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Coronavírus e Metapneumovírus Aviários em Aves Domésticas e Silvestres do Brasil: Caracterização Molecular e Filogenética.

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Orientadora: Profa. Dra. Clarice Weis Arns

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SUMÁRIO

Lista de Siglas e Abreviaturas

SIDA: Síndrome da imunodeficiência adquirida.

aMPV (avian metapneumovirus): Metapneumovírus Aviário.

BIG: bronquite infecciosa das galinhas.

ELISA (enzyme linked immuno sorbent assay): ensaio imunoenzimático.

hMPV (human metapneumovirus): Metapneumovírus Humano.

IgA: Imunoglobulina do isotipo A.

IgY: Imunoglobulina do isotipo Y.

IgM: Imunoglobulina do isotipo M.

ORFs (open reading frame): Região de leitura de frames.

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

PhCoV (Pheasant Coronavirus): Coronavírus dos faisões.

PIB: (GDB – gross domestic product): Produto interno bruto.

RER: Retículo endoplasmático rugoso.

RNA: Ácido ribonucleico.

RNAm: Ácido ribonucleico mensageiro.

RNAi (RNA interference): Interferência por RNA.

RSV (respiratory syncytial virus): vírus respiratório sincicial.

RT (reverse transcriptase): Transcriptase reversa.

SARS: Síndrome aguda respiratória sistêmica.

SCI: Síndrome da cabeça inchada.

siRNA (short interfering RNA): RNA pequeno interferente

SN: Soroneutralização.

TOC (tracheal organ culture): Cultivo de anel traqueal.

TCoV (turkey coronavirus): Coronavírus dos perus.

TRT (turkey rhinotracheitis): Rinotraqueíte em perus.

UTR (untranslated region): região não traduzida.

VBI: vírus da bronquite infecciosa.

bRSV: (bovine respiratory syncycial virus) Vírus Respiratório Sincicial Bovino.

hRSV: (human respiratory syncycial vírus) Vírus Respiratório Sincicial Humano.

1. **Resumo:**

O Brasil figura entre os maiores produtores e exportadores do mundo de carne de frango e ovos, o que confere a avicultura a capacidade de interferir na construção do PIB nacional. Estes índices envolvem a necessidade de uma alta produtividade e consequentemente um manejo zoosanitário suficiente para atender a economicidade do setor. Este estudo focou dois gêneros virais de importância na avicultura, o *Coronavirus* e o *Metapneumovirus*, objetivando verificar a presença de vírus em aves silvestres e sinantrópicas da avifauna brasileira, visando compreender melhor a eco-epizootiologia das respectivas morbidades de que são agentes etiológicos. Para tanto se coletou 134 amostras através de swabs traqueais e cloacais de 53 aves silvestres brasileiras de 20 espécies diferentes e de 14 pombas (*Columba livia*), que não possuíam sinais de doença. Além destas aves, foi também objeto de estudo amostras de galinhas e frangos comerciais oriundos de vários estados da federação nos anos de 2003 a 2009, com sintomas respiratórios. O material genético dos vírus encontrados foi extraído e submetido à reação de *nested* RT-PCR, destinado a amplificar a região hipervariável do gene que codifica a proteína S_1 dos coronavírus e o gene que codifica a proteína G dos metapneumovírus. Os amplificados foram então sequenciados e analisados construindo-se árvores filogenéticas e matrizes de similaridade através de recursos de bioinformática. Foi possível recuperar seis (06) amostras de coronavírus de *Columba livia* e vinte e três (23) de galinhas domésticas. No estudo filogenético se observou dois grandes clusteres; um agrupando-se com o genótipo Massachusetts e outro com D207; das amostras de pomba 5 agruparam-se com Massachusetts; 1 amostra de galinha e uma de pomba agruparam com o genótipo Connecticut e 1 de galinha com o Arkansas. As amostras oriundas de pomba e as de galinha compartilharam uma similaridade que chegou a 100%, para algumas delas. Quanto aos metapneumovírus recuperou-se vírus de 7 aves silvestres, 7 de pombas e 15 de galinhas domésticas, sendo que estas amostras agruparam-se em dois grandes clusteres, um com o subtipo A (5 de aves silvestres, 2 de pombas e 1 de galinha) e outro com o subtipo B (2 de aves silvestres, 5 de pombas e 14 de galinha). A identidade genética entre os amplificados de galinha e de aves não domésticas chegou a 100%. Tanto para os coronavirus como para os metapneumovirus o isolamento de vírus com um de seus genes mais variáveis apresentando até 100% de identidade com os das galinhas domésticas, pode representar uma

possível recuperação dos vírus atenuados utilizados nas vacinações de rotina, ou mesmo a passagem dos vírus patogênicos para estes animais, fazendo com que estes mantenham o agente no ambiente. Aves de diferentes espécies podem ainda funcionar enquanto um ambiente seletivo para estes vírus, provocando a fixação de novos genótipos virais, o que seria uma provável explicação para o fato destes agravos continuarem a ocorrer em granjas de todo o mundo em detrimento da existência de vacinas, além da possibilidade de surgimento de vírus recombinantes capazes de infectar humanos, como é o caso da SARS.

Abstract:

Avian Coronavirus and Metapneumovirus in Brazilian Domestic and Wild Birds: Molecular Characterization and Phylogenetic.

Brazil is among the largest producers and exporters of poultry meat and eggs in the world, which gives the poultry industry the capacity to interfere in the construction of national GDB. These indexes involve the need of a high productivity and consequently a sufficient zoosanitary management for the economy of the sector. This study focused on two important viral genera for this, *Coronavirus* and the *Metapneumovirus*, to verify the presence of virions in wild birds and synanthropic ones of Brazil, seeking a better understanding of those diseases and your ecoepizootiology. For that, were collected one hundred thirty-four samples (tracheal and cloacal swabs) from fifty three Brazilian wild birds of twenty different species and fourteen from pigeons (*Columba livia*), without symptoms, and samples of chickens from several states of the federation in the years from 2003 to 2009, with swollen head syndrome. The virus genetic material found was subjected to the reaction of nested RT-PCR, to amplify the hypervariable region of the gene encoding the coronavirus S_1 subunit of the S protein and the gene encoding the G protein of metapneumovirus. The amplified were then sequenced and analyzed by constructing phylogenetic trees and similarity matrices using bioinformatics resources. Were isolated six (6) coronavirus from *Columba livia* and twenty three (23) from domestic fowl. In the phylogenetic analysis, two major clusters were observed one grouped with Massachusetts genotype and the other with D207; samples of five pigeons grouped with Massachusetts as well, one chicken sample and one from pigeon were grouped with genotype Connecticut and other from chicken with Arkansas genotype. The samples from pigeons and chickens shared a similarity which reached 100%. As for metapneumovirus, virus was recovered from seven wild birds, seven pigeons and fifteen from chickens. These samples were grouped into two major clusters; with subtype A (five from wild birds, two from pigeons and one from chicken) and with subtype B (two wild birds, 5 pigeons and 14 chickens). The genetic similarity between chicken amplicons to non domestic birds reached 100%. For both coronavirus and metapneumovirus, virus isolation from non domestic birds and their great similarity (reached 100%) with those

from chickens, may represent a possible recovery of the attenuated virus used in routine vaccinations, or even the passage of pathogenic viruses for these animals, causing them to keep the agent in the environment. Birds of different species can also function as a selective environment for these viruses, leading to the establishment of new viral genotypes, which would be a likely explanation for the fact that these injuries continue to occur on farms around the world, even with the use of vaccines, besides the possibility of emergence of recombinant viruses capable of infecting humans, as is the case with SARS.

2. Introdução:

2.1 – Avicultura no Brasil:

A importância econômica e social da avicultura brasileira coloca o setor em evidência no âmbito nacional e internacional, visto que o Brasil tem alcançado frequentemente o posto de maior exportador de carne de frango em receita cambial. Somando-se as produções, norte-americana e a brasileira, suplementadas ainda pela produção de alguns outros países latino-americanos, como o México, o continente americano concentra atualmente cerca de metade da produção mundial (UBA, 2010).

No ano de 2009, em detrimento da crise internacional, o Brasil produziu 7.297 milhões de toneladas de carne de frango, sendo que 66,7% foram destinadas ao mercado interno, e o restante foi exportado, influenciando a construção do PIB brasileiro. O estado da federação com maior produção foi o Paraná, seguido por Santa Catarina, São Paulo e Rio Grande do Sul. Já a produção de ovos foi igualmente importante atingindo a marca de 61,6 milhões de caixas de 30 dúzias em 2009, sendo que 52% foram destinados ao consumo interno. Neste cenário a região sudeste aparece como a mais importante produtora de ovos, seguida pela sul, nordeste e centro-oeste (UBA, 2010).

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

2.2 - Bronquite infecciosa das galinhas.

A bronquite infecciosa das galinhas (BIG) representa um dos agravos à saúde de difícil controle e mais comuns nas aves em criações comerciais, em todo mundo (Gelb et al., 2005). Trata-se de enfermidade contagiosa podendo atingir, rapidamente, uma grande quantidade de animais e nestes, uma infinidade de órgãos de origem ectodérmica, assumindo diversas formas clínicas, de acordo com os tecidos acometidos. Os sinais clínicos mais comumente observados estão relacionados ao aumento das secreções nasal e ocular, quantidade aumentada de muco na traquéia, diminuição no ganho de peso e na conversão alimentar, diarreia, espirros, alterações nos ovos. Além das lesões diretamente causadas pelo vírus; proporcionam, ainda, uma porta de entrada para outros agentes microbianos (Matthijs et al., 2003). Normalmente a letalidade é baixa, porém em aves jovens, ocasionalmente atinge índices preocupantes, além de poder causar baixa na produtividade sem que os animais apresentem sinais sensíveis à acuidade clínica convencional (Cavanagh, Naqi, 2003; Cavanagh, 2005; Ignjatovic et al., 2006; Cavanagh, 2007).

Ao exame *post mortem* os achados macroscópicos mais frequentes são a presença de exsudato seroso, catarral ou caseoso na traquéia, fossas nasais, brônquios e, eventualmente sacos aéreos. Em aves de postura, o rompimento dos ovos em formação no interior do oviduto pode provocar o aparecimento de seu conteúdo na cavidade celomática. Quando a cepa possui nefrotropismo, os rins podem apresentar-se pálidos e edemaciados, com deposição de uratos nos ureteres (Lovato, Dezengrini, 2007).

O agente etiológico desta enfermidade foi o primeiro da família *Coronaviridae* isolado (1934) sendo, portanto, o protótipo para estudo desta família que possui dois gêneros: *Coronavirus* e o *Torovirus*. Possuem o maior genoma conhecido dentre os vírus RNA e seu nome é dado devido à presença de espículas no envelope viral, dando aspecto de coroa (figura 1). Replicam-se por um processo que envolve a produção de RNAs mensageiros subgenômicos, o que, associado aos "erros" cometidos pela RNA-polimerase, contribui para uma alta freqüência de recombinações e mutações, resultando numa grande variedade genômica, antigênica e consequentemente de sorotipos (Bochkov et al., 2007; Cavanagh, 2005; Ignjatovic et al., 2006; Cavanagh, 2007; Lee, Jacwood, 2000; Lovato, Dezengrini, 2007).

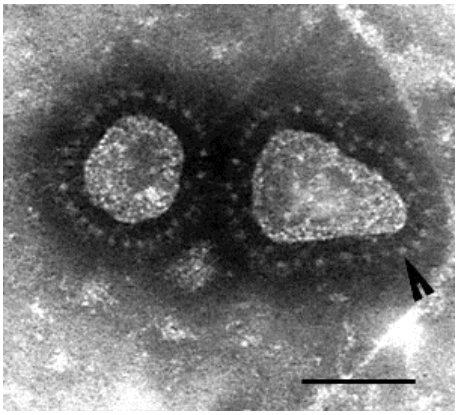

Figura 1: Eletro microfotografia de coronavírus, demonstrando o aspecto de coroa.

Fonte: http://www.vet.uga.edu/vpp/archives/ivcvm/1998/steffens/index.php

O vírus da bronquite infecciosa (VBI) é envelopado com um genoma composto por fita simples de RNA de sentido positivo, simetria helicoidal, apresenta forma que vai de esférica a pleomórfica, sendo que seu envelope tem aproximadamente 120 nm de diâmetro, com projeções glicoproteicas (espículas) de 20 nm. O genoma possui 27.600 nucleotídeos, e codifica três proteínas estruturais majoritárias: a glicoproteína de superfície (S - espícula), a proteína de membrana (M), a do nucleocapsídeo (N) e uma quarta estrutural denominada pequena proteína de membrana (E), que está associada com o envelope viral em pequenas quantidades, sendo essencial para a formação do vírion (figura 2). O gene do complexo replicase, situado próximo a extremidade 5', ocupa aproximadamente dois terços do genoma (Cavanagh, 2005; Cavanagh, 2007; Lovato, Dezengrini, 2007).

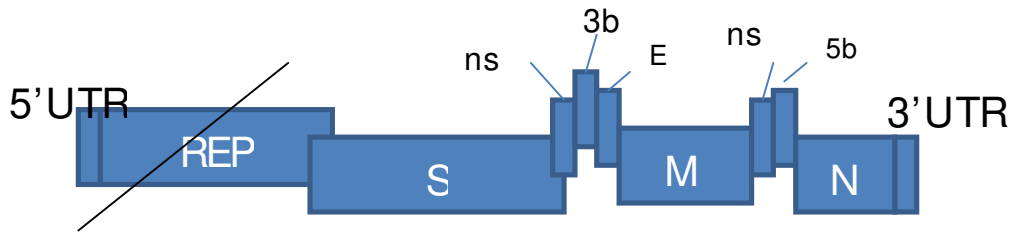

Figura 2: Organização genômica do VBI: UTR 5'e 3' – regiões não codantes; Rep – gene da RNA polimerase; S,M, N e E – genes das proteínas estruturais; ns 3a, 3b, 5a e 5b proteínas não estruturais.

A replicação do VBI ocorre inicialmente nas células ciliadas da mucosa das vias aéreas superiores e em células secretoras de muco, porém na continuidade do processo infeccioso, outras células epiteliais em outros órgãos poderão ser infectadas (Dhinakar-Raj, Jones, 1997). Após a adesão e fusão do vírion a membrana das células alvo, através da proteína S, a replicação ocorre inteiramente no citoplasma. Nesse processo, o RNA genômico se liga aos ribossomos para sintetizar a enzima RNA polimerase-RNA dependente, formando uma fita de sentido negativo de RNA. Esta serve como molde para a síntese de um novo RNA genômico e dos RNAs mensageiros subgenômicos. Estes são produzidos por um mecanismo de transcrição descontínuo, passando a funcionar como *replicons* e podendo gerar genomas recombinantes, quando da co-infecção celular por outro Coronavírus (Lai, Cavanagh, 1997).

A tradução de cada RNAm vírus específico produz apenas um peptídeo sendo que a proteína de nucleocapsídeo (N) e as proteínas não estruturais são sintetizadas em polissomos na matriz citoplasmática e as glicoproteínas S e M em polissomos ligados ao retículo endoplasmático rugoso (RER). A glicoproteína S é inserida co-traducionalmente para o interior das membranas do RER e aí ocorre a glicosilação na cadeia crescente de polipeptídeo. A proteína S é então transportada do complexo de Golgi para a membrana citoplamática e cerca de dois a três glico-peptídeos S constituem a espícula (Tomley et al., 1987).

A glicoproteína S possui uma massa molecular de 180 Kd, está ancorada no capsídeo viral e possui três domínios; um externo (maior), um transmembrana e um pequeno interno. Já a M está presente em várias cópias e possui um pequeno domínio externo (apenas 10% está exposto), um com três passagens através da membrana e um grande domínio interno. Esta interage com o nucleocapsídeo, atua na morfogênese e brotamento dos vírions e forma o revestimento do núcleo (core). A pequena proteína E também está envolvida na morfogênese viral e a N está ligada ao RNA por intermédio de um de seus domínios e também se associa a M na morfogênese viral (Cavanagh, 2007; Lovato, Dezengrini, 2007) (figura 3).

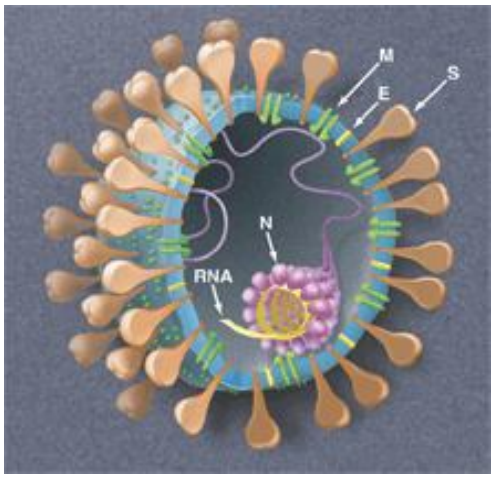

Figura 3: Esquema tridimensional do VBI mostrando aspectos de seu genoma e de suas proteínas estruturais.

Fonte: http://www.sciencemag.org/cgi/content/summary/300/5624/1377

A proteína da espícula (S) é clivada posteriormente a sua transcrição, dando origem aos fragmentos S_1 (cerca de 500 aminoácidos) e S2 (600 aminoácidos); o primeiro é mais externo enquanto o segundo ancora a glicoproteína ao capsídeo viral. Sem dúvida a glicoproteína S é uma das mais estudadas nesta família, principalmente devido ao variado papel que assume; sendo a

responsável tanto pela adesão do vírion aos receptores da célula alvo, quanto pela sua fusão à membrana celular. Fornece ainda, uma importante característica fenotípica ao vírion, uma vez que os anticorpos neutralizantes são baseados na estrutura do fragmento S_1, sendo que alterações de apenas 2 a 3% na composição de seus aminoácidos podem determinar o surgimento de um novo sorotipo viral. A S_1 representa a parte da partícula viral mais predisposta às pressões seletivas conferidas pelo sistema imune dos hospedeiros e consequentemente o gene que a codifica possui uma das maiores taxas de evolução (Cavanagh, et al., 1988; Cavanagh, et al, 1992; Cavanagh, 2006; Mckinley et al., 2008).

Amostras de campo recuperadas acusam a existência de uma mistura de variantes genéticas (*quasiespecies*), sendo que algumas destas podem ser fixadas pelo processo de seleção natural dando origem a novos genótipos, ou mesmo sorotipos virais (Lee et al., 2001; Jackwood et al., 2003; Mckinley, 2008). As características industriais da produção avícola, com uma grande quantidade de aves alojadas em um mesmo espaço, além da co-circulação de mais de um sorotipo, sem dúvida também tem um papel importante no aparecimento e fixação de mutações do VBI (Capua et al., 1999; Cavanagh et al., 1999; Schikora et al., 2003; Jackwood et al.,2005, Montassier et al., 2008).

A base da imunidade das aves ao VBI ainda não é muito bem entendida, uma vez que os níveis de anticorpos séricos reativos com antígenos virais não estão necessariamente relacionados com proteção. Após a apresentação do antígeno vacinal à ave a resposta é a padrão, com um pico inicial de IgM diminuindo após o aumento das IgG. No segundo desafio o isotipo IgM tem um pico ao mesmo tempo que a IgG, porém declina mais rapidamente. De outro modo, o aparecimento de células T citotóxicas está relacionado com a diminuição da infecção e da sintomatologia clínica. A transferência destas células, a partir de animais infectados, parece proteger a ave receptora em exposições futuras ao VBI. Outra resposta estudada é aquela relacionada à produção do Interferon I, uma vez que trabalhos demonstram que este reduz a replicação viral em culturas de células. A aplicação em galinhas, apesar de apresentarem resultados controversos, parece diminuir a severidade da doença (Mocket, Cook, 1986; Martins et al., 1991; Collisson et al., 2000; Pei et al., 2001; Cavanagh, 2007).

A utilização de vacinas com vírus atenuados é prática corriqueira na indústria avícola mundial e sem dúvidas, as mais empregadas são aquelas compostas pelo sorotipo Massachusetts. Normalmente estes imunobiológicos promovem uma proteção aos sorotipos homólogos mas não aos heterólogos, como é o caso dos novos sorotipos que são freqüentemente descritos na literatura especializada. Outro problema é o da contaminação ambiental por vírus atenuados, que torna difícil a discriminação entre cepas vacinais e cepas de campo, além de poder ser recuperado em outras aves, sinantrópicas e/ou silvestres. Assim sendo a eco-epidemiologia do agente etiológico se torna muito mais complexa, uma vez que pode evoluir em diferentes espécies de aves, podendo, potencialmente gerar um maior número de variantes genéticas e sorológicas (Cavanagh et al., 1997; Meulemans et al., 2001; Farsang et al. 2002, Cavanagh, 2005). Segundo Toro et al (2006) um outro fator importante a ser considerado nas falhas de vacinação é o co-infecção com patógenos que levam as aves a imunodeficiência, como o caso do vírus da anemia das galinhas e o da doença de gumboro.

Em se tratando de aves domésticas em sistema de criação comercial as infecções por coronavírus não são uma exclusividade das galinhas. Num passado recente foi descoberto nos Estados Unidos, em perus, um membro deste grupo causando gastroenterite, além de queda de produtividade, que foi denominado de *turkey* coronavirus (TCoV). Posteriormente este foi encontrado no Reino Unido, no Brasil e na Itália. Estudos demonstraram que o TCoV é geneticamente e antigenicamente relacionado ao VBI (Fabricant, 1998; Cavanagh et al., 2001; Guy, 2000, 2003;). Da mesma forma, um vírus associado a doenças respiratórias e renais, foi isolado de faisões (PhCoV – *Pheasant* Coronavirus) e o seu nível de similaridade com o VBI é parecido ao encontrado com o TCoV, com exceção a similaridade da proteína S, que entre PhCoV e o VBI é maior. A diferença genética entre o PhCoV e o VBI é próxima àquela observada dentre os sorotipos do vírus da bronquite infecciosa (Cavanagh et al., 2002; Cavanagh, 2005). A inoculação do TCoV em galinhas, apesar da replicação no trato gastrointestinal não provocou sintomatologia alguma; da mesma forma com o PhCoV (Ismail et al., 2003). Segundo Cavanagh (2005) isto poderia ser devido ao pequeno número cepas até então testadas em laboratório e que nas condições de campo, outros fatores poderiam propiciar uma doença em galinhas por estes novos vírus.

A literatura internacional nos mostra que uma série de novos vírus, semelhantes geneticamente ao VBI, foi encontrada em diferentes espécies de aves no mundo. As evidências nos

conduzem a acreditar que o VBI pode ter uma infinidade de outros hospedeiros, que não os galiformes domésticos, e que sua eco-epidemiologia pode ser muito mais complexo do que se imagina. Pesquisadores chineses sequenciaram o genoma completo de vírus recuperados do pavão doméstico (*Pavo cristatus*), da perdiz (*Alectoris* sp.), da galinha d'Angola (*Numibia meleagris*) e de anatídeos (*Anas* sp) e todos apresentaram uma organização genômica igual a do VBI, além disto, alguns coronavírus recuperados de pavão apresentaram 99% de identidade com a cepa vacinal H 120 (Cavanagh, 2005).

Cepas virais antigenicamente relacionadas com o VBI, isoladas de galinha d'Angola ao serem inoculadas nestas primeiras e em frangos fizeram com que ambos apresentassem sinais respiratórios e fezes aquosas. Soros obtidos de galinhas de postura (várias vezes vacinadas contra o VBI) neutralizaram o vírus obtido das galinhas d'Angola mostrando uma estreita relação fenotípica entre este e o VBI (Ito et al., 1991; Cavanagh, 2005). Isolados de patos criados em quintais na China mostraram aproximadamente 90% de identidade com vários sorotipos de VBI, inclusive cepas nefropatogênicas. Estes quando inoculados em galinha causaram doença inclusive com o envolvimento renal (Liu et al, 2005; Cavanagh, 2005). De forma semelhante, como o descrito acima vírus recuperados de pombos na Austrália, causaram lesões características do VBI, quando inoculados em ovos embrionados e foram neutralizados por soros de galinhas vacinadas (com um sorotipo australiano de VBI). Quando este mesmo vírus foi inoculado em pombos e galinhas, apenas estas últimas manifestaram sintomatologia compatível com a bronquite infecciosa das galinhas (Barr et al., 1988).

Jonassen et al. (2005), por outro lado, mostraram que coronavírus recuperados de 2 espécies de anatídeos (*Anser anser* e *Anas platythynchos*) e de pombas (*Columbia livia*) apesar de pertencerem ao grupo 3 apresentaram diferenças importantes na sequência de nucleotídeos entre si e entre os outros vírus deste grupo conhecido. Alguns apresentavam mais genes e outros insertos na região hipervariável da UTR 3', porém nenhum deles foi recuperado com sucesso após a inoculação na membrana corioalantóidea de ovos embrionados de galinha. Gough et al. (2006) isolaram um vírus do papagaio amazônico da cara verde (*Amazon viridigenalis cassin*) a partir de cultura de células primárias que apresentava fenótipo compatível aos coronavírus, o que foi confirmado pela utilização de um "pan-coronavírus" RT-PCR, que amplificou parte do gene que codifica a proteína não-estrutural RNA-dependente RNA polimerase. A sequência obtida de 66 aminoácidos

apresentou 66 a 74% de similaridade com as sequências dos coronavírus dos Grupos 1, 2 e 3. Porém, muitos outros pares de primers que produzem produtos na reação de PCR correspondendo aos genes 3, 5, N e região 3`UTR do VBI, TCoV e PhCoV, não amplificaram nenhum dos genes estudados, sugerindo haver um novo coronavírus, sendo pela primeira vez descrito nesta espécie de psitacídeo. Estes achados reabrem a discussão sobre a diversidade dos coronavírus pertencentes ao Grupo 3 e a sua real importância no entendimento da epizootiologia da bronquite infecciosa das galinhas.

No Brasil, em detrimento da importância da BIG, poucos pesquisadores têm se debruçado sobre o tema e, normalmente, os que o fazem obtém resultados desafiantes; como no caso de Villarreal et al. (2007) que em um universo de 119 amostras, coletadas em 9 estados da federação localizaram em 5 delas, coronavírus assemelhados ao grupo II, onde normalmente são classificados aqueles de bovinos, suínos e humanos (mamíferos). Este resultado é no mínimo intrigante, pois até então todos os vírus desta família isolados de aves encontrava-se somente no grupo III (exclusivo de aves). Vários outros estudos evidenciam a circulação de mais de um genótipo no Brasil (Villarreal et al., 2010; Felippe et al., 2010) e, em alguns casos, testes de proteção *in vitro* indicaram que os soros obtidos a partir de aves vacinadas foram ineficientes na neutralização da ação de alguns dos vírus isolados (Chacón et al., 2008). Abreu et al. (2008) analisando parte do gene que codifica a proteína S observaram uma grande diversidade entre os isolados brasileiros, principalmente quando da modelagem das proteínas resultantes e da análise das regiões mais expostas da S_1.

Quando o foco são os vírus de genoma RNA a preocupação pode extrapolar os limites da economia da indústria, uma vez que a grande maioria dos agravos emergentes saúde humana emergentes, incluindo SIDA, ebola, influenza aviária e finalmente a SARS (coronavírus), tiveram por origem a transmissão entre espécies animais até atingirem potencial zoonótico. No caso específico dos vírus da família *Coronaviridae*, principalmente devido ao surgimento da SARS na China, inúmeros estudos envolvendo espécies animais silvestres têm sido realizados. Observa-se, assim, que a diversidade desta família é muito maior do que se imaginava, não só nas aves não domésticas, mas também em mamíferos, e dentre estes nos chirópteros (morcegos), que possuem os coronavírus mais antigos e teoricamente com o mesmo potencial de dispersão (capacidade de voar) que as aves (Jonassen et al., 2005; Vijgen et al., 2005; Woo et al., 2006; Tang et al., 2006; Vijaykrishna et al., 2007; Felippe et al., 2007).

2.3 – Metapneumovirose Aviária (Rinotraqueíte dos Perus).

A rinotraqueíte dos perus é uma importante enfermidade na criação comercial destas aves, surtos em criações levam a uma morbidade de 100%, porém a mortalidade pode estar abaixo de 1%, ou mesmo exceder os 50% quando da presença de fatores agravantes, como as infecções secundárias (Gough et al., 1988, Jirjis et al., 2000). A enfermidade é caracterizada por espirros, estertores traqueais, edema dos seios infraorbital, nasal e muitas vezes frontal, com descarga ocular. A descarga nasal pode se tornar mucopurulenta devido infecção bacteriana secundária. Apesar da replicação do vírus ocorrer na traquéia e pulmões, ela é mais limitada ao trato respiratório superior, onde as partículas virais podem ser detectadas mais facilmente (Cook, 2000).

O aparecimento dos sinais clínicos é rápido e a infecção pode se disseminar em 24 horas (Stuart, 1989). Normalmente, ocorre recuperação total das aves dentro de 14 dias. Porém, como em outras infecções respiratórias em aves domésticas, o manejo precário ou ocorrência de infecção bacteriana secundária podem provocar o agravamento dos sinais clínicos, como aerossaculite, pericardite, pneumonia, e também aumento da morbidade e da mortalidade (Cook et al., 1991). Observações experimentais e de campo sugerem que o metapneumovírus pode facilitar a infecção pelo *Ornithobacterium rhinotracheale* e exacerbar infecções pelo *Mycoplasma gallisepticum* (Hafez, 1988; Naylor, Jones, 1993).

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

Nas galinhas, a metapneumovirose aviária é uma a doença normalmente associada a outros agentes etiológicos envolvidos na chamada "Síndrome da Cabeça Inchada" (SCI), que normalmente se manifesta por um importante inchaço dos seios nasais infra e periorbital e

frequentemente pode levar a torcicolo ou mesmo opistótomo. Normalmente a minoria das aves de uma granja é afetada, porém nesta espécie a mortalidade pode ser maior do que 15% (Picault et al., 1987; Buys et al., 1989; Pattison et al., 1989; Steenhuisen, 1989; Tanaka et al., 1995). A produção de ovos pode também ser afetada tanto em infecções naturais como artificiais.

A SCI é relatada tanto em frangos de corte como em matrizes e, nessas últimas, a evidência do papel do Metapneumovírus aviário como agente etiológico primário da enfermidade é maior (Cook, 2000). Porém, ele não é o único agente associado à SCI. Outros também foram descritos como o vírus da bronquite infecciosa (Morley, Thomson, 1984) e *Escherichia coli* (APEC) (Droual, Woolcock, 1994).

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

O metapneumovírus aviário (aMPV, avian metapneumovirus) é o agente etiológico da metapneumovirose aviária e da rinotraqueíte dos perus. O aMPV, que era classificado no gênero *Pneumovirus*, foi recentemente designado no Gênero *Metapneumovirus* pertencendo à família *Paramyxoviridae*, subfamília *Pneumovirinae*, (Randhawa et al., 1997; Bäyon-Auboyer, et al., 1999; Easton, et al., 2004; Munir, et al., 2006). Os metapneumovírus são envelopados e apresentam o genoma com aproximadamente 13.000 nucleotídeos (Ling, Easton et al., 1992; Fenner, Gibbs et al., 1993). Os vírions do aMPV são partículas pleomórficas de 80 a 600 nm e nucleocapsídio helicoidal com projeções de 13 nm na sua superfície (figura 4) (Collins, et al., 1986; Gough, Collins, 1989; Hafez, Weiland, 1990).

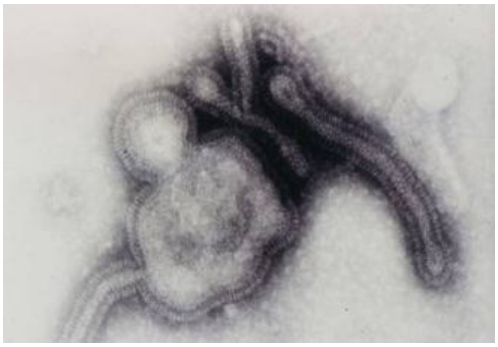

Figura 4: Eletro microfotografia do Metapneumovírus aviário; pleomorfismo.
Fonte: http://www.liv.ac.uk/researchintelligence/issue33/apv.htm

Estes vírus possuem o genoma formado por RNA de fita simples negativa, e contém oito genes dispostos na seguinte ordem: nucleoproteína (N), fosfoproteína (P), matriz (M), fusão (F), segunda matriz (M2), proteína hidrofóbica (SH), glicoproteína G (G), polimerase (L) (3'-N-P-M-F-M2-SH-G-L-5')(Easton et al., 2004). O RNA genômico está associado às proteínas N, L e P, que formam o complexo da RNA-polimerase. Este complexo é necessário para a replicação e transcrição viral (Barik, 2004; Easton et al., 2004). A proteína L é o principal componente deste complexo e se liga na porção 3´ das sequências promotoras do RNA genômico. Entretanto, a proteína L é incapaz de iniciar a transcrição ou replicação na ausência da proteína P, que oferece para a proteína L o prolongamento necessário para promover sua liberação e produção de transcritos inteiros (Dupuy, Dobson et al., 1999). A proteína N está intimamente associada com o RNA genômico, e oferece resistência à ação das RNAses (Barik, 2004) e induz a formação de uma estrutura hélica ao genoma viral (Easton et al., 2004).

A proteína M nos vírus de RNA de fita simples negativa 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 das membranas viral e celular (Walsh, Hruska, 1983). A proteína F é sintetizada primeiramente como F0, que se torna ativa por uma clivagem em F1 e F2 assim que a proteína chega ao complexo de Golgi (Collins 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 (figura 5) (Olmsted, Collins, 1989).

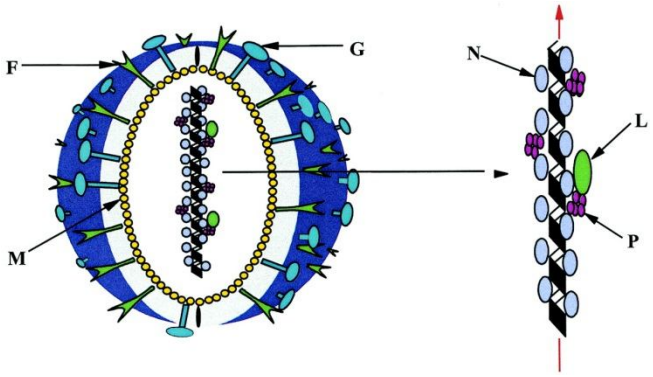

Figura 6: Esquema tridimensional do aMPV mostrando aspectos de seu genoma e de suas proteínas estruturais.
Fonte: http://cmr.asm.org/cgi/content/full/17/2/390

As proteínas acima apresentadas são as mesmas existentes nos vírus classificados no Genero *Pneumovirus*, exceção feita a ausência das proteínas não estruturais NS1 e NS2 nos *Metapneumovirus* (Randhawa et al., 1997) e a localização do gene que codifica a proteína G no genoma; a qual nos *Pneumovirus* localiza-se entre os genes que codificam as proteínas SH e F e em *Metapneumovirus* entre a SH e a L na extremidade 5´ (figura 6) (Ling et al., 1992; Yu et al., 1992; Randhawa et al., 1997; Collins, Crowe, 2007).

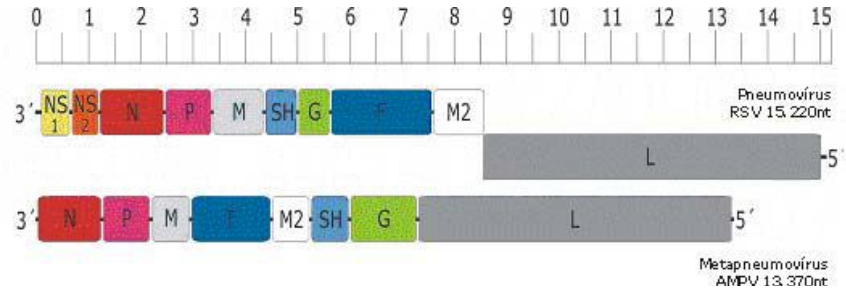

Figura 6. Organização genômica dos vírions pertencentes ao gênero *Pneumovirus* e *Metapneumovírus*.
Fonte: Ferreira, 2007.

No processo de adsorção a proteína G dos metapneumovírus se liga ao receptor celular, quando ocorre uma mudança conformacional da glicoproteína F para a liberação do peptídeo de fusão. Em seguida, o complexo da RNA-polimerase (proteínas N, L e P) é liberado no citoplasma (Lamb, Kolakofsky, 1996; Easton, Domachowske et al., 2004). Os metapneumovírus codificam e empacotam a sua própria RNA polimerase, enquanto que o RNAm é sintetizado apenas quando ocorre o desnudamento viral na célula infectada. A replicação viral ocorre após a síntese do RNAm e requer a produção contínua de proteínas virais. O antigenoma sintetizado é utilizado como molde para as cópias de RNA genômico (-), assim como todos os eventos da replicação viral, a montagem também ocorre no citoplasma (Lamb, Kolakofsky, 1996; Easton, Domachowske et al., 2004).

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

O aMPV pode ser dividido em quatro subtipos (A, B, C e D) baseados na sequência genética que codifica a proteína G (Bäyon-Auboyer et al., 2000; Toquin et al., 2003). Estes vírus foram primeiramente isolados em perus na África do Sul em 1978 (Buys et al., 1980). Durante a década de 80 aMPV foi detectado no Reino Unido (McDougall, Cook, 1986; Wilding, et al., 1986; Wyeth, et al., 1986), França (Giraud, et al., 1986), países baixos, Espanha, Hungria, Itália (Cook, et al., 1993), Israel (Weisman, et al., 1988) e Alemanha. Inquéritos sorológicos indicaram que em um primeiro momento em 1986 o vírus ocorria apenas em criações de perus, porém em 1988 foi considerado endêmico nas galinhas em toda a Alemanha (Hafez, Löhren, 1990).

Os vírus inicialmente isolados na África do Sul eram antigenicamente relacionados aos aMPV A (Cook et al., 1993). Todos os isolados encontrados no Reino Unido até 1994 também pertenciam a este subtipo (Juhasz, Easton, 1994; Naylor et al., 1997), porém a partir deste ano o subtipo B também passou a ser encontrado (Cavanagh et al., 1997[b]; Naylor et al., 1997; Cavanagh et al., 1999). Na Europa Continental os subtipos A, B e D eram prevalentes desde o final dos anos 80 (Juhasz, Easton, 1994; Naylor et al., 1997; Bäyon-Auboyer et al., 2000; Hafez et al., 2000). Apesar do aMPV D ter desaparecido a partir do ano de 1984 os subtipos A e B estão presentes hoje, não só na Europa (Van de Zande et al., 1998; Catelli et al., 2004) mas também na África (Owoade et al., 2008), América do Sul (Arns, 1992, 1995; Dani et al., 1999; D'Arce et al., 2005; Chacón et al., 2007; Coswig et al., 2010) e Ásia (Mase et al., 2003; Owoade et al., 2008).

A América do Norte (EUA) foi considerada livre de aMPV, até o aparecimento de um surto em perus no ano de 1996 onde os isolados foram classificados como subtipo C (Panigrahy et al., 2000; Seal, 2000). Este surto inicial, no estado do Colorado, foi controlado com base em rígidas medidas de biossegurança, após cerca de 10 meses de seu início, porém em 1997, o aMPV C foi detectado em Minnesota, estado com maior produção de perus nos Estados Unidos (Goyal et al., 2000; Panigrahy et al., 2000; Lwamba et al., 2002). Nos dias atuais este subtipo é endêmico neste estado apresentando uma soroprevalência de 40% nas criações de perus (Alkhalaf et al., 2002; Shin et al., 2002; Goyal et al., 2003; Bennett et al., 2004). O aMPV C tem sido diagnosticado em estados vizinhos ao de Minnesota, no entanto só este subtipo tem sido encontrado no país até então e em perus (Panigrahy et al., 2000; Seal, 2000; Gough, 2003). Recentemente o aMPV C foi encontrado na França em anatídeos selvagens (Toquin et al., 2006) e em faisões na Coreia (Lee et al., 2007). Porém, os isolados franceses apresentaram diferenças genômicas quando comparados aos aMPV C de ocorrência natural nos Estados Unidos (Toquin et al., 2006; Padhi, Poss, 2009).

As sequências deduzidas de aminoácidos das proteínas virais estão mais relacionadas dentre os subtipos A, B e D, enquanto o C é mais distante dos demais (Seal, 1998; Seal, 2000; Shin et al., 2002; Njenga et al., 2003). A sequencia deduzida de aminoácidos da proteína G é de 391 aminoácidos para o subtipo A, 414 para o B e 389 para o D. No que diz respeito ao subtipo C existem na literatura dados conflitantes sobre a sua extensão variando de 252 a 585

aminoácidos (Govindarajan et al., 2004; Govindarajan, Samal, 2005; Bennett et al., 2005; Lee et al., 2007; Velayudhan et al., 2008).

No final da década de 80 vacinas atenuadas de aMPV começaram a ser utilizadas no continente europeu, porém a doença em animais vacinados continuou a ser diagnosticada (Cook et al., 1989; Williams et al., 1991). Apesar de existirem evidências de que muitos destes casos ocorreram por um processo de vacinação inadequado ou mesmo infecções por subtipos virais diferentes daqueles utilizados na vacina (Naylor[a] et al., 1997; Van de Zambe et al., 1998), alguns apareceram por variantes originadas do vírus vacinal (Catelli et al., 2006). Por outro lado, alguns surtos da doença foram relatados posteriormente a vacinações corretamente realizadas e pelo mesmo subtipo diagnosticado. Como o exemplificado no estudo de Banet-Noach et al. (2009), onde a vacinação com o subtipo B não foi suficiente para proteger os animais contra as cepas de campo; o autor sugere que a substituição de aminoácidos na proteína G possa ser a causa do ocorrido.

Um estudo realizado na Itália envolvendo isolados de aMPV no país entre 1987 e 2007 mostrou que os mais recentes apresentavam mutações no gene que codifica a proteína G e que estas foram suficientes para alterar alguns aminoácidos (Cecchinato et al., 2009). Catelli et al. (2010) observaram num desafio experimental de animais vacinados na Itália com as cepas de campo recentemente isoladas que a vacina produziu apenas imunidade parcial, sugerindo que estas mudanças de aminoácidos ocorreram em regiões importantes e que alteraram o fenótipo antigênico do vírus.

Anticorpos neutralizantes em perus e galinhas, infectados experimentalmente com cepas virulentas de aMPV, aparecem por volta dos 6 dias pós-infecção atingindo um pico no intervalo de 10 a 14 dias (Jirjis et al., 2000; Aung et al., 2008). Estudos mostram que anticorpos neutralizantes com alta especificidade podem ser detectados em até 12 semanas após a infecção (Jones et al., 1988), assim como anticorpos do isotipo IgA podem ser encontrados em mucosas, lágrimas, secreção nasal, bile em galinhas e perus infectados pelo aMPV (Ganapathy et al., 2005; Liman, Rautenschlein, 2007; Cha et al., 2007).

Estudos de campo mostram que a presença de anticorpos reagentes com antígenos do aMPV não conferem necessariamente proteção a infecção ou doença (Sharma et al., 2004; Kapczynski et al., 2008); concordando com esta assertiva os anticorpos adquiridos da mãe também não conferem proteção aos perus jovens desafiados com cepas virulentas do aMPV

(Naylor[b] et al., 1997; Catelli et al., 1998). O mesmo é também encontrado em outros da família *Pneumovirinae* como o hMPV (Metapneumovírus Humano), bRSV (Vírus Respiratório Sincicial Bovino) e hRSV (Vírus Respiratório Sincicial Humano) (Kimman et al., 1987; Kimman et al., 1988; Belknap et al., 1991; Plotnicky-Gilquin et al., 2000; Alvarez, Tripp, 2005).

No que diz respeito a infecções envolvendo os aMPV dos subtipos A, B e D em espécies de aves diferentes dos perus e das galinhas, o único estudo foi o de Catelli et al. (2001), que encontraram anticorpos em faisões de criações comerciais e de vida livre reativos aos aMPVs dos subtipos A e B. Outrossim, nenhum estudo envolvendo as aves silvestres para os subtipos acima é encontrado na literatura científica indexada.

No entanto o subtipo C tem sido encontrado em uma grande variedade de espécies de aves não domésticas, como pombas, ganso canadense, gansos da neve, marrecos de asas azuis, pardais e gaivotas (Shin et al., 2000; Bennett et al., 2002; Bennett et al., 2004; Turpin et al., 2008), porém nenhum sinal clínico foi relacionado a infecção pelo aMPV nestas espécies. Os surtos de aMPV C nas criações de perus no estado americano de Minnesota apresentaram padrões sazonais com aumento da casuística na primavera e no outono, porém a contribuição das espécies silvestres enquanto reservatórios de vírus durante estas estações é ainda muito discutida (Shin et al., 2002; Goyal et al., 2003). No Brasil, até então, nenhuma detecção de aMPV foi realizada em nenhuma espécie de ave não doméstica.

Assim como no caso dos Coronavírus, especula-se sobre o potencial zoonótico dos Metapneumovírus, uma vez que o hMPV (metapneumovírus humano) possui uma estreita semelhança com o aMPV C, que é geneticamente mais próximo a este do que aos demais subtipos do aMPV (van den Hoogen et al., 2001; van den Hoogen et al., 2002; Njenga et al., 2003). A similaridade das sequências de aminoácidos das proteínas F, L, M, M2, N e P, entre o hMPV e o aMPV C varia entre 68 a 88% enquanto a do aMPV C com o aMPV A ou B é de 52 a 78% (Seal, 1998; van den Hoogen et al., 2001; van den Hoogen et al., 2002; Shin et al., 2002). Em contraste existe uma baixa homologia das proteínas SH e G entre o hMPV e o aMPV C, o que pode ser a causa do tropismo por espécies animais diferentes destes vírions (van den Hoogen et al., 2002). Autores como de Graaf et al. (2008) sugerem que o hMPV e o aMPV C se originaram de um ancestral comum a cerca de 200 anos atrás, além disso, recentemente Nagajara et al (2007) induziram doença respiratória detectável clinicamente em perus inoculados com o

vírus humano (hMPV), o que sem dúvida deixa franca a discussão sobre a capacidade destes dois vírus de ultrapassarem a barreira de espécie.

3. Objetivos:

3.1 Objetivos Gerais

Pesquisar a presença de vírus dos gêneros Coronavírus e Metapneumovírus em aves domésticas, sinantrópicas e selvagens.

3.2 Objetivos específicos

- Coletar swabs cloacais e traqueais de aves domésticas (aves de granja, de várias regiões do Brasil), selvagens e sinantrópicas da região de Campinas;

- Sequenciar os genes codantes das proteínas mais variáveis dos vírus encontrados e comparar suas sequncias com aquelas depositadas no GeneBank;

- Avaliar a percentagem de similaridade entre os replicons dos vários vírus encontrados, com aqueles depositados no GeneBank;

- Realizar estudos de bioinformática e construção de árvores filogenéticas, comparando os vírus recuperados e isolados com aqueles depositados no GeneBank.

4. Resultados:

Os resultados são apresentados em forma de publicação.

O primeiro artigo (artigo I) foi aceito e publicado na *Avian Disease*s e o segundo (artigo II) foi submetido a revista *Avian Pathology.*

Artigo I

Genetic Diversity of Avian Infectious Bronchitis Virus Isolated from Domestic Chicken Flocks and Coronaviruses from Feral Pigeons in Brazil Between 2003 and 2009

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Genetic Diversity of Avian Infectious Bronchitis Virus Isolated from Domestic Chicken Flocks and Coronaviruses from Feral Pigeons in Brazil Between 2003 and 2009

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SUMMARY. To detect the presence of infectious bronchitis virus or avian coronavirus, a nested reverse transcriptase PCR (RT-PCR) method was developed with the aim of amplifying a fragment of 530 bases, comprising the gene coding S1 protein. In the first step, all samples were submitted to RNA extraction, RT-PCR, and nested PCR. Next, only the positive nested-PCR samples were propagated in specific-pathogen-free (SPF) embryonated chicken eggs for virus isolation. Positive samples were then sequenced and analyzed using a molecular phylogeny approach. Tracheal swab samples were collected from 23 different domestic chickens distributed in three regions of Brazil, in the period between 2003 and 2009. Also analyzed were six swab samples (tracheal and cloacal) from asymptomatic pigeons (*Columba livia*), caught in an urbanized region in southeastern Brazil. The study revealed two major phylogenetic groups: one clustered with the Massachusetts vaccine serotype and another joined with the D207 strain. Interestingly, samples grouped with the Connecticut and Arkansas serotypes were also found. Pigeon isolates clustered with the Massachusetts serotype showed significant similarity (close to 100%) to those obtained from chickens. Only one pigeon isolate was seen to be grouped with the Connecticut serotype, and no correlation was observed between sample grouping and region origin. Understanding the diversity of genotypes and eco-epizootiology of the disease in different environments is expected to be helpful for vaccine production aimed at the main circulating variants. In this respect, one could also expect benefits in the management of other bird species that may act as avian coronavirus reservoirs.

RESUMEN. Diversidad genética de los virus de la bronquitis infecciosa aviar aislados de parvadas de pollos domésticos y de los coronavirus de palomas silvestres en Brasil aislados entre el 2003 y el 2009.

Para detectar la presencia del virus de la bronquitis infecciosa y coronavirus aviar, se desarrolló una prueba de transcripción reversa y reacción en cadena de la polimerasa (RT-PCR) anidada con el objetivo de amplificar un fragmento de 530 bases, que incluye al gene que codifica la proteína S1. En la primera etapa, se extrajo el ARN de las muestras y se llevaron a cabo la prueba de RT-PCR y la amplificación por PCR anidada. Únicamente las muestras positivas después de la PCR anidada fueron inoculadas en huevos embrionados de pollo libres de patógenos específicos para realizar el aislamiento de los virus. Posteriormente, las muestras positivas fueron secuenciadas y analizadas utilizando un enfoque de filogenia molecular. Se recolectaron muestras de hisopos traqueales de 23 parvadas diferentes de pollos domésticos distribuidos en tres regiones de Brasil, en el período comprendido entre los años 2003 y 2009. También se analizaron muestras de hisopos traqueales y cloacales de seis palomas (*Columba livia*) que no presentaban signología clínica y que fueron capturadas en una región urbana en el sureste de Brasil. El estudio reveló dos grupos filogenéticos principales: uno agrupó a la vacuna serotipo Massachusetts y el otro se agrupo con la cepa D207. Resultó interesante que también se encontraron muestras que se agruparon con los serotipos Connecticut y Arkansas. Los aislamientos de paloma se agruparon con el serotipo Massachusetts y mostraron una similitud importante (cerca del 100%) con los aislamientos obtenidos de pollo. Sólo un aislamiento de paloma se agrupó con el serotipo de Connecticut y no se observó correlación entre la forma en que se agruparon las muestas y la región de origen. El estudio de la diversidad de genotipos y de los aspectos ecológicos y epidemiológicos de la enfermedad en diferentes ambientes se espera que sea de utilidad para la producción de vacunas dirigidas a las principales variantes que se encuentran circulando. En este sentido, también se podría esperar obtener beneficios en el manejo de otras especies de aves que pueden actuar como reservorios del virus de la bronquitis infecciosa aviar.

Key words: coronavirus, pigeon, chicken, genetic diversity, phylogeny, nested PCR

Abbreviations: bp = basis pair; CAM = chorioallantoic membrane; cDNA = complementary DNA; IBV = infectious bronchitis virus; kb = kilobases; MEM = minimum essential medium; RT-PCR = reverse transcriptase–polymerase chain reaction; S = spike protein; SARS = severe acute respiratory syndrome; SPF = specific pathogen free; UTR = untranslated region

Avian infectious bronchitis virus (IBV), a member of the family *Coronaviridae*, order *Nidovirales*, is a highly infectious pathogen of domestic fowl (6,43). IBV is an enveloped virus that replicates in the cell cytoplasm, and its genome is constituted by a nonsegmented positive-stranded RNA of 27.6 kb (3). All coronaviruses maintain a set of essential genes, including those that encode the polymerase (Pol), spike (S), small membrane (E), membrane (M), and nucleocapsid (N) proteins in the invariable order 5′-Pol-S-E-M-N-3′ and a 3′ untranslated region (UTR) (6). In addition to these essential genes, IBV contains group-specific or accessory genes that encode small proteins (23). Although the coronaviruses are traditionally separated into three groups (4,23) based on genetic and antigenic characteristics, many other groups and subgroups have recently been proposed (13,18,40,46). These viruses have been associated with diseases in several warm-blooded animals, including humans (22,42). Coronaviruses from group I and II have been found to infect several mammalian species, including humans, pigs, cows, dogs, horses, cats, and rodents. Group III viruses have been found to infect poultry. This group includes the chicken IBV, the turkey coronavirus, and the pheasant coronavirus (22,43). Although IBV indeed causes respiratory disease, it also replicates at many nonrespiratory epithelial surfaces, where it may cause pathology (6), and represents one of the most economically significant diseases of

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Table 1. Brazilian samples utilized in this study with their respective regions of origin, host, and GenBank number.

Brazilian samples	Host	Country region	GenBank no.
COLUMBA/BRAZIL/2007/UNICAMPT6	Pigeon	Southeastern	HM561878
COLUMBA/BRAZIL/2006/UNICAMP64C	Pigeon	Southeastern	HM561879
COLUMBA/BRAZIL/2007/UNICAMP65T	Pigeon	Southeastern	HM561880
COLUMBA/BRAZIL/2007/UNICAMP67T	Pigeon	Southeastern	HM561882
COLUMBA/BRAZIL/2006/UNICAMP63C	Pigeon	Southeastern	HM561881
COLUMBA/BRAZIL/2007/UNICAMP66T	Pigeon	Southeastern	HM561883
IBV/BRAZIL/2003/UNICAMP31422	Chicken	Southeastern	HM561884
IBV/BRAZIL/2004/UNICAMP31298	Chicken	Southeastern	HM561885
IBV/BRAZIL/2004/UNICAMP703	Chicken	Southern	HM561887
IBV/BRAZIL/2004/UNICAMP706	Chicken	Northeastern	HM561886
IBV/BRAZIL/2006/UNICAMP788	Chicken	Southern	HM561888
IBV/BRAZIL/2007/UNICAMP801	Chicken	Southern	HM561889
IBV/BRAZIL/2007/UNICAMP805	Chicken	Southeastern	HM561890
IBV/BRAZIL/2007/UNICAMP810	Chicken	Southeastern	HM561891
IBV/BRAZIL/2008/UNICAMP816	Chicken	Southeastern	HM561896
IBV/BRAZIL/2008/UNICAMP818	Chicken	Southern	HM561898
IBV/BRAZIL/2008/UNICAMP820	Chicken	Northeastern	HM561893
IBV/BRAZIL/2008/UNICAMP821	Chicken	Southeastern	HM561901
IBV/BRAZIL/2008/UNICAMP830	Chicken	Southeastern	HM561897
IBV/BRAZIL/2008/UNICAMP832	Chicken	Southeastern	HM561894
IBV/BRAZIL/2008/UNICAMP836	Chicken	Southeastern	HM561895
IBV/BRAZIL/2008/UNICAMP846	Chicken	Southern	HM561899
IBV/BRAZIL/2008/UNICAMP857	Chicken	Southern	HM561892
IBV/BRAZIL/2008/UNICAMP861	Chicken	Southern	HM561900
IBV/BRAZIL/2008/UNICAMP882	Chicken	Southeastern	HM561902
IBV/BRAZIL/2008/UNICAMP890	Chicken	Southern	HM561903
IBV/BRAZIL/2009/UNICAMP897	Chicken	Northeastern	HM561904
IBV/BRAZIL/2009/UNICAMP901	Chicken	Southern	HM561905
IBV/BRAZIL/2009/UNICAMP940T	Chicken	Southeastern	HM561906

the intensive poultry industry. In young chicks, respiratory disease or nephritis lead to mortality, reduced weight gain, and condemnation at processing; whereas, in chickens of laying age, the disease is subclinical and results in reduced egg production and aberrant eggs (6,32).

Since IBV was first described by Schalk and Hawn in the 1930s (14), many serotypes have been identified worldwide and new variants have arisen, many of them as a result of vaccination programs (28). Vaccines are generally effective in controlling the clinical disease; however, escape mutants or variants continue to cause clinical disease and production problems in vaccinated flocks (8,20). The continuing appearance of new IBV variants is associated with the high evolution rate, expressed as the accelerated rate at which viable mutations accumulate in the genome (12,24,25).

Coronavirus has also been isolated from wild bird species and racing pigeons, which could constitute an important environment for virus evolution (17,22,31,46). The other problem is the possibility of recombination events within coronaviruses, as may be the case for the severe acute respiratory syndrome (SARS) virus. In fact, some authors have argued that there is evidence for recombination events in the evolution of this virus, involving both mammalian and avian coronaviruses (36,37).

Molecular epizootiology can be very important to understand the coronavirus dynamics in various correlated avian species and to improve its control. Avian populations, such as, e.g., pigeons, which are asymptomatic for respiratory diseases, could be critical in this context, because they can transport the virions. In this way, they provide an important environment for virus evolution. The main objective of the present study was to better understand the genetic diversity aspects of avian coronavirus in Brazilian commercial poultry and pigeons. The study was based on molecular characterization and phylogenetic analysis performed on partial sequences of the S_1 gene of IBV isolated from chicken (*Gallus gallus domesticus*; historical 5-yr series) and coronavirus from feral pigeon (*Columba livia*).

MATERIALS AND METHODS

Viruses. In the period from 2003 to 2009, a total of 23 positive samples, isolated from 102 tracheal swab pools from IBV symptomatic chicken, were obtained from the main poultry-producing regions of Brazil; namely, the southern (11), southeastern (6), and northeastern (1) regions (Table 1). In addition, there were six positive samples from 12 pigeons with no symptoms of IBV disease (cloacal and tracheal swabs) captured at the city of Campinas (São Paulo State, southeastern region of Brazil) in 2006 and 2007. These pigeons were identified and released after completion of tracheal and cloacal swabs. All swabs were resuspended on 0.2 ml of minimum essential medium (MEM), from which 0.1 ml was used for further molecular studies and subsequent inoculation in embryonated specific-pathogen-free (SPF) eggs (positive samples by reverse transcriptase (RT)-PCR).

RNA extraction and viral nucleic acid amplification. The swabs were resuspended in 0.1 ml of MEM in an Eppendorf tube and centrifuged at 3000 RPM for 5 min. RNA was extracted from the supernatant using a High Pure Viral Nucleic extraction kit™ (Roche Diagnostics™, Manheim, Germany) and the cDNA was synthesized using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA). Both procedures were performed according to the manufacturer's instructions for use with random hexamers. All samples were investigated for IBV through the amplification of specific genome fragments. The amplification of the variable region of the S1 protein gene was the target for the polymerase chain reaction.

The first step of nested RT-PCR was carried out using forward primer S7 (5′-TACTACTACCAGAGTGC(C/T)TT-3′) annealing at the

Table 2. Names, hosts, and accession numbers of the different coronavirus strains from the GenBank used in this study.

Name	Host	Accession no.	Name	Host	Accession no.
H52	Chicken	AF352315	JMK	Chicken	L14070
Mass/4/UNAM	Chicken	EU526411	Clark-333	Chicken	AF201732
H120	Chicken	M21970	PA/Wolgemuth/98	Chicken	AF305595
Massachusetts-41	Chicken	DQ664534	ARK99	Chicken	L10384
Ma5	Chicken	AY561713	PP14	Chicken	M99452
Conn/B13dpvcontact	Chicken	EU283060	AL/4614/98-arkdpi	Chicken	DQ458217
Conn/B6dpvcontact	Chicken	EU283059	Jilin-China-vacina	Chicken	AY839144
Conn/Bvial2	Chicken	EU283058	Variant-3330-spike	Chicken	AF201733
Conn/75/UNAM	Chicken	EU526408	SE-17	Chicken	M99484
316/74/UNAM	Chicken	EU526406	Holte	Chicken	L18988
CU510	Chicken	AY61716	4/91-attenuated	Chicken	AF093793
Egypt/Zag/07-03	Chicken	EU368594	4/91-pathogenic	Chicken	AF093794
Conn/Cvial2	Chicken	EU283062	Vic	Chicken	DQ490221
L2	Chicken	AB363961	Gray	Chicken	L18989
Connecticut46	Chicken	L18990	D207	Chicken	M21969
2994/02	Chicken	AY606324	UK123/82	Chicken	X58067
Conn/43/UNAM	Chicken	EU526403	UK7/93	Chicken	Z83979
Conn/1/UNAM	Chicken	EU526410	UK7/91	Chicken	Z83975
Conn/Bvial1	Chicken	EU283057	SARS-CUHKtc55NS	Human	DQ412629
V5-90	Chicken	DQ490218	Pigeon	Pigeon	DQ099734
IBV/Brazil/2005/USP-1	Chicken	DQ355995	Pigeon PSH050513	Pigeon	PSH050513

20,455 IBV genome position (Massachusetts 41 type) and a reverse primer, S6 (5′-ACATC(T/A)TGTGCGGTGCCATT-3′) annealing at the 21,008 position (2) with amplified products of 572 bp. PCR was carried out in a 50 µl mixture containing 5 µl of 10× PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl), 1.5 µl of 0.2 mM $MgCl_2$, 1.5 µl of a 10 mM dNTP mixture, 2.5 µl of each primer (10 mM), 5 µl of cDNA, and 0.2 µl (5 U/µl) of *Platinum*™ DNA Polymerase (Invitrogen Ltd., California, USA). The amplification was preheated for 5 min at 95 C, followed by 35 cycles, each consisting of 1 min at 95 C (denaturation), 1 min at 55 C (annealing), and 1 min at 72 C (extension). After completion of the 35 cycles, a final extension of 7 min at 72 C was performed.

A second step of nested PCR was carried out with the amplified products of the first-round using forward primer S9 (5′-ATGGTTGG CATTT(A/G)CA(C/T)GG-3′; 20,486 genome position) (2) and the reverse primers S5 (5′-GTGCCATTGACAAAATAAGC-3′; 20,995 genome position) (2). The nested PCR was performed using nested primers S9-S5 corresponding to the amplified products of 530 bp. The amplifications were carried out in a thermal cycler PCR System 9,700 (Gene Amp, Applied Biosystems, Perkin-Elmer, CA). The PCR products were run on 1% agarose gel and visualized under UV light after staining with ethidium bromide.

Passage in embryonated SPF chicken eggs. The positive samples, after RT-PCR and sequencing, were inoculated 0.1 ml onto the chorioallantoic membrane (CAM) of embryonated SPF chicken eggs (9 days old). The eggs were incubated and observed daily for viability. After 1 wk the embryos were evaluated for lesions typical of IBV (stunting and curling). This process was repeated five times. In all passages, control samples were taken for molecular investigations (RT-PCR and sequencing).

Sequencing and phylogenetic analysis. PCR products were sequenced three times each, in both the forward and reverse directions using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The BioEdit software, version 7.0.5.2 (16), was used to manipulate the retrieved nucleotide and amino acid sequences. The sequence alignments were performed using the Clustal W software version 1.83 (41) using full alignment and 1000 total replications on the bootstrap in order to ensure a higher level of confidence for the analysis (41). Phylogenetic analyses were performed using neighbor-joining as implemented in the MEGA version 4 software package (39) based on the Kimura two-parameter distance estimation method. Bootstrap resampling was performed for each analysis (1000 replications). Reference IBV nucleotide sequences were retrieved from the GenBank database (Table 2), including the complete Massachusetts type genome (accession number AY851295) utilized to estimate the nucleotide positions. The IBV nucleotide sequences obtained have been submitted to GenBank.

RESULTS

Clusters and similarity of studied S1 fragment from different samples. The chicken samples demonstrated two principal groups, one similar to the Massachusetts strain (IBV/Brazil; 2006/UNICAMP788, 2008/UNICAMP836, 2008/UNICAMP861, 2008/UNICAMP818, 2009/UNICAMP901, 2009/UNICAMP897, 2008/UNICAMP890, 2008/UNICAMP832, 2008/UNICAMP820, 2008/UNICAMP857, and 2008/UNICAMP821) and the other different from vaccine strains used in Brazil (IBV/Brazil; 2007/UNICAMP801, 2008/UNICAMP846, 2003/UNICAMP31422, 2004/UNICAMP31298, 2004/UNICAMP706, 2004/UNICAMP703, 2009/UNICAMP940T, 2008/UNICAMP882, 2007/UNICAMP810, and 2008/UNICAMP816), which grouped to the D207 strain. Five pigeon samples were similar to the Massachusetts group in the chickens (Columba/Brazil; 2006/UNICAMP63C, 2006/UNICAMP64C, 2007/UNICAMPT6, 2007/UNICAMP67T, and 2007/UNICAMP65T). One pigeon sample (Columba/Brazil/2007/UNICAMPT66) and another from chickens (IBV/Brazil/2007/UNICAMP 805) were similar to the Connecticut strain (not used in Brazil as vaccine), and one sample from the chickens (IBV/Brazil/2008/UNICAMP 830) was very different from the others and grouped with the Arkansas strain (Fig. 1).

The similarity of the S1 fragment nucleotide sequence from coronavirus isolated from pigeon and chicken ranged from 100% to 51.7% (100% to 22.6% for the amino acid sequence). In just the chickens, this range was from 100% to 71.8% (100% to 50.6% for the amino acid sequence), while in pigeons it was from 100% to 60.8% (100% to 33.3% for the amino acid sequence). When the intercluster similarity was considered, the Massachusetts type ranged from 77.3% to 69.1% (62.2% to 46.2% for the amino acid sequence) for the D207 group, 94.1% to 60.4% (88.6% to 33.9% for the amino acid sequence) for the Connecticut type, and 72% to 67.4% (57.4% to 49% for the amino acid sequence) for the Arkansas type.

With regard to the isolation chronology, the Massachusetts type was isolated from 2006 to 2009 in chickens and from 2006 to 2007 in

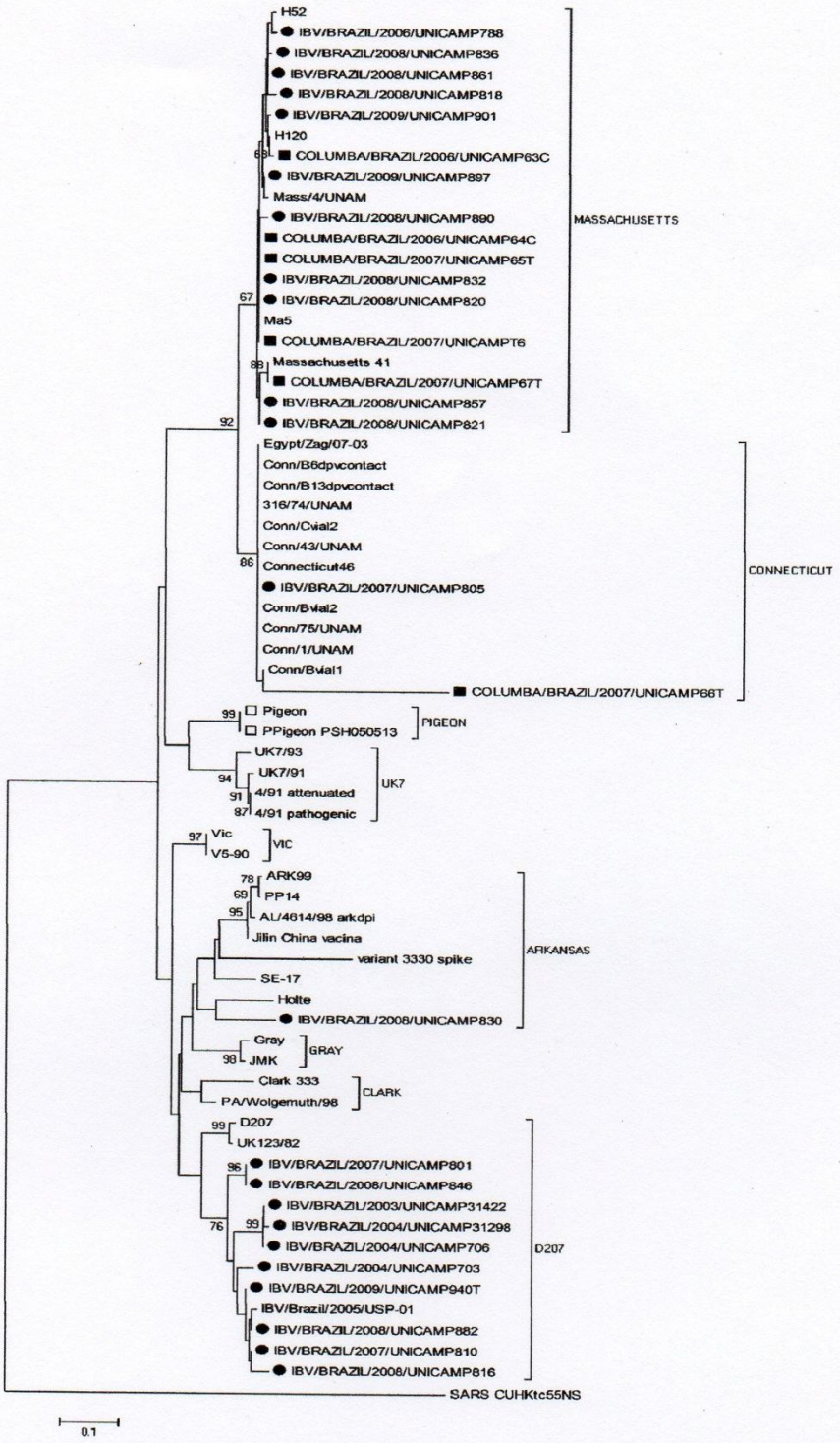

Fig. 1. Phylogenetic tree showing partial S1 gene interrelationships between avian coronavirus (GenBank), Brazilian chicken field samples (dark dot), and feral pigeons from this study (dark square) and from other studies (white square). Phylogenetic analyses were performed using neighbor joining method, Kimura two-parameter distance estimation method. Bootstrap resampling was performed for each analysis (1000 replications). The SARS coronavirus S1 genome virus was included as an outgroup.

pigeons. The D207 type was isolated since 2003 only in chickens. No correlation was observed between sample grouping and region origin.

Passage in SPF eggs. All samples used in this study, both those of chicken and pigeon, when passed in embryonated SPF eggs, caused lesions consistent with IBV, notably dwarfing and body curling.

Samples and country region. When the isolates were considered by region of origin, it was observed that of those from the southern region (9), six grouped with the Massachusetts type and three with the D207 cluster. Of those from the southeastern region (11), three grouped with the Massachusetts type, three with the D207 cluster, one with the Arkansas type, and one with the Connecticut type. Among the three samples from the northeastern region, two grouped with the Massachusetts type and one with the D207 cluster.

DISCUSSION

The genetic diversity found in the viruses isolated from chickens in this study was relatively higher than that reported by other molecular studies concerning Brazilian samples in which only viruses corresponding to the D207 group were described (44,45). These studies showed no Massachusetts group, perhaps due to the screening method chosen to evaluate only the samples that were amplified by RT-PCR of the UTR of their genomes. Thus, it is interesting to consider the possibility of differences in these and others genome regions among the different virus clusters found in Brazil; certainly others studies should be performed to verify this hypothesis. In a characterization of IBV isolated after an outbreak in Brazil in the late 19th century, a variety of serotypes was also observed including the presence of the Massachusetts type (10). Another retrospective Brazilian IBV study, using the restriction fragment length polymorphism technique and predicted N protein amino acid composition, also showed great diversity (classified in six genotypes), mainly after official vaccination (1). Besides these Brazilian cases, virus diversity has also been described in other countries (5,19,21,27,30,33). Recent studies involving the complete sequencing of coronavirus isolated from chickens have demonstrated the existence of viruses with different genomic organizations (11,26,29).

Of the 23 strains of chickens used in this study, 11 grouped with the Massachusetts serotype. This may represent a possible vaccine origin virus used for production of attenuated vaccines currently in Brazil. Our findings have shown lesions characteristic of IBV when passaged in embryonated SPF eggs (stunting and curling) and isolated from chickens with swollen head syndrome. All six isolates from pigeons have also caused the same injury when passed in chicken eggs SPF. Other investigations *in vivo* for studying the pathogenicity of these isolates for chickens certainly need to be made. In any case, even if the viruses isolated were from a vaccine origin, it is especially important to consider that most coronaviruses isolated from healthy pigeons showed regions of the gene coding for S1 with a similarity close to 100% with IBV isolates. One can conjecture that pigeons or other synanthropic birds, as well as wild ones, could actively participate in the eco-epidemiology of IBV.

These avian species could constitute a host range in which the virus can evolve, as well as a space for recombination between the wild-type coronavirus and IBV vaccine. As a consequence, new variants with different pathogenic potential may be produced (15). For example, in one study, coronavirus isolates with high similarity to the Massachusetts serotype were nonpathogenic to peafowls but pathogenic for chickens. In an earlier study, coronaviruses were encountered in seven wild birds (four ducks, one swan, one red knot, and one Eurasian oystercatcher); of these, three of the duck samples and the one from the swan grouped with the H120 (Massachusetts) vaccine strain, and those authors also conjectured about the possibility of these being revertant attenuated vaccine strains (17). Another study involving the S1 coronavirus gene isolated from pigeons also showed genetic and antigenic similarities with the Massachusetts genotype, but in this case the virus causes pancreatitis in this species (35). Yet another study found coronavirus different from the Massachusetts type in pigeons as well (22).

A small number of isolates have appeared only once since 2004, namely IBV/Brazil/2007/UNICAMP805, which grouped with the Connecticut type, and a sample IBV/Brazil/2008/UNICAMP830 which grouped together with the Arkansas type. This is noteworthy, because, to our knowledge, it has never been reported before. In Brazil, Massachusetts-type virus is used in preventive live vaccine. One explanation for the occurrence of the Arkansas and Connecticut serotypes could be the use of unauthorized live vaccines. Another important finding is the absence of isolated virions in pigeon samples within the D207 group. This could mean either that the virions are not adapted to pigeon or the number of samples was not large enough.

In this study the samples were collected in the main regions producing chicken and eggs in Brazil (south, southeast, and northeast) and in all these the presence of isolates from groups D207 and Massachusetts was observed. This shows that there is apparently no predominance of one or another group among the different regions of the country. Only in the southeastern region were isolates observed that were grouped with Arkansas and Connecticut types, an occurrence that is difficult to assess since only two viruses were found.

An overall investigation of the eco-epizootiology of IBV must include the possibility of virus evolution or transport by other animal species, especially those that often coexist in the farm environment. Moreover, the dynamics of the attenuated vaccine virus in the environment must also be considered for the control of avian infections. A better understanding of these issues would aid in the development of more effective vaccines and vaccination programs, as well as improved livestock health management practices. Also, in light of recent examples such as SARS and the newly discovered bat and wild mammal coronaviruses (34,40,46), a direct benefit would be the decreased emergence of new coronaviruses that pose a threat to the health of poultry and humans.

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Artigo II

Presence and phylogenetic studies of avian metapneumovirus (aMPV) recovered from feral pigeon and wild birds in Brazil.

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(aMPV in pigeons and wild avian species).

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Abstract

The aim of this study was to determine whether Avian Metapneomovirus (aMVP) related virus were present in wild and synanthropic birds in Brazil. Therefore, we analyzed samples from wild bird, feral pigeon and domestic chickens in order to perform phylogenetic comparison. To detect the presence of avian metapneumovirus, a nested RT-PCR was developed with the aim of amplifying a fragment of 270 bases for subtype A and 330 for subtype B, comprising gene coding G glycoprotein. Positive samples aMPV subtype A and B were found in 7 (13.2%) different asymptomatic wild birds and pigeon (50%) with were received to Bosque dos Jequitibás Zoo Triage Center, located in Brazil. Also analyzed, were positive samples from 15 (12.9%) domestic chickens with swollen head syndrome from several regions of Brazil. The positive samples from wild birds, pigeon and domestic chickens clustered in two major phylogenetic groups: some with aMPV subtype A and other with B. The similarity of G fragment nucleotide sequence, from aMPV isolated from chickens to no domestic avian species, ranged than 100 to 97.5% (100 to 95 for amino acid). Some positive aMPV samples with were taken from wild birds classified to the Orders *Psittaciformes*, *Anseriformes* and *Craciformes* clustered with subtype A and from the *Anas* and *Dendrocygma* genera (*Anseriformes* Order) to B. The understanding of the ecoepizootiology of the avian metapneumovirus is very important, especially if this involves the participation of nondomestic bird species, which would add complexity to their control in farms and implementation of vaccination programs.

Keywords: avian metapneumovirus, subtype A and B, phylogeny, feral pigeon, wild birds, Brazil

Introduction

Avian metapneumovirus belongs to the subfamily *Pneumovirinae* of the *Paramyxoviridae* family, of which four types (A, B, C and D) have been recognised based on genetic and serological properties (Juhasz & Easton, 1994; Bayon-Auboyer *et al*., 2000; Seal, 2000). Since the first detection in South Africa, in 1978, aMPV has induced infections in turkeys worldwide resulting in an acute rhinotracheitis, characterized by coughing, nasal discharge and conjunctivitis in turkeys (TRT). Moreover, aMPV has been also involved in the aetiology of multifactorial diseases such as swollen head syndrome in chickens (SHS) (Buys & De Preez, 1980; McDougall & Cook 1986; Pringle, 1999; van den Hoogen *et al*., 2001).

Isolates belonging to different subtypes of aMPV has been reported worldwild. Since the early 1990s, the great majority of aMPV detected have been of subtypes A and B, and were

reported in Israel (Banet-Noach, *et al*., 2005), Mexico (Decanini *et al*., 1991), Jordan (Roussan *et al*., 2008), Brazil (Dani *et al*., 1999; D'Arce *et al*., 2005), Japan (Tanaka *et al*., 1995; Mase *et al*., 2003), and many European countries (Giraud & Bennejean, 1986; Lister & Alexander, 1986; McDougall & Cook, 1986; Wilding *et al.*, 1986; Naylor & Jones, 1993; Naylor, *et al.*, 1997; Hafez, *et al*., 2000). Subtype C was first detected in the USA in 1996 and is more closely related to the human metapneumovirus (hMPV) than to any other aMPV (Govidanrajan & Samal, 2004, 2005; Govidanrajan *et al*., 2004; Toquin *et al*., 2003; Yunus *et al*., 2003; Lwamba *et al*., 2003; van den Hoogen *et al*., 2002; Graaf *et al*., 2008). Finally, the subtype D was once reported in France (Bayon-Auboyer *et al*., 1999).

Metapneumoviruses contain a non-segmented, negative-sense RNA with an approximately 13,000 nt long genome, and consists of eight viral genes arranged as follows: nucleoprotein – phosphoprotein – matrix – fusion - second matrix - small hydrophobic – glycoprotein - large polymerase (3'-N-P-M-F-M2-SH-G-L- 5'), flanked by a leader and trailer at the '3 and 5' ends, respectively (Drummond *et al*., 2002). The N protein intimately wrapped with the viral RNA genome forms a nucleoprotein complex (N-RNA) and becomes RNAses resistant. The N-RNA complex is an essential component of the polymerase complex (associated with the P and L proteins), which is responsible for the synthesis of all viral RNA, including mRNA, replicate intermediates, and the progeny RNA genomes (Easton *et al*., 2004).

The envelope of aMPV contains two glycoproteins, F protein, a disulphide-linked glycoprotein, and G protein with is responsible for the attachment of the virus particle to the receptor on the host cell (Drumond & Raumbaut, 2007). Comparing with the other proteins codified by the aMPV genome, G protein is known to be the most variable (Drummond *et al*., 2005), because it is a major target for neutralizing antibodies together with F protein (Dar *et al*., 2001).

After attachment of the virion to the cell surface, the F protein mediates the fusion of viral envelope and cell membrane. The F protein is highly conserved among aMPV isolates and the peptide is efficiently recognized by the chicken immune system, since the immunization with the F protein induces protective immune responses against virus challenge (Gonvidarajan *et al*., 2004).

In United States, approximately 80% of the aMPV (subtype C) outbreaks occur in spring, corresponding to the period of wild bird migration. This fact leads to hypothesis that wild birds

could be involved in transmitting the virus to poultry. In support of this, aMPV RNA was isolated from many wild avian species (Velayudhan, *et al*., 2008; Padhi & Poss, 2009). In Brazil (subtypes A and B) the role of wild and synanthropic birds in ecopidemiology of swollen head syndrome is not clear yet neither if the aMPVs like virions can be isolated from this avian species. This information could be of great importance for the control and prevention of this disease in this and other countries.

Materials and Methods

Samples from wild bird, feral pigeon and domestic chickens. Tracheal and cloacal swabs from 53 different wild free living species of birds and a total of 14 different pigeon were analyzed. The wild avian species and feral pigeons were received to Bosque dos Jequitibás Zoo Triage Center, located at Campinas City, São Paulo State in Brazil. The samples were collected immediately after the arrival of the birds (in the quarantine period). Both wild birds as pigeons had no symptoms of respiratory disease or swollen head syndrome at the moment of collection, which was held in the years between 2005 and 2008. In addition, were also analyzed samples from commercial poultry in order to perform the comparison between different groups of birds. Samples of tracheal swabs from 116 domestic chickens (*Gallus gallus domesticus*) with respiratory symptoms or swollen head syndrome were obtained from poultry main regions of Brazil between 2008 and 2009. All these samples were tested in parallel for infectious bronchitis virus (IBV), with a nested RT-PCR (Felippe *et al.*, 2010).

Virus samples. Viruses of two vaccines used commercially in Brazil, from subtype A (vaccine A) and another from subtype B (vaccine B) were recovered and submitted to the same RT-PCR and sequencing protocol of the samples from commercial chicken, wild birds and pigeons. The strains were propagated in Chicken Embryo Related (CER) cell line (Coswig, et al, 2010).

RNA Extraction and Viral Nucleic Acid Amplification. Swabs from both wild birds as pigeons and chickens were suspended in 1 ml of MEM (minimum essential medium, Sigma-Aldrich®) and centrifuged for 5 minutes at 3,500 x g to sediment the cellular debris. RNA was extracted from the supernatant using High Pure Viral Nucleic extraction kit™ (Roche Diagnostics™, Manheim, Germany). cDNA was synthesized using High Capacity cDNA kit

(Applied BiosystemsTM, Foster City, CA). Both procedures were performed according to the manufacturer's instructions for use with random hexamers. All samples were investigated for aMPV using amplification of specific genome fragments. The amplification of G protein gene was the target for polymerase chain reaction. The first step of nested RT-PCR was carried out with the forward primer, G1 (5' gggacaagtatct/cc/at/g 3') and the reverse primer, G6 (5'ctgacaaattggtcctgatt 3') (Juhasz & Easton, 1994; Cavanagh *et al*., 1999) corresponding to amplified products of 440 bp and was carried out with 50 µl mixture, containing 5 µl of 10X PCR buffer (10 mM Tris–HCl Ph 8.0, 50 mM KCl), 1.51, 5µl of $MgCl_2$ (0.2mM), 1.5µl of a 10 mM dNTPs mixture, 2.5 µl of each primer (10 mM), 5 µl of cDNA, and 0.2µl (5U/µl) of *Platinum*™ DNA Polymerase (Invitrogen Ltd., California, USA). In the amplification of first PCR reaction, the cDNA was pre-heated for 2 minutes at 94 ºC, following for 30 cycles, each of them composed of 30 seconds at 94 ºC (denaturation), 45 seconds at 58 ºC (annealing), and 1 min at 72 ºC (extension). After completion of the 30 cycles, a final extension of 7 min at 72ºC was performed. The nested PCR for aMPV subtypes A and B was the same, except only difference of the annealing temperature (57 ºC for A and 59 ºC for B subtypes). A second step of Nested PCR was carried out from the amplified products of template the first-round and using G8-A (5'cactcactgttagcgtcata 3') and G5 (5' caaagaa/gccaataagccca 3') primers, for subtype A and G8-B (5'tagtcctcaagcaagtcctc 3') and G5 for subtype B (Juhasz & Easton, 1994; Cavanagh *et al*., 1999). Amplifications were carried out in a thermal cycler PCR System 9700 (Gene Amp, Applied Biosystems, Perkin-Elmer, California, USA). The PCR products were run on 1% agarose gel and visualized under UV light after staining with ethidium bromide.

Sequencing and Phylogenetic Analysis. RT-PCR products were sequenced three times each, both in forward and reverse directions using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied BiosystemsTM). The BioEdit software, version 7.0.5.2 (Hall, 1999), was used to manipulate the nucleotide and amino acid retrieved sequences. The sequence alignments were performed using the Clustal W software, version 1.83 (Thompson *et al*., 1994) using full alignment and a number of 2,000 total replication on the bootstrap in order to ensure a higher level of confidence to our analysis (Thompson *et al*., 1994). Phylogenetic analyses were performed using neighborjoining as implemented in MEGA version 4 software package (Tamura *et al*., 2007) based on the Kimura two parameter distance estimation method. Bootstrap

resampling was performed for each analysis (500 replications). Reference aMPV nucleotide sequences were retrieved from the GenBank database.

Results|

Positive aMPV from wild bird, feral pigeon and domestic chicken. A total of 07 (13.2%) positive aMPV samples were detected from different wild species of free living birds in Brazil. Among these positive samples, 05 birds (four different species) were belong to subtype A (*Anas bahamensis, Penelope superciliaris, Aratinga leucophtalmus, Neochen jubata*) and 02 birds/species belong to subtype B (*Anas bahamensis*, *Dendrocygma viduata*). From feral pigeon, a total of 07 (50%) positive aMPV were detected from samples of different specimes of *Columba livia.* Among these positive samples, 02 belong to subtype A and 05 to subtype B. A total of 15 (12.9%) aMPV positive samples (01 belong to subtype A and 14 belongs to subtype B) were detected from commercial chicken from different regions in Brazil between 2008 and 2009 (table I). All these samples were negative by nested RT-PCR for Infectious Bronchitis Virus (IBV).

Similarity of studied G fragment from different samples. The similarity of G fragment nucleotide sequence, from aMPV subtype A, isolated from *Gallus gallus domesticus* (recovered in this study) to feral pigeons was 100%, from wild avian species ranged than 100 to 97.5 % (100 to 95 % for amino acid sequence). The similarity for nucleotide sequence to vaccine A subtype from chickens and nondomestic avian species isolates ranged than 99.1 to 96.6 % (97.5 to 92.5 % for amino acid sequence) (table II). In subtype B the similarity from chickens to feral pigeons ranged than 100 to 98.5 % (100 to 97 % for amino acid sequence), from wild avian species than 100 to 98.5 % (100 to 97 % for amino acid sequence). The similarity to subtype B vaccine from chickens and nondomestic avian species ranged than 100 to 99 % (100 to 98.5 % for amino-acid sequence) (table III).

Phylogenetic analysis. The majority of chicken´s samples clustered with subtype B (aMPV-B/Brazil/UNICAMP-874, aMPV-B/Brazil/UNICAMP-895, aMPV-B/Brazil/UNICAMP-886, aMPV-B/Brazil/UNICAMP-916, aMPV-B/Brazil/UNICAMP-871, aMPV-B/Brazil/UNICAMP-917ES, aMPV-B/Brazil/UNICAMP-870, aMPV-B/Brazil/UNICAMP-872, aMPV-B/Brazil/UNICAMP-817, aMPV-B/Brazil/UNICAMP-888, aMPV-B/Brazil/UNICAMP-880,

aMPV-B/Brazil/UNICAMP-889, aMPV-B/Brazil/UNICAMP- 897, aMPV-B/Brazil/UNICAMP-917) and only one (aMPV-A/Brazil/2009/UNICAMP-915) with subtype A. Two wild avian positive samples clustered together with subtype B UNICAMP (Dendrocygma/Brazil/2006/UNICAMP-35T; Anas/Brazil/2006/UNICAMP-48T). Only one chicken sample clustered with subtype A (aMPV-A/Brazil/2009/UNICAMP-915), but five wild avian species samples grouped in this cluster (Anas/Brazil/2005/UNICAMP-27C, Anas/Brazil/2006/UNICAMP-33T, Penelope/Brazil/2005/UNICAMP-3C, Aratinga/Brazil/2005/UNICAMP-10C, Neochen/Brazil/2006/UNICAMP-29C). With respect to samples from feral pigeons two clustered with A subtype (Columba/Brazil/2007/UNICAMP-60C; Columba/Brazil/2007/UNICAMP-67C) and five with B one (Columba/Brazil/2007/UNICAMP-61T; Columba/Brazil/2008/UNICAMP-PT4; Columba/Brazil/2008/UNICAMP-PC4; Columba/Brazil/2008/UNICAMP-PC5 e Columba/Brazil/2008/UNICAMP-PT7) (figure 1).

Sequences and submission GenBank number. chicken/A/BR/121/95 (AY842243.1); chicken/A/BR/119/95 (AY842242.1); aMPV/B/Brazil-07/USP-08G (EU140749.1); aMPV/B/Brazil-07/USP-06G (EU140747.1); aMPV/B/Brazil-07/USP-05G (EU140746.1); aMPV/B/Brazil-05/USP-03G (DQ786398.1); aMPV/B/Brazil-05/USP- 04G (DQ786399.1); aMPV/B/Brazil-05/USP-02G (DQ786397.1); aMPV/B/Brazil-05/USP-01G (DQ786396.1); aMPV/A/Brazil-05/USP-02G (DQ786395.1); aMPV/A/Brazil-07/USP-03G (EU140745.1); aMPV/A/Brazil-05/USP-01G (DQ667150.1); aMPV/B/Brazil-06/USP-07G (EU140748.1); chicken/B/Brazil/27A-07/2007 (FJ828952.1); chicken/B/Brazil/23-07P/2007 (FJ828951.1); chicken/B/Brazil/25A-07/2007 (FJ828953.1); strain Mn-1a (AY590692.1); strain Colorado (AY590691.1); C Minnesota 7 (AY198394.1); C Minnesota 2A (AY192393.1); FR/85/02 (AJ288946.1); FR/85/1 (AJ251085.1); strain 1556 (L34030.1); CVL14/1 (L34032.1); strain 872S (L34034.1); strain 6574 (L34033.1); strain 2119 (L34031.1) and Human IA23-2004 (DQ312464.2).

Discussion

The co-circulation to Metapneumovirus of subtypes A and B, in commercial flocks in Brazil were previously described in other studies (D´Arce *et al*., 2005; Chacón *et al*., 2007). In

this study was observed one higher amount of virus recovery from subtype B (n=14) than subtype A (n=1), suggesting that subtype B are more prevalent in Brazilian commercial breeding chickens or easier retrieval. All viral RNA came from chickens with swollen head syndrome or with respiratory symptoms with were previously tested for infectious bronchitis virus (all negative), suggesting that these viruses were pathogenic and not a vaccine strain.

The aMPV subtype B recovery in this study clustered in one group, distinct from other Brazilian samples. Only the isolated aMPV/B/Brazil-05/USP-01G (unpublished data, deposited in GenBank), grouped with the samples from this study, corroborating with Villarreal *et al.* (2009) that suggests that the subtype B in Brazil could be represented of at least two subpopulations in a possible geographic pattern.

The fragments of RNA amplified in samples from pigeons and wild birds, raise the discussion about the possibility of these viruses are Metapneumovirus, especially if presented 100% of similarity from chicken´s ones, in the gene that encoding the G protein, the most variable in Metapneumovirus genus. Even those samples that showed any difference clustered with subtype A or B in the phylogenetic tree.

In the specific case of samples with clustered to subtype A, free-living birds of different orders of Brazilian fauna were found with the virus (*Psittaciformes*, *Anseriformes* and *Craciformes*) representing 4 different zoological Genera (*Anas*, *Aratinga*, *Neochen* and *Penelope*) and in B only the Order *Aseriformes* family *Anatidae* with 2 genera (*Anas* and *Dendrocygma*). Except the *Anatidae* family, which are waterfowl the other avian species have distinct ecological niches. None of the species in which viruses were recovery belong to the list of migratory birds from IBAMA (Brazilian Official Organ Responsible for Fauna and Flora), but this list contains 4 species of the Genus *Anas.*

In the case of metapneumovirus subtype C, infection has been confirmed either by virus isolation, positive RT-PCR or detection of aMPV-specific antibodies in several wild bird species, including rock pigeons, Canada geese, blue winged teals, swallows, house sparrows, ring billed gulls, Muscovy and Pekin ducks, mallards, ostrich (Toquin *et al*., 1999; Shin *et al*., 2000; Shin *et al*., 2001; Bennett *et al*., 2002; Turpin *et al*., 2003; Bennett *et al*., 2004; Turpin *et al*., 2008). At the expense of large genotypic difference between subtype C and other, in the case of Brazil there was no information on the recovery of metapneumovirus in nondomestic birds, perhaps because that possibility had not been considered yet.

The similarities of RNA recovered from pigeons and wild animals with the vaccine strain (99.1 to 96.6 for A subtype and 100 to 99.5% for B), suggesting a provable environmental contamination from this attenuated virus. Previous studies demonstrated the persistence and loss of attenuation from vaccine strains in the environment after vaccination, which would favor the contamination of nondomestic birds (Catelli *et al*., 2006). The infection of birds other than the target species could represent different selective challenges for the vaccine virus, with could return for poultry production with a new pathogenic profile. Curiously a retrospective study of 20 years performed with subtype B in Italy and other European countries showed changes in the G protein of field strains after introduction of vaccine to this subtype, suggesting that the vaccine virus might somehow could interfered with the phenotype of field strain (Cecchinato *et al*., 2009).

Another possibility being considered was the virus recovery from wild birds, come from different origins and exhibit G protein similar to the aMPV. However, studies of evolution involving know avian Metapneumovirus (A, B, C, D) indicate that the similarity of the G is low, ranged from 5.6 to 28.7%, also suggest that, due to high rate of non-synonymous substitutions between isolates this is a positively selected gene (Alvarez *et al*., 2003). What makes remote the possibility of a virus originating from other bird species have similar G protein to the aMPV.

Certainly studies of pathogenicity in chickens and turkeys should be conducted, as well as study of other genes of virus recovered in this study.

Anyway the idea that they were metapneumovirus of subtype A and B, pathogenic or from vaccine origin, in wild birds is very important, which could demonstrate the importance of this group of animals in ecoepidemiology of swollen head syndrome of chickens or even rhinotracheitis in turkeys and the necessity to consider these factors not only to the choice of vaccines to be used, as well as those involving zoosanitary barriers that impede the access of other birds to the environment of farm animals.

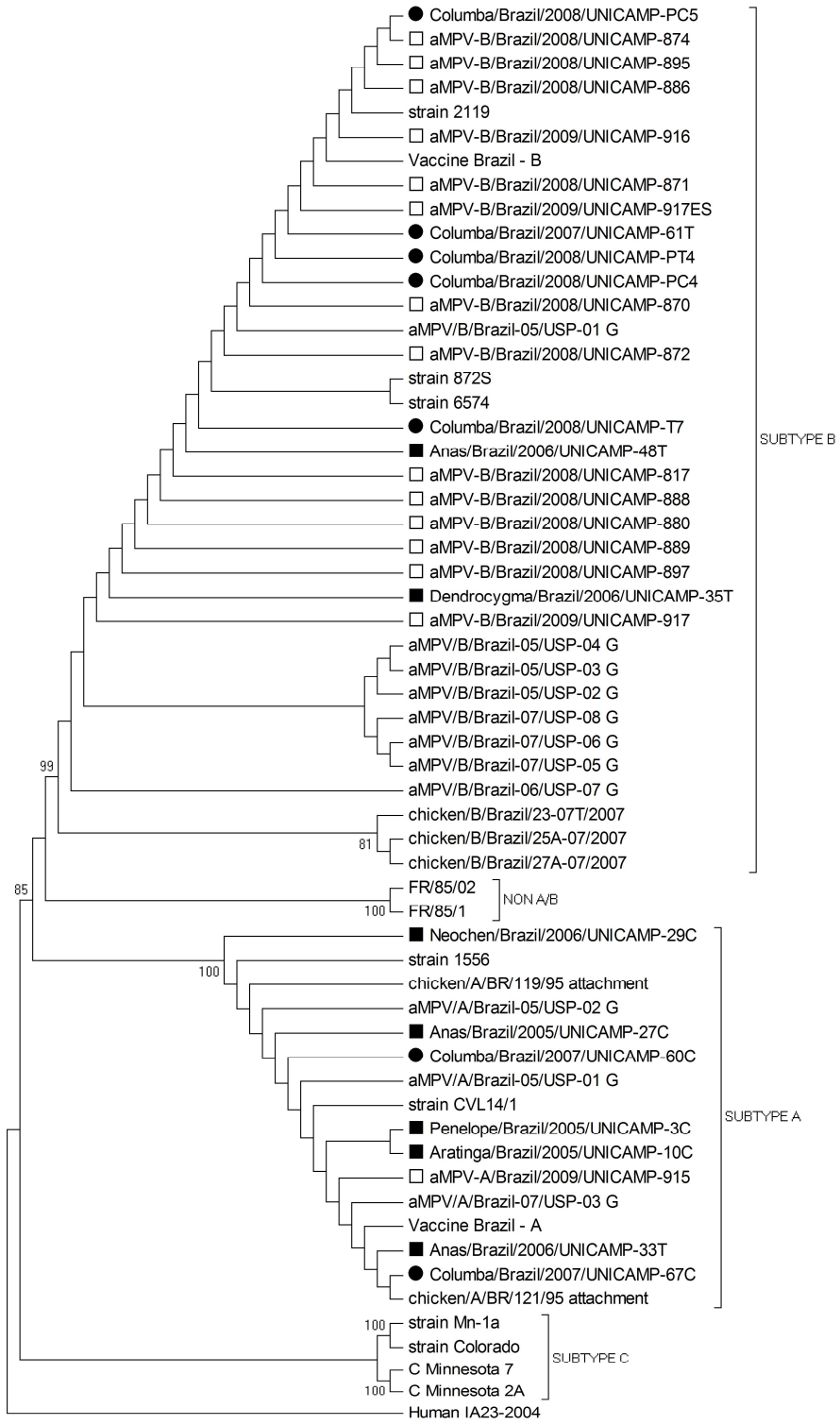

Figure 1. Phylogenetic tree showing partial G gene inter-relationships between avian metapneumovirus (from GenBank), brazilian chicken field samples (white square), feral pigeons (dark dot) and wild avian species (dark square).

Table I: Names and abbreviation from samples, corresponding avian species and aMPV subtypes.

Sample Name	**Abbreviation**	**Avian Species**	**Subtype Cluster**
Anas/Brazil/2006/UNICAMP-33T	33T	*Anas bahamensis*	A
Penelope/Brazil/2005/UNICAMP-3C	3C	*Penelope superciliaris*	A
Aratinga/Brazil/2005/UNICAMP-10C	10C	*Aratinga leucophtalmus*	A
Neochen/Brazil/2006/UNICAMP-29C	29C	*Neochen jubata*	A
Anas/Brazil/2005/UNICAMP-27C	27C	*Anas bahamensis*	A
Anas/Brazil/2006/UNICAMP-48T	48T	*Anas bahamensis*	B
Dendrocygma/Brazil/2006/UNICAMP-35T	35T	*Dendrocygma viduata*	B
Columba/Brazil/2007/UNICAMP-60C	60C	*Columba livia*	A
Columba/Brazil/2007/UNICAMP-67C	67C	*Columba livia*	A
Columba/Brazil/2007/UNICAMP-61T	61T	*Columba livia*	B
Columba/Brazil/2008/UNICAMP-PT4	PT4	*Columba livia*	B
Columba/Brazil/2008/UNICAMP-PC4	PC4	*Columba livia*	B
Columba/Brazil/2008/UNICAMP-PC5	PC5	*Columba livia*	B
Columba/Brazil/2008/UNICAMP-PT7	PT7	*Columba livia*	B
aMPV-A/Brazil/2009/UNICAMP-915	915	*Gallus gallus domesticus*	A
aMPV-A/Brazil/2009/UNICAMP-874	874	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-895	895	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-886	886	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-916	916	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-871	871	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-917 ES	917ES	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-870	870	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-872	872	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-817	817	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-888	888	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-880	880	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-889	889	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-897	897	*Gallus gallus domesticus*	B
aMPV-A/Brazil/2009/UNICAMP-917	917	*Gallus gallus domesticus*	B

Table II. Identity matrix of G gene partial nucleotide and amino acid (italic) sequences of metapneumovirus subtype A, from brazilian chicken field samples, feral pigeon and wild avian species.

Seq->	**Vac-A**	**915**	**60C**	**67C**	**29C**	**27C**	**33T**	**3C**	**10C**	**31T**
Vac-A	ID	*97.5*	*97.5*	*97.5*	*92.5*	*95.0*	*95.0*	*95.0*	*92.5*	*97.5*
915	99.1	*ID*	*100*	*100*	*95.0*	*97.5*	*97.5*	*97.5*	*95.0*	*100*
60C	99.1	100	ID	*100*	*95.0*	*97.5*	*97.5*	*97.5*	*95.0*	*100*
67C	99.1	100	100	ID	*95.0*	*97.5*	*97.5*	*97.5*	*95.0*	*100*
29C	96.6	97.5	97.5	97.5	ID	*92.5*	*92.5*	*92.5*	*90.0*	*95.0*
27C	98.3	99.1	99.1	99.1	96.6	ID	*95.0*	*95.0*	*97.5*	*97.5*
33T	98.3	99.1	99.1	99.1	96.6	98.3	ID	*95.0*	*92.5*	*97.5*
3C	98.3	99.1	99.1	99.1	96.6	98.3	98.3	ID	*92.5*	*97.5*
10C	97.5	98.3	98.3	98.3	95.8	99.1	97.5	97.5	ID	*95.0*
31T	99.1	100	100	100	97.5	99.1	99.1	99.1	98.3	ID

Note: In top of this table the sequences are writing only to the final numbers (Abbreviation).

Table III. Identity matrix of G gene partial nucleotide and amino acid (italic) sequences of metapneumovirus subtype B, from brazilian chicken field samples, feral pigeon and wild avian species.

Seq>	**V-B**	**817**	**886**	**880**	**870**	**871**	**872**	**874**	**888**	**889**	**895**	**897**	**917ES**	**917**	**916**	**61T**	**PT4**	**PC4**	**PC5**	**T7**	**34C**	**48T**	**41C**	**35T**
Vac-B	ID	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
817	99.5	ID	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
886	100	99.5	ID	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
880	100	99.5	100	ID	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
870	99.5	99.0	99.5	99.5	ID	*98.5*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
871	99.0	98.5	99.0	99.0	98.5	ID	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*97.0*	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*97.0*	*98.5*	*97.0*
872	100	99.5	100	100	99.5	99.0	ID	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
874	100	99.5	100	100	99.5	99.0	100	ID	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
888	99.5	99.0	99.5	99.5	99.0	98.5	99.5	99.5	ID	*100*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
889	100	99.5	100	100	99.5	99.0	100	100	99.5	ID	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
895	100	99.5	100	100	99.5	99.0	100	100	99.5	100	ID	*100*	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
897	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	ID	*100*	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
917 ES	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	ID	*100*	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
917	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	ID	*100*	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
916	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	ID	*98.5*	*100*	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
61T	99.5	99.0	99.5	99.5	99.0	98.5	99.5	99.5	99.0	99.5	99.5	99.5	99.5	99.5	99.5	ID	*98.5*	*98.5*	*98.5*	*98.5*	*98.5*	*97.0*	*98.5*	*97.0*
PT4	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	ID	*100*	*100*	*100*	*100*	*98.5*	*100*	*98.5*
PC4	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	100	ID	*100*	*100*	*100*	*98.5*	*100*	*98.5*
PC5	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	100	100	ID	*100*	*100*	*98.5*	*100*	*98.5*
T7	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	100	100	100	ID	*100*	*98.5*	*100*	*98.5*
34C	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	100	100	100	100	ID	*98.5*	*100*	*98.5*
48T	99.5	99.0	9.95	99.5	99.0	98.5	99.5	99.5	99.0	99.5	99.5	99.5	99.5	99.5	99.5	99.0	99.5	99.5	99.5	99.5	99.5	ID	*98.5*	*97.0*
41C	100	99.5	100	100	99.5	99.0	100	100	99.5	100	100	100	100	100	100	99.5	100	100	100	100	100	99.5	ID	*99.5*
35T	99.5	99.0	99.5	99.5	99.0	98.5	99.5	99.5	99.0	99.5	99.5	99.5	99.5	99.5	99.5	99.0	99.5	99.5	99.5	99.5	99.5	99.0	99.5	ID

Note: In top of this table the sequences are writing only to the final numbers (Abbreviation).

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5. Considerações Finais:

Sem dúvidas a biologia molecular abre novos horizontes ao estudo de muitos agravos à saúde tanto dos animais como do homem. O entendimento fenotípico da diversidade viral, através da utilização de anticorpos, recebe nos dias de hoje um importante reforço; o da avaliação das diferenças genotípicas que, sem dúvida oferece subsídios importantes à epidemiologia molecular e ao entendimento do surgimento de novos vírus ou mesmo do processo de adaptação destes para novos hospedeiros. A utilização de ferramentas de bioinformática sobremaneira amplia os horizontes e oferece uma base para um estudo mais detalhado e matematicamente confiável de sequências de nucleotídeos encontradas, assim como modelos de proteínas por eles gerados. O estudo do VBI e do aMPV, seguramente não foge a regra, e a literatura especializada dos últimos 10 anos nos traz uma sucessão de surpresas e consequentes avanços necessários para o entendimento da diversidade dos diferentes gêneros virais. O olhar sobre o VBI e o aMPV isolados de aves não domésticas, assim como aqueles vacinais mudou; hoje existe uma grande preocupação, como nos trabalhos de Vijaykrishna et al. (2007); Catelli et al. (2006); Dolz et al. (2008); em se entender qual seria a dinâmica destes vírus no ambiente. E se existe uma dispersão para outras espécies de aves, uma convergência adaptativa destes de outras aves para a galinha, ou mesmo qual seria o papel da recombinação e surgimento de mutações neste emaranhado de vírus aviários, para o surgimento de novas variantes. A primeira epidemia humana do século XXI, a síndrome aguda respiratória (SARS), atingiu cerca de 3.000 pessoas em 26 países diferentes, por ironia um coronavírus emergente, levou pesquisadores do mundo inteiro a estudar esta família e ao entendimento de que a ecologia destes é muito complexa e envolve várias espécies animais. De forma semelhante trabalhos como o de Nagaraja et al. (2007) mostra a capacidade do metapneumovirus humano, em infecções experimentais, causar doenças em perus, o que sem dúvidas agrega preocupação sobre um possível potencial zoonótico dos metapenmovirus aviários, principalmente aqueles do subtipo C. Neste cenário o entendimento da ecoepidemiologia das enfermidades respiratórias da galinha, além de importância em saúde pública, pode ser uma poderosa ferramenta para seu controle, principalmente se considerarmos a possibilidade da vigilância “epidemiológico-molecular”, afinada a localizar o surgimento e a origem de novos genótipos e lançar mão de uma série de

medidas, inclusive a de se adaptar as vacinas, de forma dinâmica, a cada nova realidade encontrada.

6. Conclusões:

- A presente pesquisa revelou que aves sinantrópicas (*Columba livia*) são portadoras de vírus assemelhados ao VBI. O estudo com o gene que codifica a proteína S_1 apresentou até 100% de identidade com o VBI isolado a partir de galinhas com sintomas de Bronquite Infecciosa.

- As amostras de pombas (*Columba livia*) mostrou que são portadoras de vírus assemelhados ao da vacina atenuada normalmente utilizada no Brasil. Apresentando até 100% de identidade no gene que codifica a porção hipervariável de S_1.

- A análise filogenética, com base no gene S_1 das amostras de pomba mostrou que a maioria se agrupou com o sorotipo Massachusetts e uma única amostra com o Connecticut.

- Nenhuma das pombas, em que foram detectados vírus assemelhados ao VBI apresentava sintomas clínicos de bronquite infecciosa.

- A análise filogenética baseada no gene que codifica o gene S_1 dos VBIs isolados de galinhas comerciais de diversas granjas do país, mostrou dois agrupamentos principais, um com o genótipo Massachusetts e outro com o D207. Além de uma amostra que se agrupou com Connecticut e outra com Arkansas.

- Em relação ao aMPV, as amostras de aves sinantrópicas (*Columba livia*) mostrou serem portadoras de vírus assemelhados ao aMPV dos subtipos A e B. O estudo com o gene que codifica a proteína G apresentou até 100% de identidade em relação aos isolados de galinhas com sintomas de Metapneumovirose aviária.

- No estudo filogenético do aMPV os vírus recuperados de *Columba livia* agruparam-se em dois clusteres; um com o subtipo A (n=2) e outro com o subtipo B (n=5).

- O estudo com as aves silvestres mostrou que cinco (05) espécies diferentes são portadoras de vírus assemelhados ao aMPV dos subtipos A e B, avaliados com genes que codificam a proteína G e apresentando até 100% de identidade com aqueles isolados de galinhas com sintomas de Metapneumovirose aviária.

- No estudo filogenético as amostras os vírus recuperados de aves silvestres brasileiras agruparam-se em dois clusteres; um com o subtipo A (n=5) e outro com o subtipo B (n=2).

- Alguns vírus recuperados de pombas, aves silvestres e galinhas apresentaram 100% de identidade com as cepas vacinais do aMPV atenuados que são utilizados na vacinação comercial de granjas no Brasil.

- Nenhuma das amostras de pombas, ou mesmo das aves silvestres em que se recuperou vírus assemelhados ao aMPV apresentavam sintomas de metapneumovirose aviária.

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