint/75/11/2873>. Doi: 10.1099/0022-1317-75-11-2873.

KEIGHTLEY, M.C. et al. Real-time NASBA detection of SARS-associated coronavirus and comparison with real-time reverse transcription-PCR. **Journal of Medical Virology**, v.77, n.4, p.602-608, 2005. Disponível em: <http://www3.interscience.wiley.com/journal/112137449>. Doi: 10.1002/jmv.20498.

LOISY, F. et al. Real-time RT-PCR for norovirus screening in shellfish. **Journal of Virological Methods**, v.123, n.1, p.1-7, 2005.

MAERTZDORF, J. et al. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. **Journal of Clinical Microbiology**, v.42, p.981-986, 2004. Disponível em : <http://jcm.asm.org/cgi/reprint/42/3/981>. Doi: 10.1128/JCM.42.3.981-986.2004.

OTSUKI, K. et al. Demonstration of serum-neutralising antibody to turkey rhinotracheitis virus in serum from chicken flocks in Japan. **Journal of Veterinary Medical Science**, v.58, n.9, p.869-874, 1996.

PABBARAJU, K. et al. Diagnosis and epidemiological studies of human metapneumovirus using real-time PCR. Journal of Clinical Virology, v.40, n.3, p.186-192, 2007. Disponível em: <http://www.journalofclinicalvirology.com/article/PIIS1386653207002740>. Doi: 10.1016/j.jcv.2007.08.004.

REED, J.I.; MUE http://www.journalofclinicalvirology.com 02740
fifth percent endpoints. **American Journal Hygiene**, v.27, p.493-497, 1938.