Universidade Federal do Rio Grande do Sul
Faculdade de Veterinária
Programa de Pós-graduação em Ciências Veterinárias

DETECÇÃO E ANÁLISE GENÔMICA DO VÍRUS DA ANEMIA INFECCIOSA DAS GALINHAS (CAV) E DO GIROVÍRUS AVIÁRIO 2 (AGV2)

Pós-graduanda: Ana Paula Muterle Varela
Orientador: Paulo Michel Roehe

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(Tese de doutorado)

Autora: Ana Paula Muterle Varela

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Ana Paula Muterle Varela

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Aprovada em 07 de abril de 2015

Aprovada por:

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Prof. Dr. Paulo Michel Roehe

Orientador e Presidente da Comissão

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Prof[a]. Dra. Ana Paula Guedes Frazzon

Membro da banca

_____

Prof[a]. Dra. Wilia M. E. Diederichsen de Brito

Membro da banca

_____

Prof. Dr. Diogenes Dezen

Membro da banca

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DETECÇÃO E ANÁLISE GENÔMICA DO VÍRUS DA ANEMIA INFECCIOSA DAS GALINHAS (CAV) E DO GIROVÍRUS AVIÁRIO 2 (AGV2)

RESUMO

O vírus da anemia infecciosa das galinhas (CAV) é um vírus pequeno, não-envelopado, com genoma circular de DNA simples fita. O vírus foi identificado pela primeira vez em 1979 no Japão e, por mais de 30 anos era o único girovírus conhecido por infectar aves. Em 2011, um novo girovírus, denominado girovírus aviário 2 (AGV2) foi identificado em soros de aves apáticas e com perda de peso. Posteriormente, genoma desse novo vírus foi identificado em aves saudáveis, bem como aves com sinais clínicos neurológicos. Especula-se que o AGV2 esteja mundialmente disseminado nos rebanhos avícolas, uma vez que genoma viral tem sido identificado em países geograficamente distantes. Tal identificação de AGV2 tem sido baseada em testes moleculares qualitativos. Dessa forma, no primeiro estudo realizado, foi desenvolvida uma duplex PCR em tempo real (dqPCR) visando detectar e quantificar simultaneamente genomas de CAV e AGV2. O ensaio mostrou ser sensível, específico e reproduzível, permitindo a detecção de no mínimo 5 cópias de genoma de CAV e 50 cópias de AGV2. Além disso, a dqPCR foi mais sensível que a técnica convencional quando aplicada para detecção destes agentes em amostras biológicas. O ensaio foi também utilizado para avaliar a presença de genomas de CAV e AGV2 em vacinas comerciais frequentemente utilizadas na avicultura. Trinta e cinco vacinas comerciais produzidas contra diferentes agentes patogênicos de aves foram analisadas. Os resultados obtidos mostram a presença de genoma de ambos os girovírus nas vacinas e enfatizam a importância da investigação aprofundada destes agentes em imunógenos. Adicionalmente, visando ampliar o conhecimento sobre o AGV2, dois estudos envolvendo análise genômica (parcial ou completa) foram realizados. Em um dos estudos, possíveis variações na sequência que codifica a proteína viral 1 (VP1) foram investigadas em amostras coletadas de aves com distintos *status* sanitários. Para tanto, penas coletadas de aves saudáveis e fígados provenientes de aves com apatia e baixo ganho de peso foram submetidos à extração de DNA e amplificação da região que codifica a VP1. Os produtos amplificados foram sequenciados e filogeneticamente analisados. Análise filogenética à nível de aminoácido deduzido permitiu a subdivisão das sequências de AGV2 em dois grupos maiores relacionados ao distinto *status* sanitário. Esses resultados podem estar associados à ocorrência de variantes do vírus, bem como à patogenicidade. Por fim, outro estudo foi realizado com o objetivo de detectar e caracterizar genomas de AGV2 presente em aves na Itália. Cem soros foram coletados de aves comerciais, aparentemente saudáveis, oriundas de 10 propriedades da região do Vêneto. As amostras de soro foram submetidas à extração de DNA e à detecção de AGV2 utilizando PCR. DNA viral foi identificado em 5 das 10 propriedades analisadas. Assim, um DNA extraído de soro representante de cada propriedade foi selecionado e submetido à amplificação do genoma completo de AGV2, sequenciamento e análise filogenética. Foi obtida uma sequência genômica completa e quatro sequências parciais do genoma. Os genomas aqui identificados apresentaram organização genômica igual aos demais girovirus conhecidos, com exceção dos girovírus 4 e 5. Além disso, a análise filogenética realizada mostrou que AGV2 e HGyV são filogeneticamente relacionados e formam um grupo distinto dentro do gênero *Gyrovirus*.

Palavras-chave: análise filogenética; girovírus; PCR em tempo real; vacina.

DETECTION AND GENOMIC ANALISYS OF THE CHICKEN ANEMIA VIRUS (CAV) AND AVIAN GYROVIRUS 2 (AGV2)

ABSTRACT

Chicken anemia virus (CAV) is a small, non-enveloped, circular single-stranded DNA virus. The virus was first identified in 1979 in Japan and, for more than 30 years, it was the only gyrovirus known to infect chickens. In 2011, a new gyrovirus, named Avian gyrovirus 2 (AGV2), was identified in sera from chicken displaying apathy and weight loss. Posteriorly, genome of this new gyrovirus was detected in healthy chickens as well as in chickens displaying neurological signs. It was speculated that AGV2 may have a widespread global distribution in poultry flocks, since its viral genome has been identified in geographical distant countries. The AGV2 identification has been based on the qualitative molecular tests. In this sense, in the first study performed, a duplex real-time PCR (dqPCR) was developed in order to detect and quantify, simultaneously, genomes of CAV and AGV2. The assay proved to be sensitive, specific and reproducible, allowing detection of a minimum of 5 copies of CAV and 50 copies of AGV2 genomes. Moreover, the dqPCR was more sensitive than conventional assay when applied for the detection of these agents in biological samples. The assay was also used to evaluate the presence of CAV and AGV2 genomes in commercially vaccines frequently used by poultry farming. Thirty five commercially vaccines produced against several avian pathogens were examined. The results obtained showed the presence of both gyroviruses genomes in vaccines and emphasize the importance of the further investigation about these agents in immunogens. In addition, in order to enhance understanding about AGV2, two other studies involving genomic analysis (partial or complete) were performed. One of the studies was carried out to investigate variations in the gene coding the viral protein 1 (VP1) from chickens samples with distinct healthy status. Thus, feathers collected from apparently healthy chickens and liver tissues collected from chickens displaying signs of apathy and low weight gain were submitted to DNA extraction and amplification of the genomic region that codes VP1. The amplification products were sequenced and phylogenetically analyzed. Phylogenetic analysis at predicted amino acid level allowed subdivision of AGV2 sequences in two major groups associated with healthy status. These findings may reflect to existence of variants of AGV2, as well as may have correlation with pathogenicity of virus. Lastly, an additional study was performed to detect and to characterize AGV2 genomes present in chicken from Italy. One-hundred serum samples were collected from healthy commercial chickens from 10 poultry farms. Serum samples were submitted to DNA extraction and to AGV2 detection by PCR. Viral DNA was identified in 5 out of 10 farms sampled. So, one DNA sample representative of each farm was selected and submitted to AGV2 complete genome amplification, sequencing and phylogenetic analysis. It was recovered one complete genome and four partial AGV2 genomes (about 83%). The genomes here identified have the same genomic organization of others gyroviruses, with exception to gyrovirus 4 and 5. Moreover, phylogenetic analysis revealed that AGV2 and human gyrovirus are phylogenetically related and form a distinct group within the *Gyrovirus* genus.

Keywords: gyrovirus; phylogenetic analysis; real-time PCR; vaccine

SUMÁRIO

INTRODUÇÃO

O Brasil é o terceiro maior produtor de frangos do mundo, estando sua produção concentrada principalmente na região sul (Paraná, Santa Catarina e Rio Grande do Sul). Embora aproximadamente 70% da produção seja destinada ao mercado interno, o país é o maior exportador mundial de carne de frango. Nos últimos quatro anos, a produção de carne de frango manteve-se em cerca de 13 milhões de toneladas por ano (UNIÃO BRASILEIRA DE AVICULTURA, 2014). Em virtude da expressiva produção de aves e visando a sanidade avícola, medidas de vigilância e defesa sanitária vêm sendo adotadas pelo Ministério da Agricultura Pecuária e Abastecimento (MAPA, 2015).

Apesar do controle sanitário aplicado na avicultura comercial, alguns agentes infecciosos continuam presentes, gerando grandes perdas econômicas. Dentre estes, encontra-se o vírus da anemia infecciosa das galinhas (CAV). A infecção por este agente causa aplasia da medula óssea, ocasionando anemia, imunodepressão e mortalidade em aves jovens, refletindo em significativas perdas econômicas para a avicultura (LUCIO et al., 1990).

Por mais de 30 anos, o CAV foi o único membro classificado no gênero *Gyrovirus*. Em 2011, um novo membro desse gênero foi identificado em aves. Este novo girovírus foi detectado a partir da análise de soros de frangos que apresentavam sinais clínicos semelhantes aos causados pelo CAV. Naquela ocasião, amostras de DNA extraído dos soros foram submetidas à amplificação por circulo rolante com a DNA polimerase do fago phi29 e com múltiplos "primers" (ACRMP). Como resultado foi obtida amplificação de um DNA com tamanho aproximado de 2,4 kb. A análise da sequência do produto amplificado mostrou que apenas 40% dos nucleotídeos possuíam similaridade com o genoma do CAV. Dada a baixa homologia, o genoma recém-identificado foi atribuído a um novo vírus, o qual foi denominado girovírus aviário 2 ou, "*avian gyrovirus* 2" (AGV2) (RIJSEWIJK et al., 2011).

Devido à recente detecção desse agente, ainda são desconhecidos dados relativos à epidemiologia, patogenia e possíveis sinais clínicos que possam estar associados a infecções com esse vírus. Especula-se a possibilidade de um efeito sinérgico entre o AGV2 e outros agentes em co-infecções, mas ainda sem confirmação experimental (ABOLNIK & WANDRAG, 2014). Além disso, até o momento, o vírus não foi isolado e ainda não estão disponíveis testes sorológicos para avaliar a resposta imune induzida por este vírus. Assim, a forma de detecção do agente tem sido restrita à pesquisa de

genomas (DOS SANTOS et al., 2012; CHU et al., 2012; PHAN et al., 2012). Essa situação reforça a necessidade da busca por alternativas eficazes que permitam aprofundar o conhecimento sobre esse vírus e evidencia a importância do aprimoramento e desenvolvimento de novas técnicas de diagnóstico e pesquisa. Com esse objetivo, um dos estudos aqui apresentado visa o desenvolvimento de uma PCR em tempo real multiplex, para simultânea detecção de CAV e AGV2. A partir disso, buscou-se aplicar essa técnica para avaliar possíveis fontes de disseminação destes agentes. Adicionalmente, visando ampliar o conhecimento sobre o AGV2, dois estudos envolvendo análise genômica (parcial e completa) foram realizados. Em um dos estudos buscou-se investigar uma possível relação entre variabilidade genômica e patogenicidade. No outro, objetivou-se detectar e caracterizar genomas de AGV2 isolados de aves oriundas da Itália, baseado na possibilidade do vírus estar presente em países europeus.

1. REVISÃO BIBLIOGRÁFICA

1.1. Histórico dos girovírus

O vírus da anemia infecciosa das galinhas (CAV) foi isolado e descrito pela primeira vez em 1979 em frangos de produção comercial (YUASA et al., 1979). Desde então, o vírus tem sido detectado por isolamento ou sorologia na maioria dos países, tanto em aves de postura quanto de corte (LUCIO, et al. 1990; HOOP, 1992; BUSCAGLIA, et al. 1994; SCHAT, 2003; TORO, et al., 2006; GHOLAMI-AHANGARAN & ZIA-JAHROMI, 2012; SNOECK et al., 2012; SHARMA et al., 2014). No Brasil, o primeiro isolamento do CAV foi reportado por Brentano e colaboradores (1991), em frangos de corte com atrofia de timo, anemia e pouco ganho de peso, gerando grandes perdas econômicas para a indústria avícola.

Em 2011, o genoma de um novo vírus relacionado com o CAV foi descrito por nosso grupo (RIJSEWIJK et al., 2011). O genoma viral foi detectado a partir de soros de frangos de criação industrial no Estado do Rio Grande do Sul que apresentavam apatia e perda de peso (RIJSEWIJK, et al., 2011). Naquela ocasião, na tentativa de identificar possíveis agentes envolvidos com os sinais clínicos observados, como o CAV, por exemplo, o DNA extraído de soros dessas aves foi submetido à amplificação por círculo rolante com múltiplos primers (ACRMP). Os produtos amplificados foram sequenciados e comparados às sequências genômicas disponíveis em um banco de dados (GenBank). Como resultado, foi observado que se tratava de um novo vírus, o qual foi denominado girovírus aviário 2, para distingui-lo do CAV, até então, o único girovírus conhecido (RIJSEWIJK et al., 2011). Posteriormente, genomas de AGV2 foram descritos em outras espécies de aves e em outros mamíferos (CHU et al., 2012; LIMA et al. 2012; PHAN et al., 2013; FEHÉR et al., 2014).

Ainda em 2011, um novo girovírus foi reportado na França, a partir da análise de suabe de pele de pessoas saudáveis e sem lesões. Este novo agente foi denominado girovírus humano (HGyV; SAUVAGE et al., 2011). Posteriormente, um estudo desenvolvido na Itália envolvendo HGyV mostrou a presença de genoma viral em amostras de sangue de pacientes transplantados e em um paciente infectado pelo vírus da imunodeficiência humana (HIV), mas em nenhuma amostra oriunda de pessoas saudáveis (MAGGI et al., 2012). Contrariamente, em um estudo realizado na França, DNA de HGyV foi detectado em amostras de sangue de doadores saudáveis, porém em

baixa frequência (BIAGINI et al., 2013). Adicionalmente, genoma do HGyV foi identificado em amostras de fezes humanas em casos de diarreia, bem como em fezes de furão doméstico (*Mustela putorius furo*) (CHU et al., 2012; PHAN et al., 2012: FEHÉR et al., 2014).

Após a identificação do AGV2 e HGyV, novos girovírus foram descritos: o girovírus tipo 3 (GyV3), o girovírus tipo 4 (GyV4), girovírus 5 (GyV5) e 6 (GyV6); o girovírus 7 (GyV7-SF), girovírus 8 (GyV8) e girovírus 9 (GyV9). O GyV3 foi descrito no Chile durante uma análise metagenômica de fezes de crianças com diarreia (PHAN et al., 2012). Na análise, genomas de CAV, AGV2 e HGyV também foram detectados tanto nas amostras provenientes de casos de diarreia como nas fezes de crianças saudáveis analisadas. O GyV4 foi identificado em fezes humanas oriundas de casos de diarreia em Hong Kong e fezes de pacientes imunocomprometidos nos Estados Unidos. O agente também foi detectado em frangos, principalmente na pele, comercializados para consumo humano em Hong Kong (CHU et al., 2012). Da mesma forma que o GyV3 e GyV4, os GyV5 e GyV6 foram identificados em amostras de fezes, nesse caso, oriundas de crianças com gastroenterite aguda na Tunísia (PHAN et al., 2013). Em 2014, um novo girovírus, denominado girovírus 7 (GyV7-SF), foi relatado como resultado de metagenômica viral em carne de frango. Nesse mesmo estudo, foram também identificados todos os girovírus descritos até o momento (ZHANG et al., 2014). Em 2015, dois novos girovírus foram identificados: GyV8 e GyV9. O GyV8 foi identificado a partir da análise por metagenômica de tecido glandular e baço de uma ave aquática, Fulmar glacial (*Fulmarus glacialis*), encalhada em São Francisco, Califórnia (LI et al., 2015). Da mesma forma, o GyV9 foi identificado como resultado de metagenômica viral, entretanto a análise foi realizada a partir de fezes de adultos com diarreia e febre (PHAN et al., 2015)

1.2. Etiologia e características moleculares

Os membros da família *Circoviridae* caracterizam-se por possuir um genoma de DNA pequeno (1.7-2.3 kb), circular, de fita simples, envolto por um nucleocapsídeo icosaédrico não envelopado, com 14 a 26 nm de diâmetro. A família *Circoviridae* é composta por dois gêneros: *Circovirus* e *Gyrovirus*. O gênero *Circovirus* compreende 11 diferentes espécies, dentre as quais são encontrados importantes patógenos de aves e suínos (International Committee on Taxonomy of Viruses – ICTV, 2015). Por outro

lado, o gênero *Gyrovirus* compreendia, até recentemente, somente o vírus da anemia infecciosa das galinhas (CAV). No entanto, com a descoberta dos novos girovírus, outras espécies estão sendo incluídas neste gênero, dentre elas o AGV2.

Contrariamente aos vírus do gênero *Circovirus*, os representantes do gênero *Gyrovirus* possuem genoma de sentido negativo (NOTEBORN et al., 1991). O genoma do CAV é constituído por 2298 ou 2319 nucleotídeos (nt) de acordo com a presença de quatro ou cinco sequências repetidas direta de 21 nt localizada na região promotora (CLAESSENS et al., 1991; NOTEBORN et al., 1991). Além das sequências repetidas diretas, existe uma sequência "TATA Box" na posição 324 da região promotora. Todas essas sequências, compreendidas entre o nucleotídeo 1 a 324, funcionam como elementos regulatórios da replicação viral e constituem assim, a porção não transcrita do genoma (NOTEBORN et al., 1991; BRENTANO, 2000). Adicionalmente, o genoma de CAV contém um sinal de poliadenilação localizado na posição 2287.

Em relação ao AGV2, o genoma é composto por 2383 nt. O vírus possui uma região 5´ supostamente não traduzida (5´UTR) com cerca de 400 nt, localizada entre o sítio de poliadenilação e o início da transcrição, a qual contém seis regiões repetidas diretas (RD) de 22 nucleotídeos. Além disso, dois sinais de poliadenilação (AATAAA) foram encontrados nas posições 27 e 39 do AGV2, onde nas posições homólogas no genoma do CAV existe somente uma sequência AATAAA. Igualmente ao CAV, uma suposta sequência "TATA box" (GATATAAG) foi identificada no genoma do AGV2 entre os nucleotídeos 415 e 422. Um sítio de início de transcrição foi postulado no nucleotídeo 440, o qual está localizado 10 nucleotídeos antes do início da fase de leitura 1 ou ORF1 (nucleotídeo 450) (RIJSEWIJK et al., 2011).

Tanto para o CAV quanto para o AGV2, seguindo a região 5´UTR, os genomas possuem três fases de leitura (ORF) parcialmente sobrepostas: ORF1; ORF2; ORF3 (Figura 1). Estas, por sua vez, são responsáveis pelas três maiores proteínas virais, codificadas no sentido senso do genoma: VP2, VP3 e VP1, respectivamente (NOTEBORN et al., 1991; SCHAT, 2009; RIJSEWIJK et al., 2011).

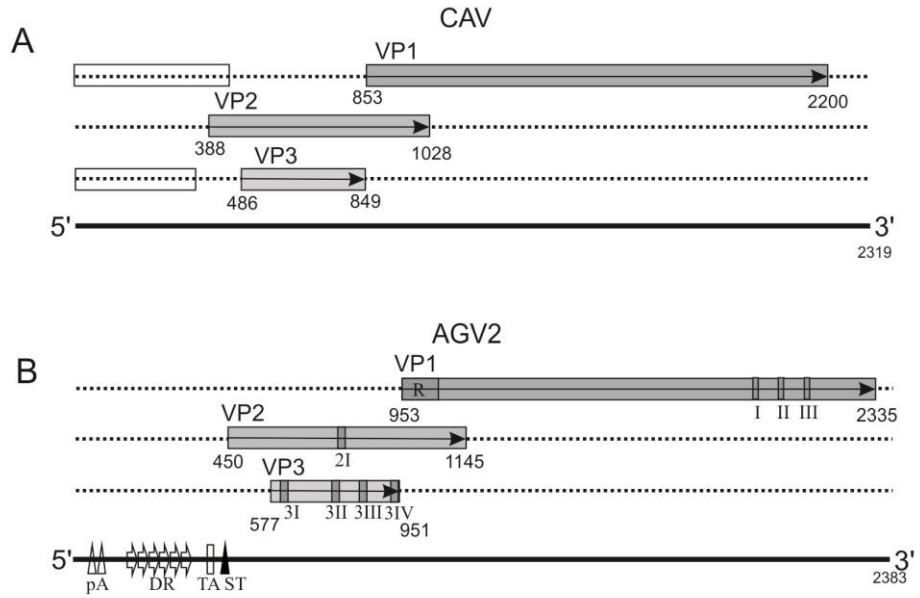

Figura 1. Representação esquemática da organização genômica de CAV e AGV2. A) Representação simplificada das ORFs de CAV indicando, através das barras horizontais cinzas, as regiões que codificam as proteínas virais. B) Organização genômica do AGV2 na qual as barras horizontais na parte superior representam os tamanhos e posições da VP1, VP2 e VP3. As regiões realçadas dentro das barras representam as posições dos motivos conservados. A linha horizontal na parte inferior representa o tamanho do genoma do AGV2 (2383 nt) indicado pelas setas verticais. As siglas pA, DR, TA, ST representam as posições do sinal de poliadenilação, regiões repetidas diretas, o TATA box e o sítio de início da transcrição, respectivamente. Adaptado de NOTEBORN et al., 1991 and RIJSEWIJK et al., 2011.

Apesar do AGV2 ser um vírus relacionado ao CAV e com semelhante organização, a comparação entre as sequências completas de nucleotídeos revelou homologia de aproximadamente 40% entre estes vírus (RIJSEWIJK et al., 2011). Assim como o AGV2, os demais girovírus até o momento descritos apresentam o mesmo tipo de organização genômica, com exceção do GyV4 e GyV5, para os quais não foi descrita uma sequência correspondente ao gene que codifica a proteína VP3 (Figura 2). Especula-se a possibilidade de que o suposto vírus humano seja oriundo do contato ou ingestão de carne de frango infectada com AGV2 (CHU et al., 2012).

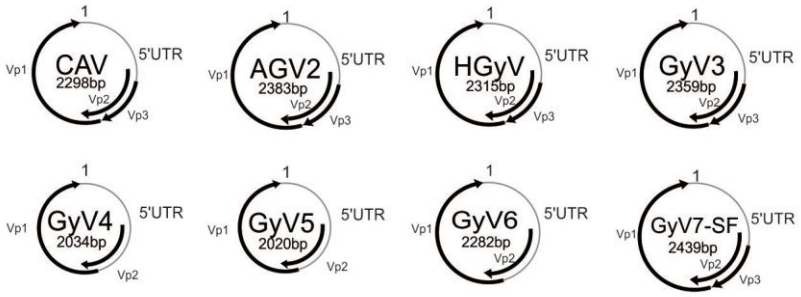

Figura 2. Representação esquemática da organização genômica dos girovírus descritos até 2014: CAV, AGV2, HGyV, GyV3, GyV4, GyV5, GyV6 e GyV7. Adaptado de PHAN et al., 2012.

1.3. Proteínas virais dos girovírus

Conforme mencionado, o genoma de CAV codifica três proteínas: VP1, VP2 e VP3. Essas proteínas são expressas em momentos diferentes durante a infecção. As proteínas VP2 e VP3 têm sido detectadas 12 horas pós-infecção, sugerindo que sejam precocemente produzidas pelo vírus durante o processo de infecção e replicação, enquanto que, a VP1 surge mais tardiamente, sendo detectada apenas 30 horas pós-infecção (DOUGLAS et al., 1995; SCHAT, 2009). Equivalentes ou análogos a essas proteínas virais também são codificadas nos genomas dos demais girovírus, com exceção do GyV4 e GyV5 para os quais não foi detectada a VP3 (SAUVAGE et al., 2011; PHAN et al., 2012; CHU et al., 2012). Para o AGV2 nenhuma proteína foi expressa e caracterizada, até o momento. Dessa forma, as informações disponíveis sobre as proteínas do AGV2 fazem analogia ao que é conhecido sobre o CAV.

A ORF1 do CAV codifica uma proteína não estrutural de 28 kDa denominada VP2, a qual é uma proteína tirosina fosfatase (*protein-tyrosine phosphatases* – PTPase) com dupla especificidade, que catalisa a remoção da fosfatase dos substratos da fosfoserina, treonina e tirosina (PETERS et al., 2002, PETERS et al., 2005). As fosfatases atuam na regulação da mitogênese, transcrição gênica, interações celulares e na resposta de citocinas dos linfócitos (ONG et al., 1997). Nesse contexto, especula-se que a atividade fosfatase da VP2 atuaria na indução de mudanças regulatórias na população de linfócitos infectados, favorecendo a replicação viral (PETERS et al., 2002; PETERS et al., 2006). Além disso, acredita-se que a VP2 seja uma proteína multifuncional, atuante na infecção, montagem e replicação do vírus (PETERS et al.,

2002). Esta proteína possui um suposto sinal de localização nuclear (NLS) e acumula-se em grande quantidade no núcleo das células infectadas (DOUGLAS et al., 1995; ADAIR, 2000). No entanto, até o momento, o mecanismo específico para a localização celular da VP2 não é bem compreendido.

Apesar da baixa identidade (40,3%) com a VP2 do CAV, a ORF1 do AGV2 também codifica a VP2, uma proteína com 231 aminoácidos que apresenta graus de similaridade variando entre 15 a 96% com os demais girovírus (CHU et al., 2012, PHAN et al., 2013). Apesar de não ter sido bem investigada, acredita-se que a VP2 do AGV2 desempenhe a mesma função da proteína do CAV uma vez que, ela contém uma sequência (CX_5R) que é altamente conservada em todas as PTPase.

A ORF2 do CAV codifica uma proteína 11 a 13 kDa denominada VP3 ou apoptina. Essa proteína é capaz de induzir apoptose de células hematopoiéticas e pode ser a responsável pela anemia observada em aves infectadas com CAV (BULLENKAMP et al., 2012). Durante a infecção de células mononucleares, a apoptina inicia uma dispersa distribuição intranuclear granular (NOTEBORN et al., 1994). Nos estágios avançados da infecção viral esta distribuição progride para a formação de agregados nucleares, fazendo com que as células se tornem apoptóticas; o DNA celular é fragmentado e condensado (LOS et al., 2009).

A apoptina possui a capacidade de desencadear apoptose de células tumorais, porém não de células normais (LOS et al., 2009; NOTEBORN, 2009). Essa toxicidade seletiva é atribuída a sua diferencial localização subcelular em células tumorais e normais. Nas células transformadas, a apoptina é encontrada predominantemente no núcleo, enquanto que nas células normais está presente principalmente no citoplasma (DANEN-VAN OORSCHOT et al., 1997; ORO & JANS, 2004; MADDIKA et al., 2005). O fato de a proteína ser encontrada tanto no núcleo quanto no citoplasma sugere que ela possui capacidade de se deslocar para dentro e para fora do núcleo. Essa mobilidade provavelmente advém do fato de a proteína possuir, na região C-terminal, uma sequência de sinal de localização nuclear bipartida (NLS1 e NLS2) e uma suposta sequência de exportação nuclear (NES) rica em leucina. Essas sequências de reconhecimento direcionariam a entrada e saída da apoptina através de poros nucleares (DANEN-VAN OORSCHOT et al., 1997; MOROIANU, 1999). Embora não completamente elucidado o mecanismo pelo qual a apoptina diferencia células tumorais das normais, a especificidade por células transformadas tornou esta proteína uma ferramenta promissora na terapia gênica contra o câncer (MADDIKA et al., 2006).

Vários estudos têm explorado tal ferramenta através da expressão da VP3 em diferentes sistemas de expressão com resultado antitumoral *in vivo* eficaz (LELIVELD et al., 2003; LACORTE et al., 2007; PAN et al., 2010).

Igualmente ao CAV, a ORF2 do AGV2 codifica uma sequência de 124 aminoácidos, correspondente à VP3. Esta sequência compartilha similaridade de 32,2% com CAV e variação na similaridade de 39,5% a 92,7% com as regiões equivalentes à sequência de aminoácidos dos demais gyrovírus (RIJSEWIJK et al., 2011; PHAN et al., 2013). Até o momento, poucas são as informações disponíveis sobre VP3 e seu caráter apoptótico. Recentemente, visando verificar a provável função proapoptótica da apoptina de HGyV, a proteína VP3 foi sintetizada fusionada com a proteína verde fluorescente (GFP) e transfectada em células carcinoma humano de colo (HCT116) e osteosarcoma. Assim como para CAV, a apoptina translocou para o núcleo com distribuição granular característica, seguida pela formação de agregados e levando a apoptose. O nível de morte celular induzido pela apoptina do HGyV foi similar ao induzido pela proteína do CAV (BULLENKAMP et al., 2012; CHAABANE et al., 2014). Adicionalmente, a proteína foi testada em fibroblastos humanos normais e em homólogos transformados com *Simian virus 40 large T antigen* (SV40-LT). No estudo foi confirmado que a apoptose ocorreu somente nos fibroblastos transformados estabelecendo, dessa forma, o caráter apoptótico da proteína do HGyV (BULLENKAMP et al., 2012). Por similitude ao HGyV, esse caráter provavelmente deve estar presente na VP3 do AGV2 uma vez que, o grau de identidade entre ambos os vírus é superior a 90%.

Em relação a ORF3 do CAV, esta codifica uma proteína estrutural de 52 kDa, denominada VP1, a qual forma o capsídeo das partículas virais (NOTEBORN et al., 1998). Substituições em nível de amino ácido podem desempenhar um papel importante na taxa de crescimento e patogenicidade do vírus (RENASCHAW et al., 1996). Além disso, a VP1 possui epítopos responsáveis pela reação imune que podem aumentar a pressão seletiva sendo assim, a região com maior variabilidade. Devido a essa variabilidade, o gene que codifica a VP1 tem sido alvo de investigações referentes à diversidade genética entre os isolados de CAV. Adicionalmente, essa proteína está associada com indução de anticorpos neutralizantes nos hospedeiros infectados. Tal característica torna a VP1 a principal candidata a antígeno para o desenvolvimento de vacinas e testes diagnósticos (NOTEBORN et al., 1998). Nesse contexto, diferentes sistemas de expressão, como baculovírus, células de plantas e *Escherichia. coli*, têm

sido utilizados com o intuito de produzir essa proteína (LACORTE et al., 2007; LEE et al., 2011; PALOMARES et al., 2012).

Assim como o CAV, a ORF3 do AGV2 também codifica a VP1, uma proteína de 460 aminoácidos que apresenta similaridade de 38,8% com a proteína do CAV e variação de 28% a 97% de similaridade com os demais girovírus (RIJSEWIJK et al., 2011; CHU et al., 2012; PHAN et al., 2013). Igualmente a outras proteínas de capsídeo, a VP1 é iniciada com uma região rica em arginina e lisina. No entanto, motivos de replicação conservados, identificados na VP1 de CAV, apresentam variações nas posições homólogas ao AGV2. Até o momento, a proteína VP1 do AGV2 ainda não teve sua expressão reportada. A dificuldade de expressão dessa proteína tem dificultado o desenvolvimento de testes sorológicos que permitam acompanhar a evolução da infecção, tanto individualmente como em rebanhos, pelo monitoramento das respostas sorológicas induzidas pelo vírus. Da mesma forma, análise molecular envolvendo a região que codifica tal proteína tem sido pouco explorada.

1.4. Replicação Viral

Inicialmente será descrita a replicação do CAV, visto que o ciclo replicativo é bastante conhecido. Em relação ao AGV2, especula-se que a replicação ocorra de forma semelhante ao CAV embora, dados biológicos sobre o agente ainda não foram descritos (Figura 3).

A replicação do genoma do CAV ocorre no núcleo da célula hospedeira, na fase S do ciclo celular (LOVATO & HENZEL, 2012). Acredita-se que o vírus replica seu DNA usando um mecanismo de replicação por circulo rolante (RCR) (TODD et al., 2001; BIAGINI et al., 2012).

Após a fase de adsorção e penetração na célula, o vírus, cujo genoma consiste de DNA fita simples de polaridade negativa, inicia uma fase replicativa na qual é sintetizada uma fita de DNA de polaridade positiva (Figura 3). Essa nova fita é complementar ao DNA viral, formando um DNA replicativo intermediário de fita dupla (BRENTANO, 2009). Após a sua síntese, o DNA replicativo é transcrito em um RNA mensageiro (mRNA) de 2,1 Kb. Este mRNA é policistrônico e contém três ORFs sobrepostas entre si, cada uma codificando uma das três proteínas do CAV: VP1, VP2 e VP3. A região promotora é responsável por sinalizar no DNA a localização de início e os níveis de transcrição do RNA. A partir do DNA replicativo intermediário, são

produzidas moléculas de DNA de fita simples circulares, correspondentes ao DNA genômico. Essas moléculas são encapsideadas por múltiplas cópias da proteína VP1 (CIACCI-ZANELLA & BRENTANO, 2012).

Inicialmente, acreditava-se que CAV transcrevia um único RNA mensageiro sem sofrer *splice*, mas estudos recentes tem demonstrado a ocorrência de *splice* para obtenção de vários transcritos durante o ciclo de vida do vírus (KAMADA et al., 2006).

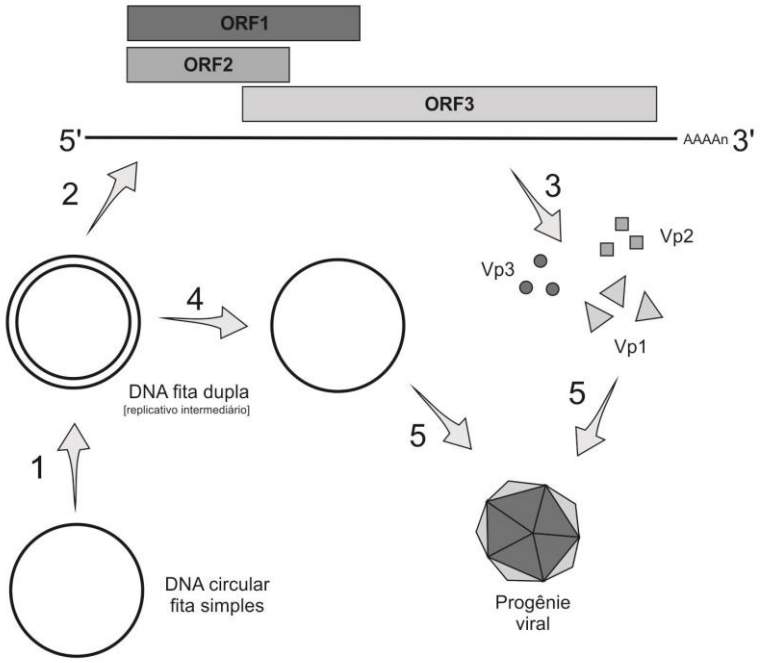

Figura 3. Ilustração esquemática do ciclo replicativo do CAV. A etapa inicial consiste na síntese de uma fita de DNA complementar ao DNA genômico (1). O DNA de fita dupla (replicativo intermediário) é transcrito pela maquinaria celular e origina um RNA mensageiro (mRNA) de 2,1 Kb (2). Este mRNA é traduzido e codifica as proteínas virais (3). O DNA de fita dupla serve de molde para a replicação, com a produção de cópias genômicas do DNA (4). Este DNA é então encapsidade por múltiplas cópias da VP1 (5). Fonte: CIACCI-ZANELLA & BRENTANO, 2012 - Adaptado de BRENTANO, 2009.

1.5. Transmissão, patogenia e sinais clínicos

1.5.1. CAV

A transmissão do CAV ocorre principalmente de forma vertical, da matriz ao embrião no ovo em formação no oviduto ou ainda, através do sêmen de galos infectados (HOOP, 1992; CIACCI-ZANELLA & BRENTANO, 2012). Essa forma de transmissão pode durar em média de três a seis semanas, com relatos de até 12 semanas, até que

anticorpos maternos neutralizantes sejam produzidos (CIACCI-ZANELLA & BRENTANO, 2012). No entanto, o agente pode também ser transmitido horizontalmente, normalmente pela via fecal-oral (ZANELLA, 2007). A excreção viral pelas fezes tem uma duração média de cinco semanas, tempo este em que o vírus pode se propagar em lotes de matrizes em fase de postura e infectar a maioria das aves do plantel. Adicionalmente, as penas das aves infectadas também têm sido consideradas uma importante fonte de infecção (DAVIDSON et al., 2008).

Aves de todas as idades podem ser infectadas, porém apenas aves jovens, entre a primeira e a terceira semana de idade, desenvolvem doença clinicamente aparente (SCHAT, 2003; SCHAT & VAN SANTEN, 2008). Em aves com idade superior a três semanas, a infecção geralmente é subclínica, sem a presença de sinais clínicos. No entanto, a infecção produz alterações na função de macrófagos e de outras células responsáveis pela fagocitose, apresentação de antígenos e produção de citocinas (ZANELLA, 2007). Dados experimentais mostram que a infecção de aves com seis semanas de idade seguem o mesmo padrão de viremia no timo, fígado e baço que as aves de um dia de idade, porém sem o desenvolvimento de lesões e sinais clínicos (KAFFASHI et al., 2006).

O quadro clínico é caracterizado por anemia, imunossupressão e perda de peso (YUASA et al., 1983; ADAIR, 2000). As lesões causadas pelo CAV são encontradas principalmente no timo, medula óssea, baço e bursa de Fabrício (SMYTH et al., 1993; TORO et al., 1997). Estas lesões são decorrentes do tropismo do vírus por hemocitoblastos, células progenitoras das séries mieloide e eritroide, bem como por células progenitoras de linfócitos T (ADAIR, 2000; MILLER & SCHAT 2004). A infecção por CAV gera um decréscimo temporal dessas células (TANIGUCHI et al., 1983) levando à ocorrência dos sinais clínicos e ao aumento da suscetibilidade a infecções bacterianas (ENGSTRÖM & LUTHMAN, 1984). A perda de hemocitoblastos prejudica a produção de eritrócitos, necessários à produção de hemácias; de trombócitos necessários à geração de células do sistema de coagulação; e, de granulócitos, precursores de células de respostas inflamatórias. Tal perda ocasiona aplasia de medula óssea, atrofia do timo, presença de hemorragia intramuscular e subcutânea, causando anemia e baixa resposta imune contra infecções secundárias que são característicos da infecção por CAV (CIACCI-ZANELLA & BRENTANO, 2012). Dessa forma, aves infectadas tornam-se mais suscetíveis a infecções bacterianas secundárias e apresentam

uma menor resposta a vacinações, reforçando o caráter imunodepressor do vírus (DE BOER et al., 1994; ADAIR 1996).

Uma vez que aves jovens são infectadas, o vírus dissemina rapidamente para vários tecidos (YUASA et al., 1983; HOOP & REECE, 1991), podendo atingindo o cérebro e o coração em menos de 2 semanas (SMYTH et al., 1993). Se a ave sobreviver à infecção por CAV, o vírus pode permanecer no cérebro por mais de 49 dias (YUASA et al., 1983) e ao longo de 12 meses em tecidos reprodutivos, mesmo após o desenvolvimento de imunidade contra o CAV (CARDONA et al., 2000).

Alguns autores têm sugerido que o CAV pode persistir como um vírus latente, o qual pode ser passado de geração para geração sem apresentar evidências da infecção (McNULTY, 1991; MILLER & SCHAT, 2004). Ocasionalmente, o vírus pode tornar a se replicar, resultando em soroconversão. Essa, por sua vez ocorre frequentemente, após o início da postura e, algumas vezes, todas as aves do lote tornam-se positivas, levando a hipótese de que a expressão do CAV pode sofrer influência da regulação hormonal do sistema reprodutor (MILLER et al., 2005). No entanto, lotes subsequentes aos positivos podem permanecer negativos (SCHAT 2003; SCHAT & VAN SANTEN, 2008). Uma vez que os anticorpos neutralizantes contra o vírus se desenvolvem, os antígenos virais desaparecem (SCHAT, 2009).

As taxas de morbidade e mortalidade e a severidade da doença causada pela infecção com CAV podem ser variáveis. Fatores como patogenicidade das cepas, título do vírus, via de infecção, idade das aves, imunidade passiva, diferenças genéticas entre as aves, presença de co-fatores infecciosos (outros vírus imunodepressores) e não infecciosos (ambiente, estresse, nutrição) influenciam na severidade do quadro clínico (ADAIR, 2000; VAN SANTEN et al., 2004).

1.5.2. AGV2

Genomas de AGV2 têm sido detectados tanto em animais saudáveis quanto doentes; no entanto, nenhuma associação com patogenicidade foi comprovada até o momento. O caráter patogênico do AGV2 - se é que existe - permanece desconhecido.

Um estudo molecular, incluindo amostras oriundas de aves saudáveis e doentes de regiões distantes geograficamente (Brasil e Holanda), foi realizado utilizando como base de comparação o primeiro genoma do AGV2 descrito (número de acesso no GenBank: HM590588). A partir desta comparação evidenciou-se o agrupamento das amostras em três grupos filogenéticos: o primeiro compreendendo sequências oriundas

de aves saudáveis, homólogos entre si; o segundo compreendendo sequências provenientes de aves com lesões cerebrais identificadas na Holanda juntamente com uma amostra de origem brasileira; e, o terceiro grupo reunindo sequências de três aves adultas com sinais clínicos de apatia e perda de peso, provenientes do Brasil (DOS SANTOS et al., 2012). Esses resultados evidenciaram a existência de variantes do AGV2, cujo diferente potencial patogênico é especulado. No entanto, futuros estudos são necessários para avaliar o papel do AGV2 como um agente etiológico.

Apesar do caráter patogênico do AGV2 não ter sido comprovado até o momento, dados experimentais sugerem que o agente é uma partícula viral completa, ativa e infecciosa, tendo a espécie *Gallus gallus domesticus* como um hospedeiro. A infectividade do vírus foi comprovada através da exposição ambiental de aves sentinelas, livres de patógenos específicos (*Specific pathogen free*, SPF), alojadas em uma instalação experimental em contato com cama de frango tratada e reutilizada. Aves negativas para o agente, ou seja, nas quais o genoma viral não havia sido detectado, mostraram ser suscetíveis à infecção após o contato com aves contaminadas (COSTA et al., 2012; ESTEVES et al., 2012; FRANCO, 2012). Dessa forma, foi possível reproduzir experimentalmente a infecção, mesmo sem ter sido realizado o isolamento viral (ESTEVES et al., 2012; COSTA et al., 2012).

Recentemente, a presença de co-infecção do AGV2 e do vírus da doença de Newcastle avirulento (NDV) em plantéis de aves com sinais neurológicos e alta mortalidade foi descrita na África do Sul. Com isso, especulou-se a possibilidade de um efeito sinérgico entre esses vírus, podendo o AGV2 atuar como um facilitador da replicação do NDV (ABOLNIK & WANDRAG, 2014). Nesse sentido, a possibilidade do desenvolvimento de doença necessitar de outros elementos também tem sido questionada. Dentre estes incluem-se nutrição deficiente, estresse, micotoxinas e co-infecções com outros microorganismos que possam contribuir no desenvolvimento de doença (FLORES et al., 2013). Caso seja comprovada, não será a primeira vez que um circovírus está envolvido no desenvolvimento de doença multifatorial, como é das doenças associadas à circovirose suína que está presente o circovírus suíno tipo 2 (GIILLESPIE et al., 2009; SEGALÉS et al., 2013; FLORES et al., 2013).

1.6. Epidemiologia

1.6.1. CAV

O CAV está presente em praticamente todos os países que possuem avicultura comercial intensiva (SCHAT, 2003). A presença de anticorpos contra o agente tem sido mais frequentemente detectada em aves de lotes de matrizes acima de 25 a 30 semanas de idade sem sinais clínicos (McNULTY, 1991). Por volta de 18 a 24 semanas, é mais frequente observar maior desuniformidade no número de aves positivas e variações maiores nos níveis de anticorpos (CIACCI-ZANELLA & BRENTANO, 2012).

Estudos epidemiológicos pesquisando a presença do CAV vêm sendo realizados no mundo todo e tem evidenciado a ampla distribuição deste agente (OWOADE et al., 2004; EMIKPE et al., 2005; BHATT et al., 2011; BIĐIN et al., 2010; GHOLAMI-AHANGARAN & ZIA-JAHROMI, 2012). Nos Estados Unidos, um estudo retrospectivo avaliando a presença de anticorpos contra CAV em soros de aves coletados entre 1959 e 2005, ressaltou a presença do vírus antes do seu primeiro isolamento (TORO et al., 2006).

No Brasil, um levantamento sorológico analisando aves de nove Estados com produção comercial intensiva de linhas de corte, abrangendo lotes de aves com diferentes idades, indicou uma elevada prevalência (89%) de CAV e a presença do agente em todos os Estados testados (BRENTANO et al., 2000). O mesmo resultado foi observado na região sul do Brasil, onde soros provenientes de lotes de matrizes de corte com e sem histórico de vacinação foram analisados, tendo sido constatada prevalência de anticorpos contra CAV de 89%. Dentre estes lotes analisados, todos tiveram pelo menos uma amostra de soro positivo, reforçando a elevada prevalência e a ampla distribuição do vírus na produção avícola. Além disso, aproximadamente 48% das matrizes possuíam títulos de anticorpos insuficientes para a proteção da progênie (CANAL et al., 2004).

1.6.2. AGV2

Devido à recente descrição do AGV2, pouco é conhecido sobre a epidemiologia deste agente. Até o momento, os poucos dados disponíveis limitam-se à detecção de genomas virais (DOS SANTOS et al., 2012, CHU et al., 2012). Genomas do vírus têm sido detectados tanto em animais saudáveis quanto doentes, obtidas de distintos locais e tipos de criação (comercial ou subsistência). Nesse contexto, dos Santos e colaboradores

(2012) mostraram que todas as amostras (4/4) de aves com sinais clínicos de retardo no crescimento, perda de peso, ou lesões cerebrais coletadas no Brasil foram positivas para AGV2. Na Holanda, genoma viral foi detectado em 42,9% (9/21) das amostras com sinais neurológicos. Adicionalmente, a presença de DNA viral encontrada variou de 60,4% (29/48) a 90,7% (98/108) nas amostras de aves saudáveis, dependendo da região geográfica analisada (DOS SANTOS et al., 2012). Dessa forma, evidenciou-se que o vírus não é restrito ao Brasil e que futuras investigações abrangendo outros países devem ser realizadas. Sua ocorrência tanto no Brasil como na Holanda sugerem que provavelmente este agente está disseminado nos rebanhos avícolas mundiais. A subsequente detecção de AGV2 em Hong Kong, África do Sul e Hungria dão suporte a esta teoria (CHU et al., 2012; ABOLNIK & WANDRAG, 2014; FEHÉR et al., 2014).

Similarmente aos demais circovírus, o AGV2 parece ser bastante resistente. Dessa forma, a detecção de genoma viral no meio ambiente pode estar associada à ampla distribuição do vírus em aves domésticas somada à possível resistência atribuída a ele (FLORES et al., 2013). Como exemplo disso, o vírus pode ser facilmente encontrado em plantéis comerciais bem como na superfície do inseto *Alphitobius diaperinus*, conhecido popularmente como cascudinho. Estes insetos já foram descrito como um reservatório de agentes patogênicos e, estão comumente presentes em camas de aviários (COSTA et al., 2012; FRANCO 2012).

Adicionalmente, genomas de AGV2 também foi identificado em outras espécies de aves, tais como o mutum de Alagoas (*Mitu mitu*; LIMA et al. 2012) e em cordornas Japaneses quail (*Coturnix japonica*; H.F DOS SANTOS, comunicação pessoal). A ocorrência desse agente em aves selvagens certamente tem implicações na manutenção e dispersão do vírus na natureza (FLORES et al., 2013), entretanto ainda não existem informações aprofundadas sobre esse tema.

1.6.2.1. Co-infecções com AGV2

Genoma de AGV2 tem sido identificado em aves co-infectadas com outros agentes. Conforme mencionado anteriormente, AGV2 foi detectado em aves com sinais neurológicos e co-infectadas com NDV avirulento (ABOLNIK & WANDRAG, 2014).

Além das aves domésticas e selvagens, genomas de AGV2 foram encontrados em amostras de fezes humanas, incluindo adultos e crianças e, envolvendo, principalmente, casos de diarreia (CHU et al., 2012; PHAN et al., 2012). Da mesma

forma, fezes de furão doméstico também continham genoma do vírus (FEHÉR et al., 2014). Curiosamente, a maioria das amostras nas quais DNA do AGV2 estava presente foi também detectado genoma de CAV e outros girovírus, como HGyV, GyV3 e GyV4 (CHU et al., 2012; PHAN et al., 2012; FEHÉR et al., 2014). Especula-se que a presença de CAV e AGV2 detectados nas amostras de mamíferos possa estar relacionada ao consumo de carne de frango. Nesse sentido, a frequente detecção de ambos os genomas em carne de frango destinada ao consumo humano tem favorecido tal hipótese (CHU et al., 2012; PHAN et al., 2012; FEHÉR et al., 2014). Do mesmo modo, dados obtidos em um estudo desenvolvido na África, em uma região onde o consumo de carne de frango é baixo, corroboram com a especulação. No estudo mencionado, amostras de fezes humanas oriundas de caso de diarreia foram analisadas e nenhum genoma de girovírus foi encontrado (BONKOUNGOU et al., 2010). Somado a isso, como genoma de CAV já foi identificado em fezes de cães e gatos, acredita-se que a presença do mesmo possa estar relacionada à dieta animal, ao consumo de produtos que contenham carne de frango na composição como, por exemplo, as rações. No entanto, apesar das especulações e das possíveis evidências, pouco é conhecido sobre a distribuição do AGV2 na avicultura bem como, sobre os fatores determinantes relacionados à infecção e/ou co-infecção com os girovírus, especialmente com CAV e AGV2. Dessa forma, estudos futuros envolvendo a detecção de CAV e AGV2 são necessários e possibilitarão conhecer a epidemiologia dos agentes e a real distribuição mundial, principalmente do AGV2.

1.7. Diagnóstico

1.7.1. CAV

O diagnóstico de infecções pelo CAV deve ser realizado com base no histórico da propriedade, aliado aos achados clínicos, lesões macro e microscópicas e à detecção de antígenos ou ácidos nucleicos virais (CIACCI-ZANELLA & BRENTANO, 2012). O isolamento viral também pode ser realizado para detecção do CAV, porém não é mais um método recomendável para o diagnóstico devido ao custo elevado e ao tempo necessário para obtenção dos resultados (BRENTANO et al., 1991; BRENTANO, 2009; CIACCI-ZANELLA & BRENTANO, 2012). No entanto, o isolamento pode ser utilizado para fins de pesquisa ou ainda como método de análise da presença de infecção ativa (CIACCI-ZANELLA & BRENTANO, 2012). Sendo assim, o CAV pode

ser propagado em cultivos de células linfoblastóides derivadas de tumores da doença de Marek, causando um efeito citopático caracterizado pelo aumento do tamanho e refratilidade das células, formação de grumos e morte celular. Adicionalmente, o isolamento pode ser realizado *in vivo*, em pintos SPF de um dia de idade, bem como em ovos embrionados com cinco dias de incubação (CIACCI-ZANELLA & BRENTANO, 2012).

A circulação do vírus pode ser evidenciada em um animal ou rebanho através da detecção de anticorpos utilizando testes sorológicos, sendo o teste imunoenzimático do tipo ELISA é mais frequentemente empregado (BRENTANO, 2009). A pesquisa por genoma tem sido incluída como diagnóstico para detecção deste agente e é, atualmente, a metodologia mais utilizada para o diagnóstico (TODD et al., 1992; MARKOWSKI-GRIMSRUD et al., 2002; CIACCI-ZANELLA & BRENTANO, 2012).

1.7.2. AGV2

Tentativas de isolamento do AGV2 a partir de amostras de soros ou macerados de tecidos de aves infectadas inoculados em células MDCC-MSB1, células primárias de fibroblasto de embrião de galinha (FEG) e ovos embrionados (dados não publicados) foram realizadas. No entanto, até o momento, em nenhuma tentativa foi obtido o isolamento viral.

A dificuldade em isolar o agente, somada à dificuldade da obtenção de proteínas virais para a confecção de testes sorológicos, tem travado o desenvolvimento de métodos de diagnóstico sorológico para detecção de anticorpos contra AGV2. Sendo assim, a identificação de AGV2 tem se limitado à detecção de ácidos nucleicos virais. Desde a primeira identificação desse agente, diferentes protocolos, visando a detecção do DNA viral, têm sido descritos (RIJSEWIJK et al., 2011; DOS SANTOS et al., 2012; ABOLNIK & WANDRAG, 2014). Contudo, a maioria dos estudos baseia-se na detecção por técnicas moleculares, principalmente em PCR convencional cuja detecção do agente restringe-se à forma qualitativa. Sendo assim, o desenvolvimento de testes quantitativos, baseados na PCR em tempo real poderá contribuir significativamente para a realização de estudos mais aprofundados, envolvendo a investigação de supostas fontes de disseminação do agente, a interação com outros patógenos, ou ainda, ampliando informações epidemiológicas.

2. OBJETIVOS

2.1. Objetivo Geral

- Contribuir para a ampliação do conhecimento do CAV e do AGV2 através de análises moleculares.

2.2. Objetivos Específicos

- Desenvolver uma duplex PCR (dqPCR) em tempo real para detecção simultânea de CAV e AGV2;

- Avaliar vacinas comerciais utilizadas na avicultura como possíveis fontes de disseminação de CAV e AGV2;

- Avaliar a variabilidade genética do AGV2 presente em aves com diferentes *status* sanitário através de análises filogenéticas;

- Detectar e caracterizar genomas de AGV2 em amostras de aves da Itália.

CAPÍTULO 1

Simultaneous detection of chicken anemia virus and avian gyrovirus 2 by duplex real-time polymerase chain reaction

Ana Paula Muterle Varela[a,b*]; Camila Mengue Scheffer[a,b]; Adriéli Wendlant[a,b]; Francisco Esmaile Sales Lima[c]; Samuel Paulo Cibulki[a,b]; Raíssa Nunes dos Santos[b]; Helton Fernandes dos Santos[a]; Thais Fumaco Teixeira[a]; Ana Cláudia Franco[b]; Paulo Michel Roehe[b]

[a]FEPAGRO – Saúde Animal – Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Estrada do Conde 6000, Eldorado do Sul, CEP 92990-000, Rio Grande do Sul, Brazil.

[b]Universidade Federal do Rio Grande do Sul (UFRGS), Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde. Av. Sarmento Leite 500, sala 208, Porto Alegre, CEP 90050-170, Rio Grande do Sul, Brazil.

[c]Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Laboratório de Microbiologia Molecular, Av. Sarmento Leite, 245, Porto Alegre, CEP 90050-170, Rio Grande do Sul, Brazil.

*Corresponding author

Laboratório de Virologia, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) - Estrada do Conde 6000, CEP 92990-000, Eldorado do Sul, Rio Grande do Sul, Brazil. E-mail address: anapaulamut@gmail.com

Artigo em fase de conclusão a ser submetido em periódico científico.

ABSTRACT

The development of a duplex real-time polymerase chain reaction (dqPCR) assay to detect and quantify simultaneously genomes of the two currently known avian gyroviruses - chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) - is reported in this study. Analytical performance included determination of limit of detection, specificity and reproducibility of the assay. In addition, the dqPCR was compared to conventional PCR assays previously describe. Twenty five DNAs samples extracted from serum and liver tissues chickens were examined by both assays and the results obtained were compared. The dqPCR showed remarkable analytical sensitivity, allowing detection of a minimum of 5 copies of CAV and 50 copies of AGV2 genomes. The assay was reproducible, with a maximum intra-assay coefficient of variation (CV) of 1.09 % for CAV and of 1.28 % for AGV2. The maximum inter-assay CV was 4.79 % for CAV and 2.1 % for AGV2. The assay allowed identification of CAV, AGV2 or both genomes in 11, 13 and 9 samples, respectively. This new test should be useful alternative for rapid and reliable simultaneous detection of CAV and AGV2 genomes.
Keywords: detection, gyrovirus, quantitative assay

1. Introduction

The genus *Gyrovirus* belongs to the family *Circoviridae* which comprises small, icosahedrical, non-enveloped DNA viruses of about 20 nm in diameter (Todd, 2011). Their genomes are circular, negative-sense, single-stranded DNA that forms a covalently closed circle (Todd, 2011). For more than 30 years, Chicken anemia virus (CAV) was the only gyrovirus known to infect chickens. CAV has a worldwide distribution, affecting all countries with an active poultry industry (Craig et al., 2009). The importance of this virus as a pathogen resides particularly on its capacity to enhance the severity of disease caused by other agents (Todd, 2000; Haridy et al., 2009; Schat, 2009).

In 2011, a new avian gyrovirus, named avian gyrovirus 2 (AGV2), was identified in serum samples of poultry displaying signs of retarded growth, during a search of CAV DNA (Rijsewijk et al., 2011). This new agent has a genome size and genomic organization resembling CAV; however, these two viruses share a nucleotide similarity of only 40% (Rijsewijk et al., 2011). Since the first description of AGV2 in diseased chickens, AGV2 genome has also been detected in in healthy birds and in co-infection with other pathogens (dos Santos et al., 2012; Chu et al., 2012; Abolnik and Wandrag, 2014; Zhang et al., 2014). Recently, co-infections with AGV2 and avirulent Newcastle disease virus (NDV) were reported in chickens displaying neurological disorders (Abolnik and Wandrag, 2014). Moreover, CAV and AGV2 co-infection has been often evidenced, including in human stool specimens (Chu et al., 2012; Smuts, 2014).

Although, so far, no association between AGV2 infections and pathological conditions has been established, the possibility of a synergistic pathogenic effect in co-infection involving AGV2 has been raising questions and requires further investigation. In addition, there is no test able to detect simultaneously both avian gyroviruses yet. So, in order to contribute to the search of AGV2 and its potential association with CAV, this study reports the development of a duplex real-time PCR (dqPCR) able to detect and quantify CAV and AGV2 genomes, when causing either single or combined infections in birds.

2. Materials and Methods

2.1 Chicken samples

A total of 25 samples (16 serum and 9 liver tissue sample) were examined in the study. Five out of the sixteen serum samples were collected from adult SPF birds (from EMBRAPA/CNPSA, Santa Catarina, Brazil). Nine serum samples were collected from adult backyard hens from small household farms in the state of Rio Grande do Sul, Brazil. In addition, nine liver tissue samples were collected from six-week-old commercial broilers displaying signs of apathy and weight loss. These tissues samples were also obtained from farms located within the state of Rio Grande do Sul, Brazil.

2.2 DNA extraction

Total DNA was extracted from sera and liver tissues using the commercially available PureLink™ Genomic DNA Mini Kit (Invitrogen™) following manufacturer's instructions. DNA extracted was quantified with a fluorometer (Qubit® 2.0; Life technologies) and the concentration of DNA was adjusted to 33 ng/ µl. All samples were stored at -20^{o}C until testing.

2.3 Primes and probes

Specific primers and probes were designed for the dqPCR with the program Primer3 (version 3.0, Geneious software), with basis on the currently available CAV (Noteborn et al., 1991) and AGV2 (Rijsewijk et al., 2011) nucleotide sequences (GenBank accession numbers NC_001427 and HM590588, respectively). The CAV primers and probe were designed targeting a 99 base pair (bp) long fragment on the viral protein 1 (VP1) coding region (Table 1). The AGV2 primers amplify a 125 bp fragment of the genomic region coding for viral protein 2 (VP2), corresponding to a region within the gene coding for the putative dual-specificity protein phosphatase (Table 1). All probes and primers were manufactured by Integrated DNA Technologies (Coralville, IA).

2.4 Construction of standard curves

The construction of the plasmid containing the complete CAV genome (pCR2.1CAV) was carried out following the same procedures previously described for the construction of the AGV2 genome-containing plasmid (pCR2.1AGV2; Rijsewijk et al., 2011). To confirm the nature of the insert, pCR2.1CAV and pCR2.1AGV2 were sequenced using M13- and CAV- or AGV2-specific primers. The amount of plasmid

was quantified by fluorimetry as above. Serial plasmid dilutions were used for plotting the standard curves of amplification.

2.5 dqPCR assay

To generate standard curves, the dqPCR was carried out in 12.5 µl containing DNA of the two standard plasmids (pCR2.1CAV and pCR2.1AGV2), 1X Platinum® quantitative PCR SuperMix-UDG (InvitrogenTM, Life Technologies, USA), 10 µM of each (forward and reverse) primers and 5 µM of each probe. Magnesium chloride concentration was adjusted to 3 mM. Amplification reactions were performed in a StepOne™ Real-Time PCR system (Applied Biosystems, Life Technologies) under the following conditions: uracil DNA glycosylase (UDG) incubation at 50ºC for 2 min; initial denaturation and *Taq* polymerase activation at 95ºCfor 2 min, followed by 40 cycles of amplification (15 s at 95ºC and 30 s at 60ºC). All real-time reactions were performed in triplicate and the reported results are averages of such triplicates. Fluorescent measurements were carried out during the elongation step. From each amplification plot, a threshold cycle (Ct) value was calculated representing the PCR cycle number in which the reporter dye fluorescence was detectable above an arbitrary threshold. The threshold was set at a level higher than the background. The magnitude of specific signals was normalized using an internal passive reference. Data analyses were performed with the aid of StepOne™ software v2.2.2 (Applied Biosystems, Life Technologies). The dqPCR was also carried out with DNA extracted from biological samples (serum and tissue samples). The load of CAV and AGV2 genomes in serum and tissue samples was determined by comparison with the standard curves.

2.6 Sensitivity and specificity determinations

The analytical sensitivity of the assay was determined by running the tests in triplicate, in three independent experiments, performed in different days. The *in silico* specificity of the primers was determined with the NCBI Primer-BLAST tool (Ye et al., 2012). The primers' specificity was also confirmed in optimized dqPCR conditions by testing DNA extracted from different bacteria: *Campylobacter jejuni* (ATCC® 35560™)*;* *Campylobacter lari* (ATCC® BAA1060D-5); *Chlamydophila psittaci* (ATCC® VR-15)*;* *Mycoplasma gallisepticum* (ATCC® 19610™); *Mycoplasma meleagridis* (ATCC® 27764™)*;* *Mycoplasma synoviae* (ATCC® qCRM-25204D™). The specificity was further checked after running the dqPCR with nucleic acids (either

DNA or reverse transcribed RNA) extracted from other avian viruses (avian pneumovirus types A and B, avian paramyxovirus types 1, 2 and 3, beak and feather disease virus, infectious laryngotracheitis virus and infectious bronchitis virus. In addition, cross reactivity assays were performed to confirm primers and probes' specificity by running assays with CAV primers and probe with plasmid pCR2.1AGV2 as template and *vice versa* (AGV2 primers and probe with plasmid pCR2.1CAV).

2.7 Intra- and inter-assay variability of the dqPCR

The reproducibility of the dqPCR was determined by testing dilutions (10^8 to 10^0 copies) of the standard plasmids pCR2.1CAV and pCR2.1AGV2. The intra-assay variability of the dqPCR was determined by comparing results of three replicates for each dilution of the assays. The inter-assay variability was determined by comparing results obtained in three different runs performed in different days. The coefficient of variation (CV) of the Ct values was determined.

2.8 PCR for CAV and AGV2

The DNA extracted from 25 samples (sera and liver tissues) were submitted to detection of CAV and AGV2 genomes by conventional PCR in independent assays, as previously described (Simionatto et al., 2005; Rijsewijk et al., 2012). The assays were performed in a Mastercycler® personal thermocycler (Eppendorf, Germany) following reaction conditions previously described (Simionatto et al., 2005; Rijsewijk et al., 2011). Negative controls (DNA extracted from feathers of SPF birds that resulted negative for CAV and AGV2 by PCR) were included in each assay; positive controls (DNA from plasmids pCR2.1CAV and pCR2.1AGV2) were included in each set of reactions. The amplicons were visualized on 1% agarose gel and stained with ethidium bromide (0.5 µg/ml) under UV light. Negative and positive controls were included in each PCR reaction. To avoid contamination, different locations were used to conduct the DNA extractions, to prepare the PCR reaction buffers and to perform PCR analyses.

3. Results

3.1 Specificity of primers

The specificity of the CAV and AGV2 primers was confirmed *in silico.* The CAV primer pair recognized only CAV sequences available at GenBank. The AGV2

primer pair recognized not only the AGV2 sequences available at GenBank, but also sequences of the highly similar HGyV, in which AGV2 shares a level of nucleotide identity above 92% (Chu et al., 2012). The specificity of the CAV and AGV2 primers was checked in the dqPCR with DNA extracted from selected non-target pathogens (see Material and Methods). No amplification was obtained with DNA from any of the non-target pathogens tested. Testing the specificity of the primers to either virus, no amplification was observed when CAV primers were tested with AGV2 genome-containing plasmid DNA; likewise, no amplification was attained with AGV2 primers on CAV genome-containing plasmid DNA.

3.2 Sensitivity of the dqPCR

The dqPCR was capable of detecting a minimum of 5 copies of CAV DNA and 50 copies of AGV2 DNA (Fig. 1A and 1B, respectively). The CAV and AGV2 standard curves (Fig. 1C and 1D, respectively) gave rise to correlation coefficients (R^2) ranging from 0.997 to 1 for both templates, with efficiencies close to 100% and slopes ranging between 3.283 and 3.322, respectively (Fig. 1C and 1D).

3.3 Reproducibility of the dqPCR

Data on the determination of the intra- and inter-assay coefficients of variation are summarized in Table 2. The maximum intra-assay CV was 1.09% for CAV and 1.28% for AGV2. The maximum inter-assay CV was 4.79% for CAV and 2.10% for AGV2.

3.4 Detection of CAV and AGV2 by dqPCR and conventional PCR

The results obtained from 25 samples (sera and liver tissues) examined by dqPCR and the conventional PCR are summarized in Table 3. By conventional PCR, nine samples (3 serum and 6 liver tissue sample) contained CAV DNA while AGV2 DNA was found in two samples (1 serum and 1 liver tissue). Co-infection with both viruses was detected in only one sample (liver tissue). When the CAV and AGV2 DNA detection were carried out by dqPCR, it was confirmed the results found by conventional PCR. In addition, the dqPCR increased the CAV, AGV2 and both viruses DNA detection to 11, 13 and 9 samples, respectively. The dqPCR allowed quantifying of viral genome recovered, which varied between 5 to 680 copies of CAV genomes and 55 to 746,141 copies of AGV2 genomes per 100 ng of DNA in the samples examined.

4. Discussion

In order to contribute to the investigation of CAV, AGV2 and its potential associations, the present study describes the development of a duplex real-time polymerase chain reaction (dqPCR) assay to detect and quantify simultaneously CAV and AGV2 genomes in a single reaction. The dqPCR was shown to be highly specific and reproducible. The range of detection for the assay showed remarkable analytical sensitivity, as this technique was able to detect a minimum of 5 copies of CAV genomes and 50 copies of AGV2 genomes. The dqPCR allowed identification of infected animals with single or mixed viruses, with enhanced sensitivity in comparison to previously available methods for genome detection. Moreover, the assay allows quantification of the genomes of both CAV and AGV2, thus making it possible to determine viral loads in both cases. In this sense, studies on such interactions will certainly benefit from the availability of the assay here reported, not only to examine potential associations between CAV and AGV2, but also other potential pathogens.

To date, no pathogenic role for AGV2 has been demonstrated and no data is available on the outcome of concurrent CAV and AGV2 infections. Likewise, it is not known whether these viruses present some form of interaction in co-infected hosts. In addition, unknown remains any potential role that AGV2 might play – if any – on the pathogenesis of CAV-associated disease. These are interesting hypotheses, since as these viruses probably share common routes for multiplication, some degree of interaction in co-infected birds might be expected. Recently, co-infections with AGV2 and avirulent Newcastle disease virus (NDV) were reported in chickens displaying neurologic signs of disease; high mortality and a possible synergistic effect between those two viruses was suggested (Abolnik and Wandrag, 2014). In this sense, the assay reported in present study may be a useful tool to examine potential associations between CAV and AGV2.

5. Conclusion

Here, it was developed a duplex real-time PCR able to simultaneously detect CAV and AGV2 genomes, either as a single agent or in co-infections. The assay proved to be practical, sensitive and reproducible. It may be a useful tool for laboratory detection and research on CAV, AGV2 and its interactions with their hosts.

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References

1. Abolnik, C., Wandrag, D.B.R., 2014. Avian Gyrovirus 2 and avirulent Newcastle disease virus co-infection in a chicken flock with neurological symptoms and high mortalities. Avian Disease, 58, 90-94.

2. Chu, D.K.W., Poon, L.L.M., Chiu, S.S.S., Chan, K.H., Ng, E.M., Bauer, I., Cheung, T.K., Ng, I.H.Y., Guan, Y., Wang, D., Peiris, J.S.M., 2012. Characterization of a novel gyrovirus in human stool and chicken meat. Journal of Clinical Virology, 55, 209–213.

3. Craig, M.I., Rimondi, A., Delamer, M., Sansalone, P., König, G., Vagnozzi, A., Pereda, A., 2009. Molecular Characterization of Chicken Infectious Anemia Virus Circulating in Argentina During 2007. Avian Dis. 53, 331–335.

4. dos Santos, H.F., Knak, M.B., de Castro, F.L., Slongo, J., Ritterbusch, G.A., Klein, T.A.P., Esteves, P.A., Silva, A.D., Trevisol, I.M., Claassen, E.A.W., Cornelissen, L.A.H.M., Lovato, M., Franco, A.C., Roehe, P.M., Rijsewijk. F.A.M., 2012.Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. Veterinary Microbiology, 155, 230–236.

5. Haridy, M., Goryo, M., Sasaki, J., Okada, K., 2009. Pathological and immunohistochemical study of chickens with co-infection of Marek‘s disease virus and chicken anaemia virus. Avian Pathology, 38, 469–483.

6. Noteborn, M.H.M., de Boer, G.F., van Roozelaar, D.J., Karreman, C., Kranenburg, O., Vos, J.G., Jeurissen, S.H.M., Hoeben, R.C., Zantema, A., Koch, G., 1991. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. Journal of Virology, 65, 3131–3139.

7. Rijsewijk, F.A., dos Santos, H.F., Teixeira, T.F., Cibulski, S.P., Varela, A.P.M., Dezen, D., Franco, A.C., Roehe, P.M., 2011. Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Archives of Virology, 156, 1097–1100.

8. Schat, K.A. 2009. Chicken anemia virus. Current Topics in Microbiology and Immunology, 331, 151–183.

9. Simionatto, S., Lima-Rosa, C.A.V., Rubin, L.L., Canal, C.W., 2005. Um protocolo de nested-PCR para detecção do vírus da anemia das galinhas. Pesquisa Veterinária Brasileira, 25(2), 106–110.

10. Smuts, H.E.M., 2014. Novel Gyroviruses, including Chicken AnaemiaVirus, in Clinical and Chicken Samples from South Africa. Advances in Virology, 29, 2014:321284.

11. Todd, D., 2000. Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathology, 29, 373–394.

12. Todd, D., 2011.Gyrovirus. The Springer Index of Viruses, 311-316.

13. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache**,** I.**,** Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC bioinformatics, 13, 134.

14. Zhang, W., Li, L., Deng, X., Kapusinszky, B., Delwart, E., 2014. What is for dinner? Viral metagenomics of US store bought beef, pork, and chicken. Virology, 468–470, 303–310

Table 1. Specific-primers and probes used for CAV and AGV2 detection by dqPCR.

Primers and probes	**Sequence (5' to 3')**	**Genome posission***
CAV foward	CCAGCTTGCGTGCTATTCAT	
CAV reverse	CGAGCAACAGTACCCTGCTA	1644 to 1742
CAV probe	JOE-ACAGCGCAAGGCACGCAAGT-BHQ-1	
AGV2 foward	CACGGGCAAGACACTAAATG	
AGV2 reverse	TATCGAGGTCGTTTCTGCTG	560 to 682
AGV2 probe	FAM -CGCTCTCGCCGACAAGCAAC-BHQ-1	

***** CAV and AGV2 (GenBank accession numbers NC_001427 and HM590588, respectively) were used as reference sequence.

Table 2. Analytical reproducibility of the duplex real-time polymerase chain reaction (dqPCR) for chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) detection in intra- and inter-assay.

		Standard Curve (number of CAV and AGV2 molecules)							
		$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^3$	$5x10^2$	$5x10^1$	$5x10^0$
Inter-assay	**CAV**								
	Ct values* (mean ± S.D.)	13.03 ±0.62	16.41 ±0.63	19.94 ±0.62	23.23 ±0.64	26.68 ±0.75	29.98 ±0.61	33.79 ±0.39	36.97 ±0.68
	CV†(%)	4.79	3.86	3.09	2.75	2.79	2.05	1.16	1.84
	AGV2								
	Ct values* (mean ± S.D.)	14.38 ±0.23	17.79 ±0.26	21.18 ±0.2	24.46 ±0.2	27.91 ±0.22	31.10 ±0.36	34.97 ±0.74	-
	CV†(%)	1.58	1.45	0.95	0.82	0.79	1.15	2.12	-
Intra-assay	**CAV**								
	Ct values* (mean ± S.D.)	12.70 ±0.01	16.17 ±0.08	19.63 ±0.10	22.96 ±0.04	26.26 ±0.29	29.51 ±0.06	33.38 ±0.14	37.29 ±1.08
	CV†(%)	0.06	0.47	0.55	0.17	1.09	0.19	0.42	1.09
	AGV2								
	Ct values* (mean ± S.D.)	14.58 ±0.2	17.93 ±0.01	21.30 ±0.03	24.59 ±0.05	28.09 ±0.05	31.27 ±017	34.42 ±0.09	-
	CV†(%)	1.38	0.09	0.16	0.19	0.19	0.54	0.26	-

*Ct: threshold cycle value; †CV: variation coefficient value expressed in percent.

Table 3. Genome detection of chicken anemia virus (CAV), avian gyrovirus 2 (AGV2) or both (CAV+AGV2) in DNA extracted from sera (n=16) or liver tissues (n=9) of birds by conventional polymerase chain reaction (PCR) or duplex real-time PCR (dqPCR) assays.

Virus detection	Method	Serum	Liver	Total*
CAV	Nested-PCR	0	3	3
	dqPCR	4	7	11
AGV2	PCR	1	1	2
	dqPCR	7	6	13
CAV + AGV2	PCR	0	1	1
	dqPCR	3	6	9

*Number of samples in which CAV, AGV2 or both genomes were detected.

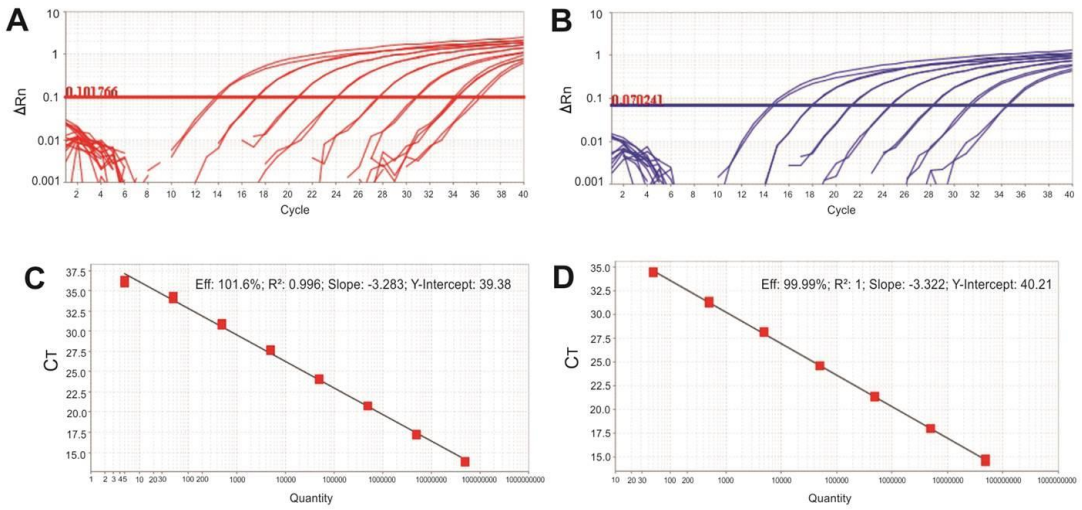

Figure 1. Amplification plots and standard curves obtained by duplex real-time PCR (dqPCR). Tenfold serial dilution of plasmids pCR2.1CAV DNA (A) and pCR2.1AGV2 (B) were amplified by dqPCR. Standard curves for CAV (C) and AGV2 (D) show amplification efficiency, R^2, values of slope and Y-interception.

CAPÍTULO 2

Chicken anemia virus and avian gyrovirus 2 as contaminants in poultry vaccines

Ana Paula Muterle Varela[a,b,*], Helton Fernandes dos Santos[a], Samuel Paulo Cibulski[a,b], Camila Mengue Scheffer[a,b], Candice Schmidt[a,b], Francisco Esmaile Sales Lima[b], Alessandra D'Avila Silva[c], Paulo Augusto Esteves[c], Ana Claudia Franco[b], Paulo Michel Roehe[a,b]

[a]FEPAGRO e Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Estrada do Conde 6000, Eldorado do Sul CEP 92990-000, Rio Grande do Sul, Brazil

[b]Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, UFRGS. Av. Sarmento Leite 500, Sala 208, Porto Alegre CEP 90050-170, Rio Grande do Sul, Brazil

[c]EMBRAPA Suínos e Aves, BR 153 Km 110, Concordia CEP: 89700-000, SC, Brazil

*Corresponding author

Laboratório de Virologia, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) - Estrada do Conde 6000, CEP 92990-000, Eldorado do Sul, Rio Grande do Sul, Brazil. E-mail address: anapaulamut@gmail.com

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Abstract

This study focuses on the detection of chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) genomes in commercially available poultry vaccines. A duplex quantitative real-time PCR (dqPCR), capable of identifying genomes of both viruses in a single assay, was employed to determine the viral loads of these agents in commercially available vaccines. Thirty five vaccines from eight manufacturers (32 prepared with live and 3 with inactivated microorganisms) were examined. Genomes of CAV were detected as contaminants in 6/32 live vaccines and in 1/3 inactivated vaccines. The CAV genome loads ranged from 6.4 to 173.4 per 50 ng of vaccine DNA (equivalent to 0.07 to 0.69 genome copies per dose of vaccine). Likewise, AGV2 genomes were detected in 9/32 live vaccines, with viral loads ranging from 93 to 156,187 per 50 ng of vaccine DNA (equivalent to 0.28-9176 genome copies per dose of vaccine). These findings provide evidence for the possibility of contamination of poultry vaccines with CAV and AGV2 and they also emphasize the need of searching for these agents in vaccines in order to ensure the absence of such potential contaminants.
Keywords: Chicken anemia virus, Avian gyrovirus 2, Vaccine contaminants

1. Introduction

Chicken anemia virus (CAV) and avian gyrovirus type 2 (AGV2) are members of the family Circoviridae, genus Gyrovirus [1,2]. CAV is the causative agent of chicken infectious anemia, an important avian disease characterized by anemia, immunosuppression and mortality in young chickens [3-5]. The virus is widely distributed in virtually all countries with significant poultry production [1]. Regarding AGV2, it was incidentally discovered by our group during a diagnostic search for CAV in chickens [2]. Infections with AGV2 have been identified in several different locations in Brazil and Netherlands, suggesting a worldwide distribution [6]. Nevertheless, to date, no evidence of AGV2 causing disease has been produced.

Since the first isolation of CAV from contaminated vaccines in Japan in 1979 [7], its role as a vaccine contaminant has been investigated [8-10]. The introduction of CAV into vaccines usually takes place vertically by infected embryonated hen's eggs. Improved vaccine production practices, such as the use of CAV-free specific pathogen-free (SPF) eggs did not eliminate the possibility of vaccine contamination [11-13]. As AGV2 bears significant similarity with CAV and it is possibly transmitted by similar routes, it might be expected that this virus could also potentially infect vaccines produced in embryonated eggs. However, as AGV2 has only recently been discovered, no studies have been performed to examine such possibility.

In view of the potential role for vaccines in the transmission and dissemination of extraneous pathogens-particularly relevant when most poultry vaccines are based on live attenuated or modified pathogens-this study was set up in order to detect CAV and AGV2 genomes in commercially used vaccines. With this aim, a duplex quantitative real-time PCR (dqPCR), capable of detecting the genomes of both viruses in a single assay, was employed [14]. The specificity of the amplified products was confirmed by cloning and sequencing.

2. Materials and methods

2.1. Vaccine samples

Thirty five commercially available vaccines, 32 prepared with live and 3 with inactivated microorganisms, were purchased commercially or obtained by donation from eight different laboratories from different countries (1 from United States of

America, 2 from Hungary, 1 from Canada, 1 from Netherlands, 2 from Germany and 28 from Brazil). All vaccines are largely employed in poultry farming and are aimed to immunize birds against several pathogens. A list with all vaccines evaluated in the present study is provided on Table 1.

2.2. DNA extraction

Total DNA was extracted from 500 µl of each of the vaccine suspensions using the PureLinK™ Genomic DNA Mini Kit (Invitrogen), following the manufacturer's instructions. The DNA extracted from the vaccines was quantified with a fluorometer (Qubit® 2.0; Life technologies) and it diluted up to 17 ng/ µl of DNA. All samples were stored at -20 °C until testing.

2.3. Duplex quantitative real time PCR (dqPCR)

The DNA extracted from the vaccines under test was evaluated by dqPCR as previously described [14]. Two plasmids were used as positive controls and to generate the dqPCR standard curves: a plasmid containing the CAV complete genome (pCR2.1CAV) [14] and a second plasmid containing the full AGV2 genome (pCR2.1AGV2) [2]. The CAV- and AGV2-specific primers (CAV forward: 5'–CCAGCTTGCGTGCTATTCAT-3'; CAV reverse: 5'-CGAGCAACAGTACCCTGCTA-3'; AGV2 forward: 5'-CACGGGCAAGACACTAAATG-3'; AGV2 reverse: 5'-TATCGAGGTCGTTTCTGCTG-3') and probes (CAV probe: 5'-JOE-93 ACAGCGCAAGGCACGCAAGT-BHQ-1-3'; and AGV2 probe: 5'-FAM - 97 CGCTCTCGCCGACAAGCAAC-BHQ-1-3') used in the assay were previously described [14]. The standard curves constructed with the plasmids mentioned above were used to estimate the number of genome copies in each vaccine sampled. The assay was capable of detecting at least 5 copies of CAV genome and 50 copies of AGV2 genome, as determined by titration of pCR2.1CAV and pCR2.1AGV2 DNA, as previously determined [14].

The determination of CAV and AGV2 genome loads in DNA extracted from vaccines was performed in total volumes of 12.5 µl using 50 ng of DNA extracted from vaccines, 6.25 µl of 2 X Platinum® quantitative PCR SuperMix-UDG (Invitrogen - Life Technologies), 200 nM of each forward and reverse primers (IDT) and 5 µM of each probe. A final concentration of 3 mM MgCl2 was used to each reaction. Amplification and detection were performed in a StepOne™ Real-Time PCR system (Life

Technologies) under the following conditions: uracil DNA glycosylase (UDG) incubation at 50 °C for 2 min; initial denaturation and Platinum® Taq activation at 95 °C for 2 min, followed by 40 cycles of amplification (15 s at 95 °C and 30 s at 60 °C). All real-time assays were performed in triplicate. The results presented are the averages of such triplicates. Fluorescent measurements were carried out during the elongation step. From each amplification plot, a threshold cycle (Ct) value was calculated representing the PCR cycle number in which the reporter dye fluorescence was detectable above an arbitrary threshold. Data analysis was performed with the StepOne software v2.2.2. All vaccine samples that were found to contain CAV or AGV2 genomes were submitted to fresh DNA extractions and were re-tested in triplicate by dqPCR to confirm the results obtained.

Standard precautions were taken to avoid the risk of cross-contamination; each step of the handling was carried out in different rooms. In addition, different displacement micropipettes and filter tips were used throughout.

2.4. Amplification by conventional PCR

DNA samples from vaccines which were found to contain CAV and/or AGV2 DNA were submitted to amplification by conventional PCR with the same primers used in the dqPCR. The amplification was performed in an Eppendorf Master cycler apparatus, in reaction mixtures containing 50 ng of vaccine DNA extract, 4 µl of 5 X Phusion HF Buffer, 5 pmol of each primer, 1 U Phusion High-Fidelity DNA polymerase (Thermo Scientific), 0.8 mM each dNTP and sterilized Milli-Q water q.s.p. 20 µl. The temperature cycling conditions consisted of 1 cycle at 98 °C for 3 min followed by 35 cycles of 98 °C for 15 s, 57 °C for 15 s and 72 °C for 15 s, plus a final extension step at 72 °C for 3 min. Five microliters of the PCR products were separated in 2% agarose gels, stained with ethidium bromide and photographed on an UV transilluminator. The specific bands were identified in comparison with a DNA ladder (Thermo Scientific GeneRuler 50 bp DNA Ladder).

2.5. Cloning and sequencing

All amplifications products obtained by conventional PCR were cloned into plasmid pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Scientific) following the manufacturer's protocol. At least three recombinant plasmids of each reaction were sequenced on both strands using pJET1.2-forward and pJET1.2-

reverse oligonucleotides as primers in a MegaBACE 500 apparatus with the Dyenamic ET terminator cycle sequencing kit (Amersham Biosciences).

2.6. Analysis of sequence data

Viral DNA sequences were initially evaluated by BLAST and subsequently aligned using the programs MEGA 5 [15] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) programs [16]. Previously published CAV and AGV2 sequences available at GenBank were used as references (AB031296.1 for CAV and JQ690763.1; HM590588.1; JQ308212.1; FR823283.1 for AGV2).

3. Results

3.1. dqPCR analysis

The results of the dqPCR performed in this study are summarized in Table 1. As expected, CAV genomes were detected in the three vaccines aimed to immunize for CAV (manufactured by laboratories B, G and H; Table 1) albeit with highly variable viral genome loads (copy numbers ranging from 2386 to 2175.381/ 50 ng; equivalent to 32 to 27,192.263 genome copies per dose of vaccine). As contaminants, CAV genomes were detected in vaccines from one producer laboratory only; six of those were live vaccines (Table 1, manufacturer D). In total, CAV viral genomes were detected in 7 out of the 35 vaccines tested (Table 1). The vaccines where CAV genomes were identified included two vaccines to Marek's disease, two vaccines to infectious bronchitis, one vaccine to fowlpox, one vaccine to egg drop syndrome (EDS-76) and one vaccine to Newcastle disease. In such vaccines, CAV genome loads ranged from 6.4 to 173.7 copies per 50 ng of vaccine DNA, equivalent to 0.07 to of 0.69 genome copies per dose of vaccine, respectively.

Regarding AGV2, such genomes were detected in nine vaccines manufactured by three laboratories (A, D and E; Table 1). These included one vaccine to fowlpox (manufacturer A), two vaccines to Newcastle disease (manufacturers D and E), one vaccine to infectious laryngotracheitis (manufacturer E), one vaccine to infectious bronchitis and four vaccines to Marek's disease (manufacturer D). Among the Marek's disease vaccines, two were prepared with serotype 1 (CVI988/Rispens) and two with serotype 3 (turkey herpesvirus - HVT) viruses. The detectable levels of AGV2 viral DNA varied from 93 to 156,187 genome copies per 50 ng of vaccine DNA (equivalent

to 0.28 - 9176 genome copies per dose of vaccine). All vaccines that contained AGV2 DNA were live vaccines.

In addition, four of the 35 examined vaccines contained DNA of both CAV and AGV2 (infectious bronchitis, Newcastle disease and Marek's disease vaccines of two serotypes; Table 1). All these vaccines were live vaccines and all were produced by a same manufacturer (Table 1).

3.2. Sequence analyses

At least three recombinant plasmids, containing either CAV or AGV2 DNA, amplified from vaccine DNA, were sequenced on both strands. These were compared with similar sequences available at GenBank (as detailed in methods). All sequences displayed a high degree of similarity to equivalent regions of CAV or AGV2 genomes (Fig. 1) and they confirmed the specificity of the results obtained at dqPCR.

In the putative CAV sequences amplified from the vaccines DNA (Fig.1, A/B), two nucleotide changes occurred at positions 849 (G/A) and 860 (G/C), some of them (CAV vaccines D; F; J; O; U) resulting in a discrete amino acid change (S/T). In sequenced clones from putative AGV2 genomes amplified from the vaccines, (Fig. 1, C/D) nucleotide changes were identified at positions 153 (A/G) and 168 (G/A). However, these did not reflect in amino acid substitutions.

4. Discussion

The present study aimed to identify genomes of CAV and AGV2 in commercially available avian vaccines. The findings obtained revealed that genomes of both agents may be detected in poultry vaccines; the specificity of the detection method was confirmed by sequence analysis. These results indicate the need to implement appropriate quality control measures to ensure absence of contamination of vaccines with such agents. Of eight manufacturer laboratories from which vaccines were tested, three had one or more vaccines that contained DNA of either CAV or AGV2, or both. One of the laboratories (manufacturer D) had the highest number of contaminated vaccines among those tested; besides, this was the only manufacturer whose vaccines were found to contain CAV genomes, besides AGV2 genomes. However, this was the manufacturer from which the greatest number of vaccines was examined; as the choice of vaccines was based essentially on the availability of the products for testing, these

proportions may have been biased by the fact that 20 out of the 35 vaccines tested were from the same laboratory. Nevertheless, it points out that this particular manufacturer seems to have a problem of CAV contamination along its vaccine production line. Regardless, these results do highlight the need for improving quality control practices to ensure the absence of such contaminants in all vaccines, particularly those where embryonated eggs are used as substrate for vaccine production, since these seem to be the main source of this type of contamination.

In the search for CAV, as expected, the vaccines meant to have CAV as immunogens did in fact contain CAV genomes, though with a considerable variation in the numbers of genome copies detected. One can speculate on how such variation might influence the vaccine's potential to induce protection. However, the main concern of the present report was the detection of CAV (and, as discussed below, AGV2) as contaminant. So, in this study, CAV genome was detected in 7 vaccines, all from the same laboratory. Contamination of vaccines with CAV has been known since 1979 [7], when the agent was first described. In Brazil, a retrospective study with commercial vaccines detected CAV contamination in vaccine batches from some laboratories until 1997, but not in 13 batches of distinct vaccines produced by five distinct laboratories from 2001 onwards [8]. However, the results obtained in the present study demonstrate the presence of CAV genomes in recently produces vaccines, substantiating the need for improved control measures to avoid CAV contamination. Although in this study only viral genomes were searched for, it is very likely that infectious virus might be present in such vaccines, as most of them were prepared with live microorganisms. This opens the possibility of potentially infectious virus to be present in the end product. It has been suggested that contamination of live vaccines with CAV may have influenced the epidemiology of chicken anemia in Brazilian and Argentinian poultry flocks [9]. In Brazil, a high prevalence of antibodies to CAV (89%) was detected in all States with intensive commercial broiler flock production [17]. Although this hypothesis might give rise to debate, it is clear that the presence of unwanted CAV might potentially introduce the virus and/or disease in flocks vaccinated with contaminated immunogens. In addition, it may lead to misleading attempts to trace the origin and evolution of the infection in the contaminated flocks; moreover, if CAV is introduced, it may lead to immune responses that may interfere with immunization programs. Therefore, this type of contamination, which involves a recognized pathogen, must be eliminated primarily to avoid introduction of the contaminant and disease and, secondarily, to avoid

unnecessary complications in flock vaccination and monitoring of the responses to vaccination.

Regarding AGV2, as this agent has only fairly recently been identified, reports on its detection in biological products have not to date been published. Nevertheless, like for CAV, the need for excluding contamination from such biological products is clear, particularly to avoid chances of dissemination of the virus. Although association between AGV2 and pathogenicity for chickens remains unclear, its presence in live vaccines may be involved in the virus' apparently wide distribution in chicken populations. As stated above, CAV can be transmitted vertically [18]; vertical transmission of AGV2 has not yet been demonstrated, but AGV2 genomes were detected in embryonic culture cells obtained from specific pathogen-free embryonated eggs (unpublished data), suggesting that vertical transmission is very likely. Therefore, contamination of embryonated eggs with such viruses must be a reason for concern in vaccine production.

Interspecies transmission of agents must also be born in mind e including a potential human involvement and since a number of live attenuated vaccines prepared in eggs are used in humans. A human gyrovirus (HGyV), which may be a contaminant from poultry origin rather than a virus of human origin, was reported nearly at the same time as the identification of AGV2 was published [19]. Subsequently, other gyroviruses were identified in samples from humans [20-22]. Thus, it is possible to speculate that perhaps one or more of such viruses might have been introduced into humans either by ingestion or handling of poultry and related products; yet it may be possible that such introduction and if it indeed happened e might have taken place by the administration of AGV2-contaminated vaccines to humans. Clearly, though, such hypothesis would require further investigation on additional vaccines, particularly on modified live attenuated vaccines for human use prepared in embryonated eggs.

The search for contaminants in vaccines was initially based on traditional techniques such as detection of antibodies or virus isolation [23]. In the course of time, genome detection technology was introduced with advantages over classical methods. Such advantages include the speed in which results can be obtained, improved accuracy and sensitivity [24]. Additionally, the possibility of detection of different contaminants in a same reaction as well as to quantify such contaminants has opened new perspectives for vaccine quality control. Genome detection technology, however, does not detect infectious virus; therefore, the results obtained here must be interpreted with

caution, as must be cautious extrapolations. Nevertheless, the sole presence of extraneous viral genomes in such biological products provides in itself substantial reason for concern.

These findings highlight the need of including the search for CAV and AGV2 in quality control testing by manufacturer laboratories, not only to improve the quality of the immunogens, but also to avoid unwanted side effects.

Contributors

Ana Paula Muterle Varela participated in all stages of the study.

Helton Fernandes dos Santos participated in execution of the work.

Samuel Paulo Cibulski participated in execution of the work.

Camila Mengue Scheffer participated in execution of the work.

Candice Schmidt participated in drafting of the paper.

Francisco Esmaile Sales Lima participated in drafting and review of the paper.

Alessandra D'Avila Silva participated in data interpretation.

Paulo Augusto Esteves participated in analysis and interpretation of data, drafting and review of the paper.

Ana Cláudia Franco participated in review of the paper.

Paulo Michel Roehe provided intellectual contribution to the study and participated in the review of the paper.

Conflict of interest

All authors contributed to the study, have approved the final version of the manuscript and declare that no competing interests exist.

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References

[1] Schat KA. Chicken anemia virus. Curr Top Microbiol Immunol 2009;331:151-83.

[2] Rijsewijk FA, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, et al. Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus gyrovirus. Arch Virol 2011;156:1097-100.

[3] Todd D. Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol 2000;29:373-94.

[4] Haridy M, Goryo M, Sasaki J, Okada K. Pathological and immunohistochemical study of chickens with co-infection of Marek's disease virus and chicken anaemia virus. Avian Pathol 2009;38:469-83.

[5] Toro H, van Santen VL, Hoerr FJ, Breedlove C. Effects of chicken anemia virus and infectious bursal disease virus in commercial chickens. Avian Dis 2009;53:94-102.

[6] Santos HF, Knak MB, de Castro FL, Slongo J, Ritterbusch GA, Klein TA, et al. Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. Vet Microbiol 2012;155:230-6.

[7] Yuasa N, Taniguchi T, Yoshida I. Isolation and some characteristics of an agent inducing anemia in chicks. Avian Dis 1979;23:366-85.

[8] Barrios PR, Marin SY, Rios RL, Pereira CG, Resende M, Resende JS, et al. A retrospective PCR investigation of avian orthoreovirus, chicken infectious anemia and fowl aviadenovirus genomes contamination in commercial poultry vaccines in Brazil. Arq Bras Med Vet Zootec 2012;64(1):231-5.

[9] Marin SYG, Barrios PR, Rios RL, Resende M, Resende JS, Santos BM, et al. Molecular characterization of contaminating infectious anemia virus of chickens in live commercial vaccines produced in the 1990's. Avian Dis 2013;57(1):15-21.

[10] Amer HM, Elzahed HM, Elabiare EA, Badawy AA, Yousef AA. An optimized polymerase chain reaction assay to identify avian virus vaccine contamination with chicken anemia virus. J Vet Diagn Invest 2011;23:34-40.

[11] Cardona CJ, Oswald WB, Schat KA. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. J Gen Virol 2000;81:2067-75.

[12] Cardona CJ, Lucio B, O'Connell P, Jagne J, Schat KA. Humoral immune responses to chicken infectious anemia virus in three strains of chickens. Avian Dis 2000;44:661-7.

[13] Miller MM, Ealey KA, Oswald WB, Schat KA. Detection of chicken anemia virus DNA in embryonal tissues and egg shell membranes. Avian Dis 2003;47:662-74.

[14] Varela APM, Scheffer CM, Santos HF, Cibulski SP, Roehe P. Simultaneous detection of chicken anemia virus and avian girovirus 2 by duplex real-time PCR. 2014 [Under review].

[15] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.

[16] Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32(5):1792-7.

[17] Brentano L, Silva BG, Sayd S, Flores SW. Anticorpos para o vírus da Anemia das Galinhas (CAV) em matrizes de corte no Brasil. Rev Bras Cienc Avic 2000;2(2):163-75.

[18] Von Bulow V, Schat KA. Chicken infectious anemia. In: Calnek BW, editor. Diseases of poultry. Ames, Iowa, USA: Iowa State University Press; 1997. p. 739-56.

[19] Sauvage V, Cheval J, Foulongne V, Gouilh MA, Pariente K, Manuguerra JC, et al. Identification of the first human gyrovirus, a virus related to chicken anemia virus. J Virol 2011;85:7948-50.

[20] Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, et al. A third gyrovirus species in human faeces. J Gen Virol 2012;93:1356-61.

[21] Chu DK, Poon LLM, Chiu SSS, Chan KH, Ng EM, Bauer I, et al. Characterization of a novel gyrovirus in human stool and chicken meat. J Clin Virol 2012;55:209-13.

[22] Phan TG, Vo PN, Sdiri-Loulizi K, Aouni M, Pothier P, Ambert-Balay K, et al. Divergent gyroviruses in the feces of Tunisian children. Virology 2013;446:346e8.

[23] Ottiger HP. Development, standardization and assessment of PCR systems for purity testing of avian viral vaccines. Biologicals 2010;38:381-8.

[24] Motitschke A, Ottiger HP, Jungb€ack C. Evaluation of the sensitivity of PCR methods for the detection of extraneous agents and comparison with in vivo testing. Biologicals 2010;38:389-92.

Table 1. Chicken anemia virus (CAV) and avian gyrovirus 2 (AGV2) genome loads in poultry vaccines.

Manufacture	Vaccine (agent)*	Vaccine (status)#	Country of production	Viral genome load##	
				AGV2	CAV
A	FPV	LV	Brazil	121	-
B	CAV	LV	United States of America	-	2,175381
B	IBV	LV	Hungary	-	-
B	IBV	LV	Hungary	-	-
C	IBV	LV	Brazil	-	-
C	AEV	LV	Brazil	-	-
C	NDV	LV	Brazil	-	-
C	TRTV/APV	LV	Brazil	-	-
C	TRTV/APV	LV	Brazil	-	-
D	IBV	LV	Brazil	-	103.2
D	IBV	LV	Brazil	2,392	173.7
D	IBDV	LV	Brazil	-	-
D	IBDV	LV	Brazil	-	-
D	IBDV	LV	Brazil	-	-
D	FPV	LV	Brazil	-	18
D	FPV	LV	Brazil	-	-
D	NDV	LV	Brazil	-	-
D	NDV	LV	Brazil	-	-
D	NDV	LV	Brazil	93	8.7
D	MDV (HVT)	LV	Brazil	-	-
D	MDV (HVT)	LV	Brazil	1,127	27
D	MDV (HVT)	LV	Brazil	156,187	-
D	MDV (HVT)	LV	Brazil	-	-
D	MDV (Rispens)	LV	Brazil	721	7
D	MDV (Rispens)	LV	Brazil	462	-
D	ACV	LV	Brazil	-	-
D	IC	IV	Brazil	-	-
D	EDS	OIV	Brazil	-	6.4
D	NDV/IBV/ IBDV	OIV	Brazil	-	-
E	IBDV	LV	Brazil	-	-
E	ILTV	LV	Canada	27,418	-
E	NDV	LV	Netherlands	178	-
F	IBV	LV	Brazil	-	-
G	CAV	LV	Germany	-	54,238
H	CAV	LV	Germany	-	2,386

*CAV - Chicken anemia virus; IBV - Infectious bronchitis virus; IBDV - Infectious bursal disease virus; FPV - Fowlpox virus; AEV - Avian encephalomyelitis virus; NDV - Newcastle disease virus; TRTV/APV - Turkey rhinotracheitis virus and Avian pneumovirus; MDV - Marek's disease virus (CVI-988/Rispens or HVT); IC - Infectious coryza; ACV - Avian coccidiosis; ILTV - Infectious laryngotracheitis virus; EDS - Egg drop syndrome. #LV - live virus; OIV - oil adjuvanted and inactivated; IV - inactivated virus. ##Data presented are the averages of the results obtained by duplex quantitative real time PCR (dqPCR) performed in triplicate and expressed as number of CAV or AGV2 genome copies per 50 ng of vaccine DNA.

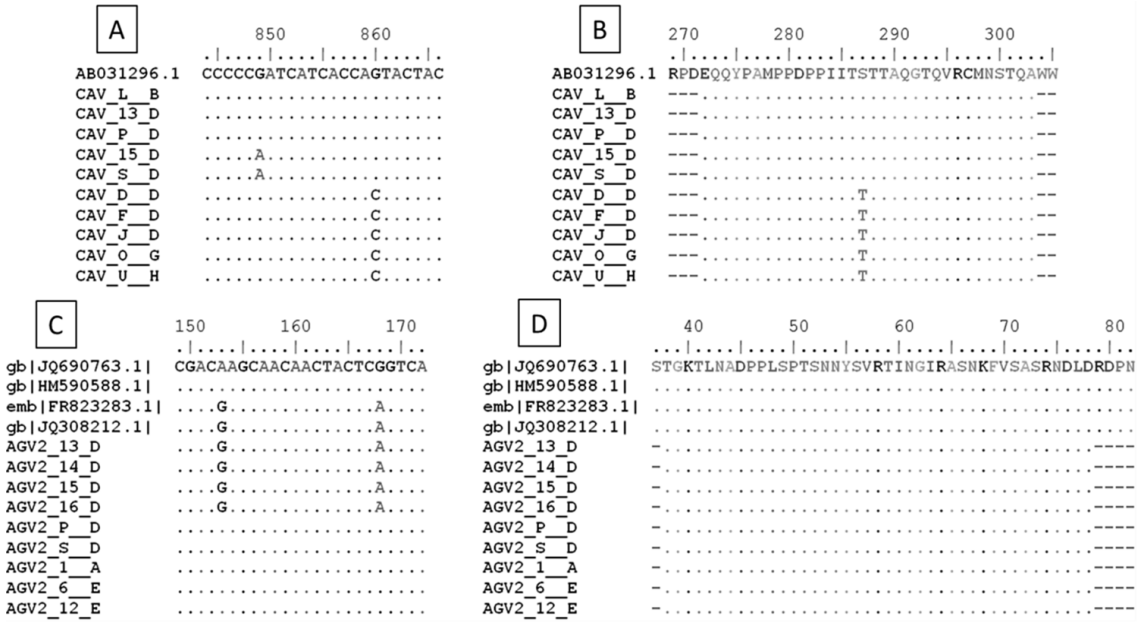

Figure 1. Comparison of nucleotide sequences in sequenced amplicons from vaccines containing CAV (A/B) and AGV2 (C/D) with similar sequences available at GenBank (accession numbers: AB031296.1 for CAV; HM590588.1; JQ308212.1; FR823283.1 for AGV2. Modifications are highlighted by the letters corresponding to the specific nucleotide changes.

CAPÍTULO 3

Phylogenetic analysis of the *VP1* gene of avian gyrovirus 2 in chickens with different health status

Ana Paula Muterle Varela[a,b*], Candice Schmidt[a,b], Helton Fernandes dos Santos[b], Paulo Augusto Esteves[c], Francisco Esmaile Sales Lima[d], Samuel Paulo Cibulski[a,b], Camila Mengue Scheffer[a,b], Alessandra D'Avila Silva[c], Ana Cláudia Franco[b], Paulo Michel Roehe[b]

[a]FEPAGRO – Saúde Animal – Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Estrada do Conde 6000, Eldorado do Sul, CEP 92990-000, Rio Grande do Sul, Brazil.

[b]Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, UFRGS. Av. Sarmento Leite 500, sala 208, Porto Alegre, CEP 90050-170, Rio Grande do Sul, Brazil.

[c]EMBRAPA Suínos e Aves - BR 153 Km 110, CEP: 89700-000, Concordia, SC, Brazil.

[d]Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Laboratório de Microbiologia Molecular, Av Sarmento Leite, 245, Porto Alegre, CEP 90050-170, Rio Grande do Sul, Brazil.

*Corresponding author:

Laboratório de Virologia, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) - Estrada do Conde 6000, CEP 92990-000 - Eldorado do Sul, Rio Grande do Sul, Brazil. E-mail address: anapaulamut@gmail.com

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ABSTRACT

Avian gyrovirus type 2 (AGV2) is a member of the genus *Gyrovirus*, family *Circoviridae*. The AGV2 genome has been detected in healthy chickens as well as in chickens displaying retarded growth, weight loss and/or neurological signs, although, to now, the association of the AGV2 with some symptoms or disease is not confirmed. Nonetheless, a possible association between disease status and different AGV2 genomic variants has been suggested. In view of such possibility, the present study was carried out to examine the variability of AGV2 genomes detected in chickens with different health status. Feather shafts from apparently healthy chickens (n= 36) and liver tissues from commercial poultry displaying low weight gain (n=71) were submitted to DNA extraction and submitted to a PCR targeting the genomic region coding for the viral capsid protein VP1. Out of the 107 samples examined, 40 were found to contain AGV2. Twenty one of these (thirteen from healthy and eight from diseased chickens) were sequenced and submitted to phylogenetic analysis. Although comparisons at nucleotide level were already discriminative, the predicted amino acid (aa) sequences more clearly revealed two groups; one included all thirteen sequences from healthy chickens, plus two VP1 sequences from diseased birds. A second cluster consisted of the remainder eight sequences from diseased birds. Interestingly, the cluster comprising sequences from healthy chickens aligned more closely to AGV2 sequences recovered from birds, whereas those from diseased chickens aligned more closely to AGV2-like viruses from mammals, including human gyrovirus (HGyV) and AGV-2 from ferrets and children. The main aa difference detected was that the VP1 from diseased chickens share a common amino acid substitution pattern in positions 270 (A/S), 310 (Q/E), 383 (Q/P), 401 (M/V) and 416 (I/L). The data presented here indicate that there may be some association between AGV2 variants and pathogenicity; in addition, pathogenicity for chickens may be related to the ability of such variants to infect mammals.
Keywords: Avian gyrovirus 2, pathogenicity, variants, phylogenetic analysis.

1. Introduction

Avian gyrovirus type 2 (AGV2) is a recently identified agent classified in the genus *Gyrovirus* of the family *Circoviridae* (Rijsewijk et al., 2011). The AGV2 genome was initially recovered from chickens displaying clinical manifestations similar to those caused by chicken anemia virus (CAV), including apathy, retarded growth rates and neurological signs (Abolnik and Wandrag, 2014; Rijsewijk et al., 2011; dos Santos et al., 2012). Nevertheless, the viral genome has also been detected in healthy chicken (dos Santos et al., 2012).

The AGV2 genome consists of a circular single-stranded molecule of DNA of about 2.4 kb (Rijsewijk et al., 2011). A 400 nucleotide-long 5' untranslated region is followed by three partially overlapping open reading frames (ORF) encoding VP1, the major structural protein, VP2, a scaffolding protein, and VP3, a non-structural protein named apoptin, all with homologs in CAV (Noteborn et al., 1991).

Little is known about AGV2 genetic variability; however, genomic analyses of the closely related CAV reveals that the VP1 gene bears the highest degree of variability between the three viral coded proteins; in addition, specific amino acid (aa) changes at positions 139 and 144 at a 13- aa long hypervariable region (HVR) in CAV VP1 (aa 139–151) were essential for virus multiplication and spread (Chowdhury et al., 2003; Renshaw et al., 1996). Subsequently, the aa at position 394 in VP1 was considered a major genetic determinant of CAV virulence (Yamaguchi et al., 2001). In addition *VP1* gene phylogenetic analysis revealed three genetically distinct CAV genotypes (I, II, and III) (Craig et al., 2009; Ducatez et al., 2008, 2006; Islam et al., 2002; Kim et al., 2010; Snoeck et al., 2012).

Preliminary analyses of AGV2 genomes so far reported revealed variability among AGV2 sequences, which in certain genomic regions may reach 15.8% (dos Santos et al., 2012). It has been suggested that such variations might be associated with differences in the pathogenic potential of variants (dos Santos et al., 2012). The present study was carried out to examine more deeply such hypothesis by comparing the genetic variability of the AGV2 *VP1* gene recovered from chickens with different health status.

2. Material and Methods

2.1 Chicken samples

One hundred and seven samples of feathers and liver tissues were collected from backyard and commercial chickens. Recovery of AGV2 genomes from either of these

sources has been demonstrated successfully (Esteves et al., 2012; dos Santos et al., 2012). Feathers were collected from apparently healthy backyard chickens and with no previous history of vaccination. In addition, liver tissues (n=71) were collected from three-weeks-old commercial chickens with signs of disease. For the purpose of the present study, diseased chickens were defined as birds displaying signs of apathy and stunted growth.

2.2 DNA extraction

Total DNA was extracted from feathers and liver samples using the commercially available PureLink™ Genomic DNA Mini Kit (Invitrogen™) following manufacturer's instructions. DNA extracted was quantified with a fluorometer (Qubit® 2.0; Life technologies) and stored at -20°C until used.

2.3 Primer design

AGV2-specific primers were designed using the Primer3 program in the Geneious software package (version 8.0.5; Kearse et al., 2012), with basis on the AGV2 nucleotide sequence (Genbank accession number NC_015396; Rijsewijk et al., 2011). Forward primer (5'-AT GGC AAG ATT ACG ACG ACG ACG-3') and reverse primer (5'-TT ACG GGT TGT ATC TGT CCG TTG-3') were selected targeting a fragment of 1383 bp along of the genomic region that codes VP1 (encompassing nucleotides 953 to 2335). The primers were synthesized commercially (IDT Integrated DNA Technologies).

2.4 Polymerase chain reaction (PCR)

PCR amplification was performed in a 20 µl total reaction volume containing 100 ng of the extracted DNA, 0.8 mM of each dNTP, 5 pmol of each forward and reverse primers, 10% HF PCR buffer (5X), 1U Phusion Hot Start DNA polymerase (Finnzymes) and sterilized Milli-Q water to complete the total volume. Reactions were performed using the following cycling profile: initial denaturation of 98°C for 3 min, followed by 35 cycles of denaturation, annealing and extension at 98°C for 30 s, 64°C for 30 s and 72°C for 30 s, respectively; and the final extension at 72°C for 3 min in Veriti® Thermal Cycler (Applied Biosystems). The amplicons were visualized on 1% agarose gel stained with ethidium bromide (0.5 µg/ml) under UV light. To avoid

contamination, different locations were used to conduct the DNA extractions, to prepare the reagents and to run the reactions.

2.5 Sequencing and sequence analyses

Twenty-one amplifications products were purified by PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Life technologies) following the manufacturer's protocol. Purified DNA products were subjected to direct nucleotide sequencing using a MegaBACE 500 apparatus with the Dyenamic ET terminator cycle sequencing kit (Amersham Biosciences). Sequences were initially evaluated by BLAST and subsequently aligned with the Geneious (version 8.0.5; Kearse et al., 2012) and MEGA package (version 5.2; Tamura et al., 2011). Multiple-sequence alignments were performed with ClustalW (Geneious) including previously published AGV2 and HGyV sequences available at GenBank (accession numbers: NC_015396, JQ690763, KF436510, KJ452213, NC_015630, KJ452214). The nucleotide and predicted amino acid sequences were determined and compared with AGV2 reference sequence (NC_015396). Phylogenetic trees were generated by neighbor-joining using the MEGA package (version 5.2; Tamura et al., 2011) with 1,000 bootstrap replications. Evolutionary distances were estimated using Kimura 2-parameter for nucleotide analysis and the Jones-Taylor-Thornton (JTT) model for amino acid analysis.

3. Results

3.1 Detection of AGV2 by PCR

The AGV2 DNA was detected in 40 (37%) out of 107 samples analyzed. Of these, 22 samples were from healthy chickens and 18 from diseased birds. A total of 21 samples, including 13 from healthy and 8 from diseased chickens were randomly selected for sequencing and further analyses.

3.2 Sequence accession numbers

The VP1 nucleotide sequences obtained in this study were submitted to GenBank under the following accession numbers: KP768319 (RS/01), KP768320 (RS/02), KP768321 (RS/03), KP768322 (RS/04), KP768323 (RS/05), KP768324 (RS/06), KP768325 (RS/07), KP795785 (RS/11), KP795786 (RS/12), KP795787 (RS/13), KP795788 (RS/14), KP795795 (RS/15) and KP795790 (RS/17) for AGV2 sequences recovered from healthy chickens; KP769414 (RS/08), KP795783 (RS/09),

KP795784 (RS/10), KP795789 (RS/16), KP795791 (RS/18), KP795792 (RS/19), KP795793 (RS/20) and KP795794 (RS/21) for AGV2 sequences from diseased chickens.

3.3 Phylogenetic analyses

Similarity between sequences of chickens with distinct health status ranged from 93.9% to 100% at nucleotide level and between 97.3% to 100% at the predicted aa level. Phylogenetic analysis of the nucleotide sequences allowed subdivision of AGV2 genomes in two major groups. One of such groups comprised all thirteen AGV2 sequences recovered from healthy chickens, plus two sequences from diseased chickens (RS/16 and 19). Two additional sequences from chickens with clinical signs (RS/08 and 09) segregated to a separate branch, though distinct from most sequences from diseased chickens, which comprised the second major cluster (Fig. 1).

Phylogenetic analyses on the predicted aa sequences (Fig. 2) confirmed the first cluster mentioned above, comprising the thirteen AGV2 sequences from healthy chickens, plus samples 16 and 19 from diseased chickens. Yet, these clustered along with previously published AGV2 sequences recovered from chickens only. The second cluster generated by aa analysis (Fig. 2) comprised six out of eight sequences (RS/08, 09, 10, 18, 20 and 21) recovered from diseased chickens. Distinctly from the first cluster, these were grouped along with AGV2-like sequences - AGV2 and HGyV sequences recovered from mammals, including humans and pet ferrets (NC_015630; KJ452214; JQ690763; KJ452213). Further analysis revealed that these share a common aa substitution pattern, characterized by substitutions at the following positions: 270 (A/S), 310 (Q/E), 383 (Q/P), 401 (M/V) and 416 (I/L) (Fig. 3). Thus, AGV2 genomes from diseased chickens all presented this same pattern, which is common to AGV2-like genomes from humans and other mammals.

4. Discussion

Due to the recent discovery of AGV2, little information on its biology is available. The virus seems widespread in chickens, and has been detected in both healthy and diseased animals (Abolnik and Wandrag, 2014; dos Santos et al., 2012). Although AGV2 was originally identified in chickens with signs of disease, to date there is no clear evidence to implicate AGV2 as a pathogenic agent. Analysis of AGV2 genomic region that codes part of the VP2 and VP3 genes in chickens with or without

neurological signs (dos Santos et al., 2012) suggests a possible association of AGV2 variants with disease. In the present study, AGV2 genomes recovered from chickens with different health status were analyzed in its genetic variability and possible association with disease. Twenty-one sequences of the gene coding for the major viral capsid protein VP1 were recovered from healthy and diseased chickens and its sequences compared. The analyses were concentrated on VP1 since previous findings with CAV indicate that, by analogy with that virus, the high genetic diversity of VP1 would make it appropriate to explore genetic variability within gyroviruses (Renshaw et al., 1996).

Regarding to the VP1 aa phylogenetic analysis, the sequences allowed grouping of the AGV2 into two major clusters; the first comprised sequences of thirteen healthy chickens, along with those of two diseased chickens These two sequences (RS/16 and 19) were homologous to sequences from healthy chickens and did not present the aa substitution pattern identified in the second cluster, with AGV2 sequences from all other diseased chickens. It is possible that these two birds were diseased due to other causes.

These findings may indicate a correlation between specific amino acid substitutions in VP1 and AGV2 pathogenicity, since by analogy for CAV, one specific amino acid substitution in the VP1 (position 394) was found to be a virulence determinant (Yamaguchi et al., 2001). It was previously suggested that differences in AGV2 pathogenicity might be related to the occurrence of AGV2 variants (dos Santos et al., 2012). The results of the present study partially support this hypothesis. Nevertheless, investigations should be carried out to further examine the role for AGV2 variants in pathogenicity.

Interestingly, the AGV2 sequences here identified in diseased chickens are more closely related to gyroviruses (AGV2 and HGyV) identified in humans and other mammals. It might be speculated that AGV2 variants pathogenic for chickens might be more prone to infect other non-avian species. However, the relationship between AGV2 from mammals and avian viruses is still undetermined and remains to be examined in the future (Fehér et al., 2014; Li et al., 2011).

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References

Abolnik, C., Wandrag, D.B., 2014. Avian gyrovirus 2 and avirulent Newcastle disease virus coinfection in a chicken flock with neurologic symptoms and high mortalities. Avian Dis. 58, 90-94.

Chowdhury, S.M., Omar, A.R., Aini, I., Hair-Bejo, M., Jamaluddin, A.A., Md-Zain, B.M., Kono, Y., 2003. Pathogenicity, sequence and phylogenetic analysis of Malaysian Chicken anaemia virus obtained after low and high passages in MSB-1 cells. Arch. Virol. 148, 2437-2448.

Craig, M.I., Rimondi, A., Delamer, M., Sansalone, P., König, G., Vagnozzi, A., Pereda, A., 2009. Molecular Characterization of Chicken Infectious Anemia Virus Circulating in Argentina During 2007. Avian Dis. 53, 331–335.

dos Santos, H.F., Knak, M.B., de Castro, F.L., Slongo, J., Ritterbusch, G.A., Klein, T.A., Esteves, P.A., Silva, A.D., Trevisol, I.M., Claassen, E.A., Cornelissen, L.A., Lovato, M., Franco, A.C., Roehe, P.M., Rijsewijk, F.A., 2012. Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. Vet. Microbiol. 155, 230-236.

Ducatez, M.F., Chen, H., Guan, Y., Muller, C.P., 2008. Molecular epidemiology of chicken anemia virus (CAV) in southeastern Chinese live birds markets. Avian Dis. 52, 68-73.

Ducatez, M.F., Owoade, A.A., Abiola, J.O., Muller, C.P., 2006. Molecular epidemiology of chicken anemia virus in Nigeria. Arch. Virol. 151, 97-111.

Esteves, P.A., Trevisol, I.M., Franco, A.C., Cornelissen, L., Silva, V.S., Rech, R., 2012. Molecular detection of Avian gyrovirus type 2 (AGV2) in specific pathogen free (SPF) chickens housed on commercial broiler litter. XXIV World's Poultry Congress: World's Poultry Science Journal, Supplement 1, expanded abstract, 325.

Fehér, E., Pazar, P., Kovacs, E., Farkas, S.L., Lengyel, G., Jakab, F., Martella, V., Banyai, K., 2014. Molecular detection and characterization of human gyroviruses identified in the ferret fecal virome. Arch. Virol. 159, 3401-3406.

Islam, M.R., Johne, R., Raue, R., Todd, D., Muller, H., 2002. Sequence analysis of the full-length cloned DNA of a chicken anaemia virus (CAV) strain from Bangladesh: evidence for genetic grouping of CAV strains based on the deduced VP1 amino acid sequences. J. Vet. Med. B Infect. Dis. Vet. Public. Health 49, 332-337.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12), 1647-1649.

Kim, H.R., Kwon, Y.K., Bae, Y.C., Oem, J.K., Lee, O.S., 2010. Molecular characterization of chicken infectious anemia viruses detected from breeder and broiler chickens in South Korea. Poult. Sci. 89, 2426-2431.

Li, L., Shan, T., Soji, O.B., Alam, M.M., Kunz, T.H., Zaidi, S.Z., Delwart, E., 2011. Possible cross-species transmission of circoviruses and cycloviruses among farm animals. J. Gen. Virol. 92, 768-772.

Noteborn, M.H., de Boer, G.F, van Roozelaar, D.J., Karreman, C., kranenburg, O., Vos, J.G., Jeurissen, S.H.M., Hoeben, R.C., Zantema, A., Koch, G., van Ormondt, H., van Der Eb, A.J., 1991. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. J. Virol. 65, 3131–3139

Renshaw, R.W., Soine, C., Weinkle, T., O'Connell, P.H., Ohashi, K., Watson, S., Lucio, B., Harrington, S., Schat, K.A., 1996. A hypervariable region in VP1 of chicken infectious anemia virus mediates rate of spread and cell tropism in tissue culture. J. Virol. 70, 8872-8878.

Rijsewijk, F.A., dos Santos, H.F., Teixeira, T.F., Cibulski, S.P., Varela, A.P., Dezen, D., Franco, A.C., Roehe, P.M., 2011. Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Arch. Virol. 156, 1097-100.

Snoeck, C.J.G., Komoyo, F., Mbee, B.P., Nakouné, E., Faou, A.L., Okwen, M.P., Muller, C.P., 2012. Epidemiology of chicken anaemia virus in Central Africa Republic and Cameroon. J. Virol. 9, 189–197.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28(10), 2731–2739.

Yamaguchi, S., Imada, T., Kaji, N., Mase, M., Tsukamoto, K., Tanimura, N., Yuasa, N., et al., 2001. Identification of a genetic determinant of pathogenicity in chicken anaemia virus. J. Gen. Virol. 82, 1233-1338.

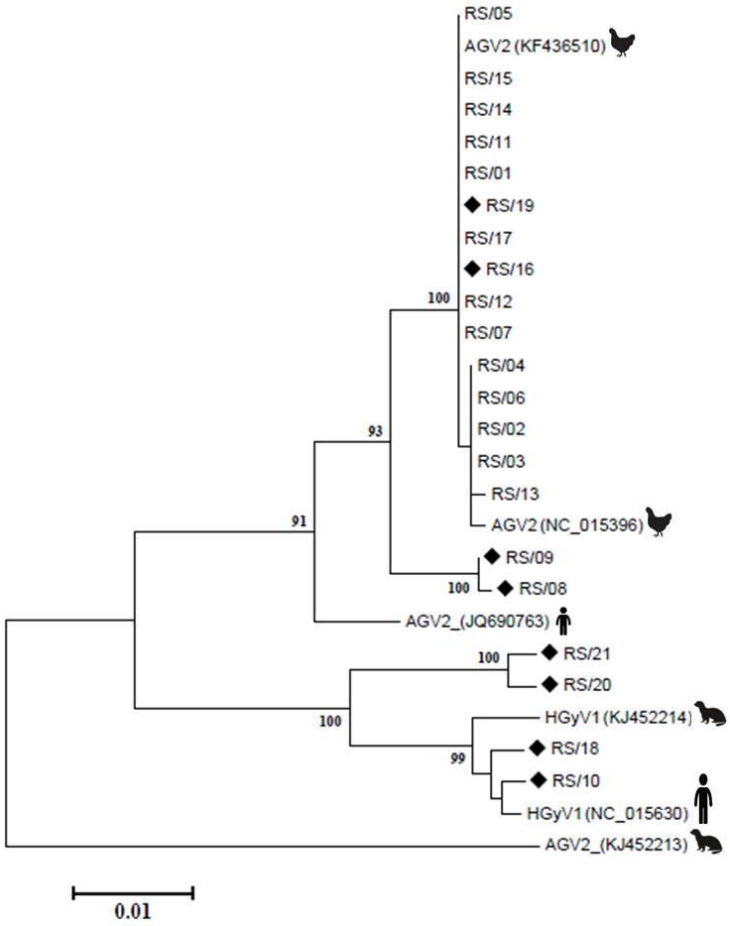

Fig. 1. Phylogenetic analysis of partial VP1 nucleotide sequences recovered from healthy and diseased chickens. AGV2 and HGyV sequences available in GenBank were included as references. VP1 sequences from diseased birds are labelled by black diamonds. Values ≥ 70 are indicated on the branches. Analysis was conducted by neighbor-joining method using MEGA5.1 software. Evolutionary distances were estimated using Kimura 2-parameter.

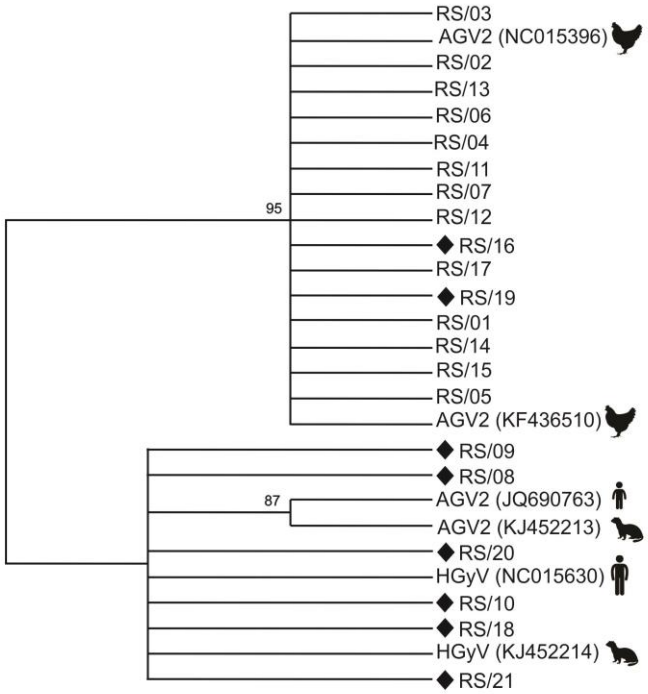

Fig. 2. Phylogenetic analysis of VP1 predicted amino acid sequences recovered from healthy and diseased chickens. AGV2 and HGyV sequences available in GenBank were included as references. VP1 sequences from diseased birds are labelled by black diamonds. Values ≥ 70 are indicated on the branches. Analysis was carried out by neighbor-joining and Jones-Taylor-Thornton (JTT) model using MEGA5.1 software.

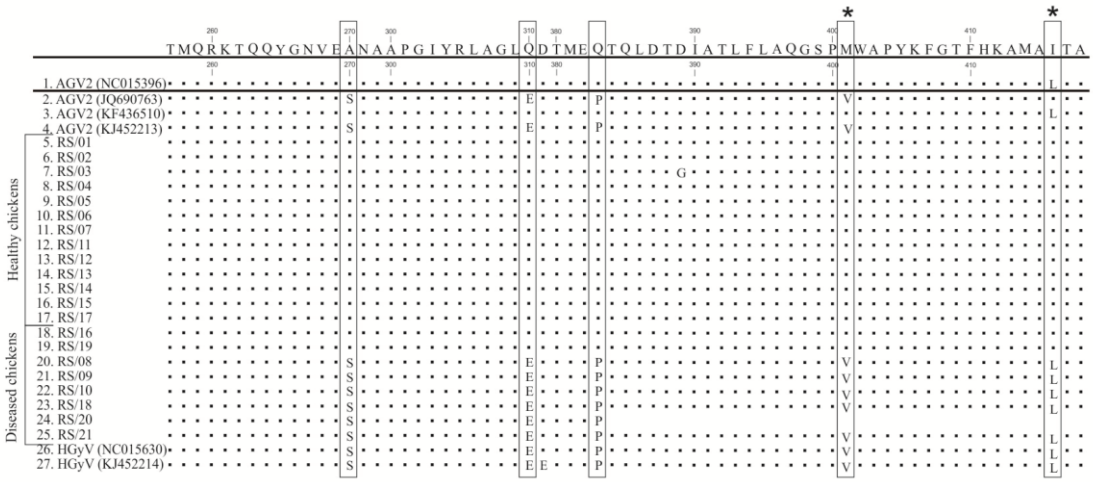

Fig. 3. Alignment of VP1 predicted amino acid sequences obtained from healthy and diseased chickens. Sequences recovered from diseased chickens shared common amino acid substitution patterns at positions 270, 310, 383, 401 and 416. These aa substitutions are highlighted. *Not available for RS/20 sequence.

CAPÍTULO 4

Molecular characterization of avian gyrovirus 2 detected in commercial poultry farms from Italy

ABSTRACT

Avian gyrovirus 2 (AGV2) is a new gyrovirus recently identified in poultry. It seems to be widespread in poultry population, since viral genomes have been detected in countries quite geographically distant. In Europe, AGV2 genomes have been detected in the Netherlands and Hungary, suggesting that virus is at least present in these European countries. In the present study, a search was conducted to examine the occurrence of AGV2 infections in commercial poultry farms in Italy. In addition, it was carried out the genomic characterization of the identified genomes. One-hundred serum samples from 10 poultry farms were submitted to DNA extraction and the AGV2 detection was performed by PCR. Viral DNA was identified in five out of the ten farms sampled. So, one DNA sample from each farm was submitted to AGV2 complete genome amplification, sequencing and phylogenetic analysis. In view of the high GC content present in the region downstream of the stop codon of VP1 of AGV2, one complete genome and four partial AGV2 genomes (about 83%) were obtained. The AGV2 genomes identified have the same genomic organization of gyroviruses, but unlike GyV4 and GyV5, whose genomes do not include the VP3-coding region. Moreover, phylogenetic analyses at amino acid level showed that AGV2 genomes recovered from Italian samples were phylogenetically more closely related to human gyrovirus (HGyV). The AGV2 sequences here obtained shared an overall degree of similarity greater than 92% with original Brazilian AGV2 genome. Whereas, regarding the others gyroviruses, the degree of overall similarity was always below 64.6%. These findings suggest that AGV2 genomes recovered from Italian samples and previously reported AGV2 and HGyV genomes are very closely related cluster with the genus *Gyrovirus.*

Keywords: genome; gyroviruses; phylogenetic analysis

Artigo em fase de conclusão para submissão na forma de *Short report* no periódico *Virus Genes*

1. Introdution

In early 2011, the genome of a small DNA virus, named avian gyrovirus 2 (AGV2), was identified in serum samples of poultry with apathy and low weight gain (Rijsewijk et al., 2011). In view of its similarity with chicken anemia virus (CAV), the genome of this new agent led to its classification as a new member of the genus *Gyrovirus*, within the family *Circoviridae* (Rijsewijk et al., 2011). This newly described genome is a circular single-stranded DNA molecule of about 2.4 kb, containing a putative 5´ untranslated region (UTR) of about 400 nucleotides (nt), followed by three partially overlapping open reading frames (ORF1, ORF2 and ORF3) which encode 3 viral proteins: VP1, VP2, and VP3 (Rijsewijk et al., 2011). Since viral genomes have been detected in countries such as Brazil, the Netherlands, Hong Kong, South Africa and Hungary, it provides evidence that AGV2 seems widespread in different geographical regions (Abolnik and Wandrag, 2014; Chu et al., 2012; dos Santos et al., 2012; Fehér et al., 2014; Phan et al., 2012). In Europe, AGV2 genomes have been detected in the Netherlands and Hungary, providing evidence to support that the virus is present in those countries (dos Santos et al., 2012; Fehér et al., 2014). In the present study, a search was conducted to examine the occurrence of AGV2 infections in commercial poultry farms in Italy. The genomes identified were subsequently sequenced and compared to other members of the genus *Gyrovirus*.

Results and discussion

A total of 100 serum samples were collected from apparently healthy poultry in 10 poultry farms (10 sera each farm) from Veneto region, Italy. Sera were pooled (two pools of five sera/farm) and DNA was extracted from 200 µl of each pool using High Pure PCR Template Preparation Kit (Roche), following manufacturer's instructions. DNA extracted was submitted to amplification by a screening PCR to detect AGV2 as follows. Each reaction mixture comprised 5 µL of DNA, 0.2 µM of each primer (Table1), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ (50mM), 1 U Platinum® Taq DNA polymerase (Invitrogen), 10% PCR buffer (10X) and DNase/RNase-free sterilized H_2O to 25 µL. The cycling conditions were: 2 min at 94 ºC, followed by 35 cycles of 45 s at 94 ºC, 30 s at 55 ºC, 2 min or 90 s at 72 ºC and a final extension step at 72 ºC. Amplifications were carried out in a MJ Mini^{TM} thermal cycler (Bio-Rad). Five microliters of the amplified products were analyzed on 1% agarose gels. Posteriorly,

DNA from serum samples of pools containing AGV2 genome (n=25) were individually extracted and submitted to PCR as above. Of these, one DNA sample, considered representative of each farm (S04/It, S53/It, S63/It, S80/It, S99/It), was submitted to two additional assays (PCR II and PCR III) aiming amplification of the entire genome. Reaction and cycling conditions for PCR II and PCR III were essentially the same as those used in the screening PCR above, except that primers and annealing temperatures (57 ºC and 54 ºC to PCR II and III, respectively). Primers set described in Table 1 design were designed with base on the available AGV2 genome sequence (GenBank accession number NC_015396; Rijsewijk et al., 2011). The PCR products were purified using ExoSAP-IT® (USB Corporation, Cleveland, OH) and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem). The nucleotide sequence editing, analysis, prediction of amino acid sequence and alignments were performed using Geneious software (version 8.0.5; Kearse et al., 2012) and MEGA package (version 5.2; Tamura et al., 2011). The AGV2 genome sequence (GenBank accession number NC_015396; Rijsewijk et al., 2011) was used as reference sequence. To determine phylogenetic relationships, all currently gyrovirus sequences available in GenBank were included in the analyses. Phylogenetic analyses of the VP1, VP2 and VP3 were performed based on the predicted amino acid sequences. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstrap replications and p-distance using Mega package (version 5.2; Tamura et al., 2011).

Genomes of AGV2 were detected in five out of the ten poultry farms sampled (8 out of 20 serum pools). Within pools, 15 out of 25 samples contained AGV2 genomes. One sample, considered representative of each farm, was sequenced to completion or near completion of the genome. Sample S53/It was sequenced to completion, encompassing a whole 2330 nucleotide genome. Four other genome sequences (S04/It, S63/It, S80/It and S99/It) were determined to near completion due to high GC content present in the region downstream of the stop codon of VP1 of AGV2. These genomes were obtained about 1970 nt (approximately 83% of the AGV2 genome) comprising the whole VP2- and VP3-coding regions, and a significant portion of VP1.

The AGV2 genomes here identified showed the same genomic organization of gyroviruses, but unlike GyV4 and GyV5, whose genomes do not include the VP3-

coding region. In the full genome sequence here obtained (S53/It) the 5' UTR region is located between the polyadenylation and the start of the transcription sites. Within that region there are six almost perfect direct repeat (DR) regions of 22 nt: GTACAGGGGGGTACGTCAc/tCAt/g in which, three nucleotide changes in the nucleotide position 19 of the first, third and fourth DRs compared to AGV2 genome reference. Such sequences were also detected in the others gyroviruses (Chu et al., 2012; Phan et al., 2013; Rijsewijk et al., 2011; Sauvage et al., 2011; Zhang et al., 2014). In addition, two canonical polyadenylation signals (AATAAA) and a putative TATA box (GATATAAG) were identified in all sequences from Italy at homologous position to AGV2 reference (Rijsewijk et al., 2011). The sequences contain also a putative transcription start site at nt 440 (GGCgGTAGGT), which is located 10 nt upstream of the start of ORF1 (nt 450), which is equally detected in HGyV. Although AGV2 also has a similar sequence, on AGV2 one nucleotide substitution (A/G) is found.

Similarly to other gyroviruses, two important motifs were identified in all AGV2 sequences here reported. One of such conserved motifs, TLX_2AQ, has been related to rolling-circle replication (RCR; Ilyina and Koonin, 1992). The presence of this motif in the AGV2 sequences suggests that viral replication occurs through the RCR model proposed for circoviruses, such as CAV and porcine circovirus 2 (Biagini et al., 2012). In the ORF1, it was found a conserved motif ($WX_7HX_3CXCX_5H$) shared by gyroviruses and anelloviruses (Takahashi et al., 2000). According to the document 2014.006a-gV from ICTV, such conserved motif plus similarities in genomic organization present in gyroviruses and anelloviruses have given ground to propose the reallocation of the genus *Gyrovirus* within the *Anelloviridae* (Lima et al., 2015).

In addition, all AGV2 genomes here identified have the sequence which codes VP3 or apoptin. This protein is reported to induce apoptosis in tumor cells, but not of non-tumor cells (Noteborn, 2009). In tumor cells the apoptin is translocated into the nucleus; whereas, in normal cells the protein is localized in the cytoplasm. This shuttle in and out of the nucleus in both normal and transformed cells is directed by one bipartite nuclear localization (NLS) and nuclear export sequence (NES) (Kuusisto et al., 2008). These recognition sequences (NLS and NES) were found in AGV2 genomes of this study. In addition, 4 out of 5 AGV2 genome sequences reported here were homologous to VP3 HGyV. Studies has been described the functional potential of HGyV apoptin with level of cell death induced by this protein similar to cell death induced by CAV-apoptin (Bullenkamp et al., 2012; Chaabane et al., 2014). In this

sense, the AGV2 genome recovered from Italian samples may also display apoptotic potential.

The overall degree of nucleotide similarity (VP1, VP2 and VP3) ranged from 95.4% to 100% with HGyV and ranged from 93.3% to 100% with AGV2; whereas, amino acid (aa) similarity ranged from 99% to 100% with HGyV and ranged from 93% to 100% with AGV2. Phylogenetic analyses on the predicted aa sequences for VP1, VP2 and VP3 confirmed this similarity and revealed that AGV2 genomes were phylogenetically more closely related to HyGV and to AGV2 than the others gyroviruses (Fig. 1).

In the VP1 phylogenetic tree, all AGV2 sequences obtained in this study clustered along with HGyV and AGV2 sequences previously recovered from human and pet ferret feces (Fig. 1). Yet these clustered separately from AGV2 reference sequence recovered from chickens. In the VP2 phylogenetic three, two main clusters were formed: one comprising 4 out of 5 sequences reported here (S04/It, S53/It, S63/It, S80/It) and previously reported HGyV VP2 sequences. One of the samples introduced here, (S99/It) displayed a distinct profile and clustered along with AGV2 VP2 sequences (Fig. 1). Regarding VP3 analyses, 4 out of 5 sequences recovered from Italian samples clustered along with HGyV. In fact, these sequences (S04/It, S53/It, S63/It, S80/It) were identical to HGyV VP3. The only discrepant genome in relation to VP3 was from sample S99/It, which was segregated in a separate branch apart from HGyV and AGV2 genomes (Fig. 1). These findings showed that although there are some genomic variation between AGV2 genomes and HGyV, these gyroviruses are phylogenetically closely. No phylogenetic distinction between viruses of human or chicken origin was found, as previously reported (Chu et al., 2012; Phan et al., 2013; Zhang et al., 2014). The finding of viruses with such degree of similarity in so evolutionarily distinct species as chickens and humans brings up the urgent need to investigate the role for such viruses in their hosts. So, in addition to bioinformatics analysis, future investigations must include attempts in virus isolation, which will give some light to better understand the relationships between these gyroviruses.

In summary, AGV2 genomes were detected in chicken flocks in Italy. The phylogenetic analysis of complete or near complete nucleotide sequences revealed that such sequences recovered from Italian samples are more genetically closely related to HGyV and to AGV2.

References

1. Abolnik C, Wandrag DB (2014) Avian gyrovirus 2 and avirulent Newcastle disease virus coinfection in a chicken flock with neurologic symptoms and high mortalities. Avian diseases 58:90-94

2. Biagini P, Bendinelli M, Hino S, Kakkola L, Mankertz A, Niel C, Okamoto H, Raidal S, Teo CG, Todd D. (2011) Family Circoviridae. In: King AM Q et al (Ed.) Virus Taxonomy: Classification and Nomenclature of Viruses (Ninth Report of the International Committee on the Taxonomy of Viruses). New York: Elsevier Academic Press 953–959.

3. Bullenkamp J, Cole D, Malik F, Alkhatabi H, Kulasekararaj A, Odell EW, Farzaneh F, Gaken J, Tavassoli M (2012) Human Gyrovirus Apoptin shows a similar subcellular distribution pattern and apoptosis induction as the chicken anaemia virus derived VP3/Apoptin. Cell death & disease 3:e296

4. Chaabane W, Cieslar-Pobuda A, El-Gazzah M, Jain MV, Rzeszowska-Wolny J, Rafat M, Stetefeld J, Ghavami S, Los MJ (2014) Human-gyrovirus-Apoptin triggers mitochondrial death pathway--Nur77 is required for apoptosis triggering. Neoplasia 16:679-693

5. Chu DK, Poon LL, Chiu SS, Chan KH, Ng EM, Bauer I, Cheung TK, Ng IH, Guan Y, Wang D, Peiris JS (2012) Characterization of a novel gyrovirus in human stool and chicken meat. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 55:209-213

6. dos Santos HF, Knak MB, de Castro FL, Slongo J, Ritterbusch GA, Klein TA, Esteves PA, Silva AD, Trevisol IM, Claassen EA, Cornelissen LA, Lovato M, Franco AC, Roehe PM, Rijsewijk FA (2012) Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. Veterinary microbiology 155:230-236

7. Fehér E, Pazar P, Kovacs E, Farkas SL, Lengyel G, Jakab F, Martella V, Banyai K (2014) Molecular detection and characterization of human gyroviruses identified in the ferret fecal virome. Archives of virology 159:3401-3406

8. Ilyina TV, Koonin EV (1992) Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic acids research 20:3279-3285

9. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

10. Kuusisto HV, Wagstaff KM, Alvisi G, Jans DA (2008) The C-terminus of apoptin represents a unique tumor cell-enhanced nuclear targeting module. International Journal of Cancer 123, 2965–2969

11. Lima FE, Cibulski SP, Dos Santos HF, Teixeira TF, Varela AP, Roehe PM, Delwart E, Franco AC (2015) Genomic Characterization of Novel Circular ssDNA Viruses from Insectivorous Bats in Southern Brazil. PloS one 10:e0118070

12. Noteborn MH (2009) Proteins selectively killing tumor cells. European Journal of Pharmacology 625, 1-3:165-73.

13. Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, Wang C, Leutenegger CM, Delwart E (2012) A third gyrovirus species in human faeces. The Journal of general virology 93:1356-1361

14. Phan TG, Vo NP, Sdiri-Loulizi K, Aouni M, Pothier P, Ambert-Balay K, Deng X, Delwart E (2013) Divergent gyroviruses in the feces of Tunisian children. Virology 446:346-348

15. Rijsewijk FA, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, Franco AC, Roehe PM (2011) Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Archives of virology 156:1097-1100

16. Sauvage V, Cheval J, Foulongne V, Gouilh MA, Pariente K, Manuguerra JC, Richardson J, Dereure O, Lecuit M, Burguiere A, Caro V, Eloit M (2011) Identification of the first human gyrovirus, a virus related to chicken anemia virus. Journal of Virology 85(15):7948-7950

17. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution 4:406-425

18. Takahashi K, Iwasa Y, Hijikata M, Mishiro S (2000) Identification of a new human DNA virus (TTV-like mini virus, TLMV) intermediately related to TT virus and chicken anemia virus. Archives of virology 145:979-993

19. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28:2731-2739

20. Zhang W, Li L, Deng X, Kapusinszky B, Delwart E (2014) What is for dinner? Viral metagenomics of US store bought beef, pork, and chicken. Virology 468-470:303-310

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	II-reverse	TGCTGAATTCTTACGGGTTGTAT CTGTCCGTTG		
PCR III	III-forward	GAAGACAGTAGCAGATGCTT	1879 to 684	1143
	III-reverse	TATCGAGGTCGTTTCTGCTG		

*Position genome was defined based on the AGV2 genome sequence (accession number: NC_015396).

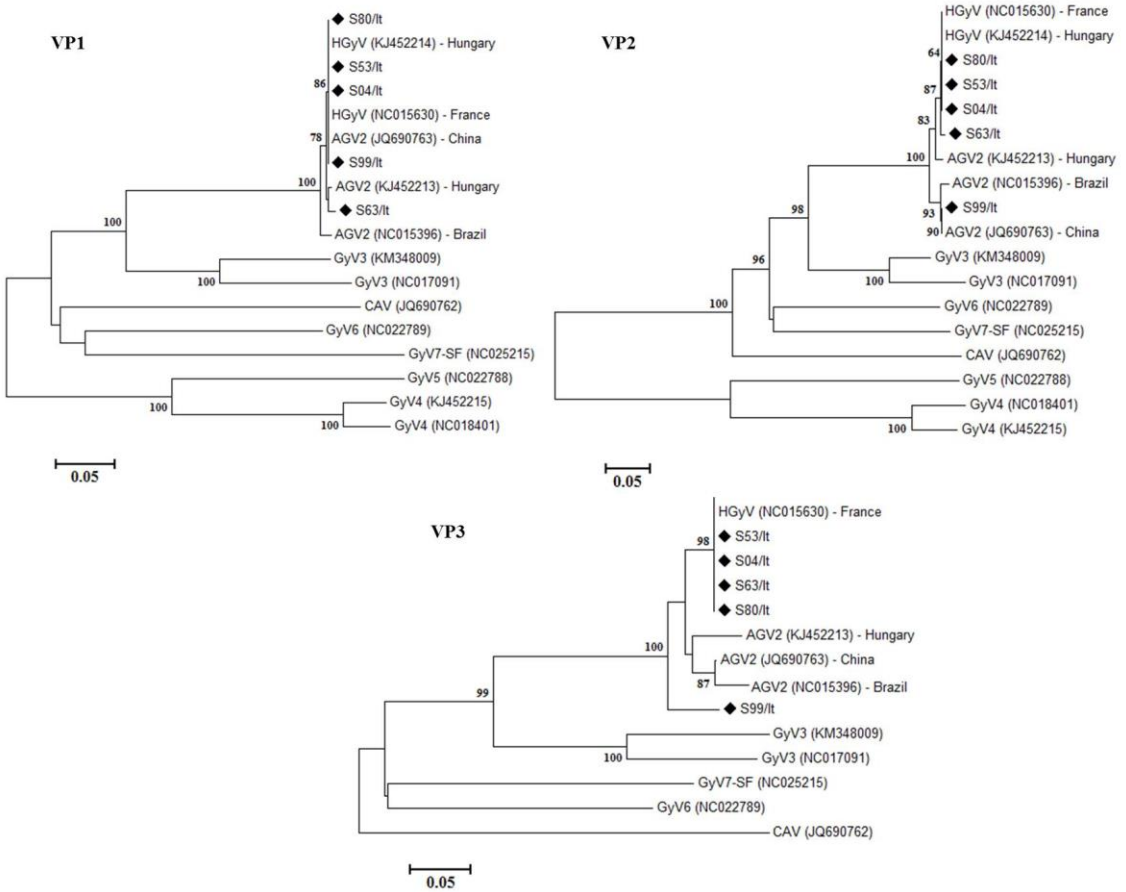

Fig.1. Phylogenetic trees generated at predicted amino acid level for VP1, VP2 and VP3 recovered from Italian samples. All currently known gyroviruses were included in analyses. Sequences identified in this study are marked with black diamond. Bootstrap values (based on 1000 replicates) for each node were shown if > 60%.

References

1. Abolnik C, Wandrag DB (2014) Avian gyrovirus 2 and avirulent Newcastle disease virus coinfection in a chicken flock with neurologic symptoms and high mortalities. Avian diseases 58:90-94

2. Bullenkamp J, Cole D, Malik F, Alkhatabi H, Kulasekararaj A, Odell EW, Farzaneh F, Gaken J, Tavassoli M (2012) Human Gyrovirus Apoptin shows a similar subcellular distribution pattern and apoptosis induction as the chicken anaemia virus derived VP3/Apoptin. Cell death & disease 3:e296

3. Chaabane W, Cieslar-Pobuda A, El-Gazzah M, Jain MV, Rzeszowska-Wolny J, Rafat M, Stetefeld J, Ghavami S, Los MJ (2014) Human-gyrovirus-Apoptin triggers mitochondrial death pathway--Nur77 is required for apoptosis triggering. Neoplasia 16:679-693

4. Chu DK, Poon LL, Chiu SS, Chan KH, Ng EM, Bauer I, Cheung TK, Ng IH, Guan Y, Wang D, Peiris JS (2012) Characterization of a novel gyrovirus in human stool and chicken meat. Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology 55:209-213

5. Danen-Van Oorschot AA, Voskamp P, Seelen MC, van Miltenburg MH, Bolk MW, Tait SW, Boesen-de Cock JG, Rohn JL, Borst J, Noteborn MH (2004) Human death effector domain associated factor interacts with the viral apoptosis agonist apoptin and exerts tumor-preferential cell killing. Cell Death Differ 11:564–573

6. dos Santos HF, Knak MB, de Castro FL, Slongo J, Ritterbusch GA, Klein TA, Esteves PA, Silva AD, Trevisol IM, Claassen EA, Cornelissen LA, Lovato M, Franco AC, Roehe PM, Rijsewijk FA (2012) Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. Veterinary microbiology 155:230-236

7. Fehér E, Pazar P, Kovacs E, Farkas SL, Lengyel G, Jakab F, Martella V, Banyai K (2014) Molecular detection and characterization of human gyroviruses identified in the ferret fecal virome. Archives of virology 159:3401-3406

8. Heilman DW, Teodoro JG, Green MR (2006) Apoptin nucleocytoplasmic shuttling is required for cell type-specific localization, apoptosis, and recruitment of the anaphase-promoting complex/cyclosome to PML bodies. Journal of virology 80:7535-7545

9. Ilyina TV, Koonin EV (1992) Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. Nucleic acids research 20:3279-3285

10. Kuusisto HV, Wagstaff KM, Alvisi G, Jans DA (2008) The C-terminus of apoptin represents a unique tumor cell-enhanced nuclear targeting module. Int. J. Cancer 123, 2965–2969

12. Lima FE, Cibulski SP, Dos Santos HF, Teixeira TF, Varela AP, Roehe PM, Delwart E, Franco AC (2015) Genomic Characterization of Novel Circular ssDNA Viruses from Insectivorous Bats in Southern Brazil. PloS one 10:e0118070

13. Noteborn, M. H. (2009) Proteins selectively killing tumor cells. European Journal of Pharmacology 625, 1-3:165-73.

14. Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, Wang C, Leutenegger CM, Delwart E (2012) A third gyrovirus species in human faeces. The Journal of general virology 93:1356-1361

15. Poon IK, Oro C, Dias MM, Zhang JP, Jans DA (2005) A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin. Journal of virology 79:1339-1341

16. Rijsewijk FA, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, Franco AC, Roehe PM (2011) Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. Archives of virology 156:1097-1100

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19. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28:2731-2739

20. Wang QM, Fan GC, Chen JZ, Chen HP, He FC (2004) A putative NES mediates cytoplasmic localization of Apoptin in normal cells. Acta biochimica et biophysica Sinica 36:817-823

21. Zhang W, Li L, Deng X, Kapusinszky B, Delwart E (2014) What is for dinner? Viral metagenomics of US store bought beef, pork, and chicken. Virology 468-470:303-310

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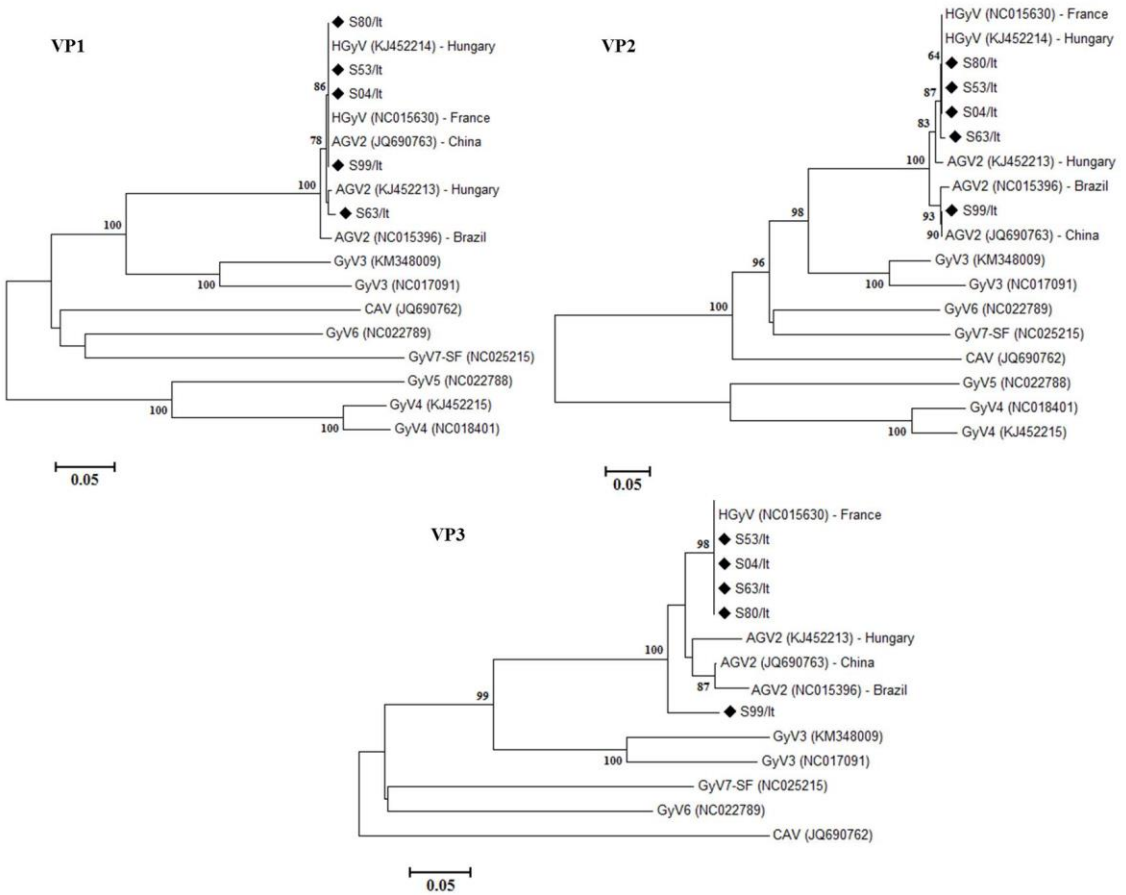

Fig.1. Phylogenetic trees generated with base on the predicted amino acid sequence of the VP1, VP2, and VP3 proteins of the Italian AGV2 sequences and all currently known gyroviruses. Sequences identified in this study are marked with black diamond. Bootstrap values (based on 1000 replicates) for each node were shown if > 60%.

CONSIDERAÇÕES FINAIS

No presente estudo foram apresentados aspectos que visam contribuir para um melhor entendimento da biologia do vírus da anemia infecciosa das galinhas (CAV) e do girovírus aviário 2, membros da família *Circoviridae*, gênero *Gyrovírus*.

Em um primeiro momento, um estudo foi conduzido com o objetivo de desenvolver uma duplex PCR em tempo real (dqPCR) que permitisse detectar e quantificar simultaneamente genomas de CAV e AGV2. Dessa forma, o teste tornaria possível a detecção desses girovírus tanto em infecções com um único agente, bem como em co-infecções. Tais co-infecções têm sido frequentemente descritas, entretanto, ainda não é conhecido o efeito, se este existir, que tal associação viral pode gerar (Phan et al., 2012; Chu et al., 2012; Zhang et al., 2014). Até o momento, o AGV2 não foi associado com patogenia, no entanto, a possibilidade do vírus atuar como um facilitador da replicação viral quando associado a outro agente tem sido especulada. A exemplo disso, pode ser citado a co-infecção com AGV2 e uma cepa avirulenta do vírus da doença de Newcastle, na qual as aves apresentavam sinais neurológicos resultando em alta mortalidade (ABOLNIK & WANDRAG, 2013). O desenvolvimento de uma técnica quantitativa poderá contribuir com a realização de estudos que visam examinar a presença de CAV e AGV2, bem como estabelecer possíveis associações entre eles.

A partir de então, a técnica desenvolvida (dqPCR) foi utilizada como método de investigação da presença de genomas de ambos agentes como contaminantes de vacinais comerciais frequentemente utilizadas na avicultura. Genomas de CAV foram detectados em 7/35 vacinas analisadas, enquanto que genomas de AGV2 estavam presentes em 9/35 vacinas avaliadas. Contaminação de vacinas com CAV são conhecidas desde a primeira descrição do agente em 1979 e vem sendo investigada ao longo do tempo. No Brasil, em um estudo retrospectivo envolvendo vacinas comerciais, verificou-se a presença de CAV em imunógenos produzidos até 1997 (BARRIOS et al., 2012). No entanto, o presente estudo demonstra que a investigação deve ser constante, uma vez que genoma viral ainda pode ser detectado em imunógenos atuais. Em relação ao AGV2, nenhuma descrição do agente como um contaminante de vacinas havia sido reportado até então. A detecção destes agentes em imunógenos representa o primeiro passo para a realização de pesquisas mais aprofundadas relacionadas à possibilidade de as vacinas representarem uma fonte de disseminações viral ou ainda, de material genético. A ampla distribuição do AGV2 em plantéis de aves saudáveis pode estar

relacionada com a utilização de imunógenos contaminados. Apesar de o estudo apresentar uma abordagem molecular, os resultados reforçam a necessidade de medidas de controle eficazes para evitar a contaminação.

Em um segundo momento, tendo em vista que genoma de AGV2 tem sido detectado em animais saudáveis e doentes, buscou-se avaliar a variabilidade genética do agente presente em aves com distinto *status* sanitário. Um estudo prévio sugeriu a possibilidade de existirem variantes do vírus as quais poderiam estar associadas à patogenicidade (Dos Santos et al., 2012). Diante desta possibilidade, um estudo foi conduzido com intuito de verificar a ocorrência destas possíveis associações. Amostras provenientes de aves com distinto *status* sanitário foram submetidas à análise filogenética da região codificadora da VP1. A análise filogenética realizada à nível de aminoácido permitiu verificar a separação das amostras em dois grupos associados ao distinto *status* sanitário. Sequências oriundas de aves doentes formaram um *cluster* separado das sequências identificadas em aves doentes, com exceção de duas amostras, e apresentaram o mesmo padrão de substituição de aminoácido. Dessa forma, estas substituições podem estar relacionadas à ocorrência de variantes do vírus. Mudanças específicas de aminoácidos têm sido determinantes no caráter patogênico do CAV, ressaltando a importância da realização de estudos que visem elucidar qual o papel dessas substituições no genoma do AGV2 (RENSHAW et al.,1996). Além disso, os resultados retomam a questão sobre a origem do AGV2 e HGyV, uma vez que sequências identificadas em aves são filogeneticamente relacionadas às sequências identificadas em humanos e furão doméstico.

Por fim, com base na especulação de que AGV2 está amplamente disseminado na população avícola e diante da possibilidade do vírus estar presente em países europeus, um estudo foi conduzido visando a detecção e a caracterização de genomas virais do AGV2 presentes na Itália. No estudo foram obtidas quatro sequências parciais, compreendendo grande parte da região codificadora do genoma, e uma sequência genômica completa. Os genomas obtidos, parciais ou completos, apresentaram organização genômica igual aos gyrovirus previamente identificados, com exceção aos girovírus 4 e 5, para os quais não foi reportada a região codificadora da VP3. Adicionalmente, análise filogenética realizada mostrou que genomas de AGV2 e HGyV são filogeneticamente distintos dos demais gyrovírus. Além disso, embora existam variações a nível de aminoácido entre os genomas analisados, genomas de AGV2 e

HGyV são filogeneticamente relacionados, formando um distinto grupo dentro do gênero *Gyrovirus*.

Embora até o presente informações sobre a biologia, epidemiologia, patogenia envolvendo AGV2 são pouco conhecidas, os estudos aqui apresentados representaram um pequeno avanço na pesquisa envolvendo esse novo girovírus identificado em aves. Os resultados e questionamentos gerados com estes estudos servirão de base para futuras investigações buscando determinar o papel das vacinas como possíveis fontes de disseminação do agente, correlações entre diversidade genômica e patogenia e a questão sobre a origem e relação existente entre girovírus identificados em humanos e em aves.

REFERÊNCIAS

ABOLNIK, C.; WANDRAG, D. B. Avian gyrovirus 2 and avirulent Newcastle disease virus coinfection in a chicken flock with neurologic symptoms and high mortalities. **Avian Diseases,** v. 58, n. 1, p. 90-4, 2014.

ADAIR, B. M. Immunopathogenesis of chicken anemia virus infection. **Development & Comparative Immunology,** v. 24, n. 2-3, p. 247-55, 2000.

ADAIR, B. M. Virus-induced immunosuppression. In: Davidson TF, Morris TR, Payne LN, editors. Poultry immunology. Abingdon, Oxfordshire: Carfax Publishing Company UK, p. 301-315, 1996.

BARRIOS, P. R. et al. A retrospective PCR investigation of avian orthoreovirus, chicken infectious anemia and fowl aviadenovirus genomes contamination in commercial poultry vaccines in Brazil. **Arquivos Brasileiros de Medicina Veterinária e Zootecnia, v.** 64, n. 1, p. 231-5, 2012.

BIĐIN, M. et al. The prevalence of antibodies against chicken anemia virus in unvaccinated broilers and broiler breeders in Croatia. **Veterinarski Arhiv,** v.8 (6), p.753-760, 2010.

BHATT, P. et al. Prevalence of chicken infectious anaemia virus (CIAV) in commercial poultry flocks of northern India: a serological survey. **Transboundary and Emerging Diseases,** v. 58, n. 5, p. 458-60, 2011.

BIAGINI, P. et al. Human gyrovirus in healthy blood donors, France. **Emerging Infectious Diseases,** v. 19, n. 6, p. 1014-5, 2013.

BIAGINI, P. Et al. Circoviridae. In: King AMQ, Adams MJ, Cartens E.B., Lefkowitz EJ, editors. Virus Taxonomy, IXth Report of the International Committee for the Taxonomy of Viruses. UK: Elsevier/Academic Press. p. 343–349, 2012.

BONKOUNGOU, I. J. et al. Epidemiology of rotavirus infection among young children with acute diarrhoea in Burkina Faso. **BMC Pediatrics,** v. 10, p. 94, 2010.

BRENTANO, L. Anemia Infecciosa das Galinhas. In: BERCHIERI JÚNIOR, A.; SILVA, E.N.; DI FÁBIO, J.; SESTI, L.; ZUANAZE, M.A.F. Doenças das Aves. 2.ed. Campinas: FACTA, 2009

BRENTANO, L. Anemia Infecciosa das Galinhas. In: Doenças das Aves. São Paulo: Facta, p.339–350, 2000.

BRENTANO, L. et al. Anticorpos para o vírus da anemia das galinhas (cav) em matrizes de corte no Brasil. **Revista Brasileira de Ciência Avícola**, v. *2*, n. 2, p. 163-175, 2000.

BRENTANO, L. et al. Isolation and identification of chicken infectious anemia virus in Brazil. **Avian Disease,** v. 35, n. 4, p. 793-800, 1991.

BULLENKAMP, J. et al. Human Gyrovirus Apoptin shows a similar subcellular distribution pattern and apoptosis induction as the chicken anaemia virus derived VP3/Apoptin. **Cell Death & Disease,** v. 3, p. e296, 2012.

BUSCAGLIA, C.; CROSETTI, C. F.; NERVI, P. Identification of chicken infectious anaemia, isolation of the virus and reproduction of the disease in Argentina. **Avian Pathology,** v. 23, n. 2, p. 297-304, 1994.

CANAL, C.W. et al. Prevalence of antibodies against chicken anaemia virus (CAV) in broiler breeders in Southern Brazil. **Pesquisa Veterinária Brasileira**, v. 24, p. 89-92, 2004

CARDONA, C. J.; OSWALD, W. B.; SCHAT, K. A. Distribution of chicken anaemia virus in the reproductive tissues of specific-pathogen-free chickens. **Journal of General Virology,** v. 81, n. 8, p. 2067-75, 2000.

CHAABANE, W. et al. Human-gyrovirus-Apoptin triggers mitochondrial death pathway--Nur77 is required for apoptosis triggering. **Neoplasia,** v. 16, n. 9, p. 679-93, 2014.

CHU, D. K. et al. Characterization of a novel gyrovirus in human stool and chicken meat. **Journal of Clinical Virology,** v. 55, n. 3, p. 209-13, 2012.

CIACCI-ZANELLA, J. R. C; BRENTANO, L. Circoviridae, in: Flores, E.F., **Virologia Veterinária**, Santa Maria-RS, 2ed, Ed. Da UFSM, cap. 14, p. 419-437, 2012.

CLAESSENS, J. A. et al. Molecular cloning and sequence analysis of the genome of chicken anaemia agent. **Journal of General Virology,** v. 72, n. 8, p. 2003-6, 1991.

COSTA et al. Detecção molecular de gyrovírus aviário tipo 2 (agv-2) em aves spf alojadas em camas comerciais e em cascudinhos presentes na cama**,** in**:** JINC – 6ª Jornada de Iniciação Científica Embrapa, SIPEX – II Seminário de Pesquisa e Extensão da UnC – Concórdia/SC, p.23, 2012.

DANEN-VAN OORSCHOT, A.A. et al. Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. **Proceedings of the National Academy of Sciences of the United States of América,** v. 94, p. 5843–5847, 1997.

DAVIDSON, I. et al. The contribution of feathers in the spread of chicken anemia virus. **Virus Research,** v. 132, n. 1-2, p. 152-9, 2008.

DE BOER, G. F. et al. Interaction between chicken anaemia virus and live Newcastle disease vaccine. **Avian Pathology,** v. 23, n. 2, p. 263-75, 1994.

DOS SANTOS, H. F. et al. Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands. **Veterinary Microbiology,** v. 155, n. 2-4, p. 230-6, 2012.

DOUGLAS, A. J. et al. Identification of a 24 kDa protein expressed by chicken anaemia virus. **Journal of General Virology,** v. 76 (Pt 7), p. 1557-62, 1995.

EMIKPE, B. O. et al. Serological evidence of chicken anaemia virus infection in Nigerian indigenous chickens. **Onderstepoort Journal of Veterinary Research,** v. 72, n. 1, p. 101-3, 2005.

ENGSTROM, B. E.; LUTHMAN, M. Blue wing disease of chickens: signs, pathology and natural transmission. **Avian Pathology,** v. 13, n. 1, p. 1-12, 1984.

ESTEVES, P. A. et al. Molecular detection of avian gyrovirus 2 (AGV2) in specific pathogen free (SPF) chickens housed on commercial broiler litter, in: XXIV Congresso Mundial de Avicultura, 2012

FEHÉR, E. et al. Molecular detection and characterization of human gyroviruses identified in the ferret fecal virome. **Archives of Virology,** v. 159, n. 12, p. 3401-6, 2014.

FLORES, E. F. et al. Emerging animal viruses: real threats or simple bystanders? **Pesquisa Veterinária Brasileira**, v. 33, n. 10, p. 1161-1173, 2013.

FRANCO A.C. Avian gyrovirus 2. Presented at the round table: Emerging viruses. XXIII Congresso Brasileiro de Virologia, Foz do Iguaçu, PR 2012.

GHOLAMI-AHANGARAN, M.; ZIA-JAHROMI, N. Serological and molecular identification of subclinical chicken anaemia virus infection in broiler chickens in Iran. **African Journal of Microbiology Research,** v. 6, n. 21, p. 4471-4474, 2012.

GILLESPIE, J. et al. Porcine circovirus type 2 and porcine circovirus-associated disease. **Journal of Veterinary Internal Medicine, v.** 23, p. 1151–1163, 2009.

HOOP, R. K. Persistence and vertical transmission of chicken anaemia agent in experimentally infected laying hens. **Avian Pathology,** v. 21, n. 3, p. 493-501, 1992.

HOOP, R. K.; REECE, R. L. The use of immunofluorescence and immunoperoxidase staining in studying the pathogenesis of chicken anaemia agent in experimentally infected chickens. **Avian Pathology,** v. 20, n. 2, p. 349-55, 1991.

ICTV (2015) The universal virus database of the International Committee on Taxonomy of Viruses. <http://www.ncbi.nlm.nih.gov/ICTVdb/>. Acesso em 06 de janeiro de 2015.

KAFFASHI, A. et al. Viral load in 1-day-old and 6-week-old chickens infected with chicken anaemia virus by the intraocular route. **Avian Pathology,** v. 35, n. 6, p. 471-4, 2006.

KAMADA, K. et al. Spliced mRNAs detected during the life cycle of Chicken anemia virus. **Journal of General Virology,** v. 87, n. 8, p. 2227-33, 2006.

LACORTE, C. et al. Assessing the expression of chicken anemia virus proteins in plants. **Virus Research,** v. 129, n. 2, p. 80-6, 2007.

LEE, M. S. et al. Production and diagnostic application of a purified, E. coli-expressed, serological-specific chicken anaemia virus antigen VP3. **Transboundary and Emerging Diseases,** v. 58, n. 3, p. 232-9, 2011.

LELIVELD, S. R. et al. Apoptin induces tumor-specific apoptosis as a globular multimer. **The Journal of Biological Chemistry,** v. 278, n. 11, p. 9042-51, 2003.

LI, L. et al. A gyrovirus infecting a sea bird. **Archives of Virology**, 2015. DOI 10.1007/s00705-015-2468-1

LIMA, F.E.S. et al. Detection of avian gyrovirus type 2 in na Alagoas curassow (mitu mitu). In: XXIII Brazilian Congress of Virology, v. 17, n. 2, 2012.

LOS, M. et al. Apoptin, a tumor-selective killer. **Biochimica et Biophysica Acta,** v. 1793, n. 8, p. 1335-42, 2009.

LOVATO, L. T.; HENZEL, A. Classificação e nomenclatura dos vírus, in: Flores, E.F., **Virologia Veterinária**, Santa Maria-RS, 2nd Ed. Da UFSM, cap. 2, p. 29-51, 2012.

LUCIO, B.; SCHAT, K. A.; SHIVAPRASAD, H. L. Identification of the chicken anemia agent, reproduction of the disease, and serological survey in the United States. **Avian Disease,** v. 34, n. 1, p. 146-53, 1990.

MADDIKA, S. et al. Cancer-selective therapy of the future: apoptin and its mechanism of action. **Cancer Biology & Therapy,** v. 5, n. 1, p. 10-9, 2006.

MADDIKA, S. et al. Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. **Journal of Cell Science,** v. 118, n. Pt 19, p. 4485-93, 2005.

MAGGI, F. et al. Human gyrovirus DNA in human blood, Italy. **Emerging Infectious Diseases,** v. 18, n. 6, p. 956-9, 2012.

MAPA (2015) Ministério da Agricultura, Abastecimento e Pecuária. http://www.agricultura.gov.br Acesso em 06 de janeiro de 2015.

MARKOWSKI-GRIMSRUD, C. J.; MILLER, M. M.; SCHAT, K. A. Development of strain-specific real-time PCR and RT-PCR assays for quantitation of chicken anemia virus. **Journal of Virological Methods,** v. 101, n. 1-2, p. 135-47, 2002.

MCNULTY, M. S. Chicken anaemia agent: a review. **Avian Pathology,** v. 20, n. 2, p. 187-203, 1991.

MILLER, M. M.; JAROSINSKI, K. W.; SCHAT, K. A. Positive and negative regulation of chicken anemia virus transcription. **Journal of Virology,** v. 79, n. 5, p. 2859-68, 2005.

MILLER, M. M.; SCHAT, K. A. Chicken infectious anemia virus: an example of the ultimate host-parasite relationship. **Avian Disease,** v. 48, n. 4, p. 734-45, 2004.

MOROIANU, J. Nuclear import and export: transport factors, mechanisms and regulation. **Critical Reviews in Eukaryotic Gene Expression,** v. 9, n. 2, p. 89-106, 1999.

NOTEBORN, M. H. Proteins selectively killing tumor cells. **European Journal of Pharmacology,** v. 625, n. 1-3, p. 165-73, 2009.

NOTEBORN, M. H. et al. Simultaneous expression of recombinant baculovirus-encoded chicken anaemia virus (CAV) proteins VP1 and VP2 is required for formation of the CAV-specific neutralizing epitope. **Journal of General Virolology,** v. 79, n. 12, p. 3073-7, 1998.

NOTEBORN, M. H. et al. A single chicken anemia virus protein induces apoptosis. **Journal of Virology,** v. 68, n. 1, p. 346-51, 1994.

NOTEBORN, M. H. et al. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. **Journal of Virology,** v. 65, n. 6, p. 3131-9, 1991.

ONG, C. J. et al. CD45 enhances positive selection and is expressed at a high level in large, cycling, positively selected CD4+CD8+ thymocytes. **Immunology,** v. 91, n. 1, p. 95-103, 1997.

ORO, C.; JANS, D. A. The tumour specific pro-apoptotic factor apoptin (Vp3) from chicken anaemia virus. **Current Drug Targets,** v. 5, n. 2, p. 179-90, 2004.

OWOADE, A. A. et al. Serologic evidence of chicken infectious anemia in commercial chicken flocks in southwest Nigeria. **Avian Disease,** v. 48, n. 1, p. 202-5, 2004.

PALOMARES, L. A., Mena, J. A., Ramírez, O. T. Simultaneous expression of recombinant proteins in the insect cell-baculovirus system: Production of virus-like particles. **Methods**, v. 56, n.3, p. 389-395, 2012.

PAN, Y. et al. Antitumor effects of a recombinant pseudotype baculovirus expressing Apoptin in vitro and in vivo. **International Journal of Cancer,** v. 126, n. 11, p. 2741-51, 2010.

PETERS, M. A. et al. Site-directed mutagenesis of the VP2 gene of Chicken anemia virus affects virus replication, cytopathology and host-cell MHC class I expression. **Journal of General Virolology,** v. 87, n. Pt 4, p. 823-31, 2006.

PETERS, M. A. Mutation of chicken anemia virus VP2 differentially affects serine/threonine and tyrosine protein phosphatase activities. **Journal of General Virolology,** v. 86, n. Pt 3, p. 623-30, 2005.

PETERS, M. A. et al. Chicken anemia virus VP2 is a novel dual specificity protein phosphatase. **Journal of Biological Chemistry,** v. 277, n. 42, p. 39566-73, 2002.

PHAN, T. G. et al. A new gyrovirus in human feces. **Archives of Virology**, 2015. DOI 10.1007/s11262-015-1210-0

PHAN, T. G. et al. A third gyrovirus species in human faeces. **Journal of General Virolology,** v. 93, n. Pt 6, p. 1356-61, 2012.

PHAN, T. G. et al. Divergent gyroviruses in the feces of Tunisian children. **Virology,** v. 446, n. 1-2, p. 346-8, 2013.

RENSHAW, R. W. et al. A hypervariable region in VP1 of chicken anemia virus mediates rate of spread and cell tropism in tissue culture. **Journal of Virolology**, v.70, n. 12, p.8872–8, 1996.

RIJSEWIJK, F. A. et al. Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. **Archives of Virology,** v. 156, n. 6, p. 1097-100, 2011.

SAUVAGE, V. et al. Identification of the first human gyrovirus, a virus related to chicken anemia virus. **Journal of Virology,** v. 85, n. 15, p. 7948-50, 2011.

SCHAT, K. A. Chicken anemia virus. **Current Topics in Microbiology and Immunology,** v. 331, p. 151-83, 2009.

SCHAT, K.A.; VAN SANTEN, V. Chicken infectious anemia. In: SAIF, Y.M., et al. (eds) Diseases of poultry. Ames: Blackwell Publishing, p 211–235, 2008.

SCHAT, K.A. Chicken Infectious Anemia. IN: SAIF, Y.M., et al. (eds). **Diseases of Poultry**. 11rd. ed. Ames: Iowa State Press; 2003.

SEGALÉS, J.; KEKARAINEN, T.; CORTEY, M. The natural history of porcine circovirus type 2: from an inoffensive virus to a devastating swine disease? **Veterinary Microbiology,** v. 165, n. 1-2, p. 13-20, 2013.

SHARMA, R. N. et al., Serological evidence of chicken infectious anemia in layer and broiler chickens in Grenada, West Indies. **Veterinary World**, v. 7, n. 2, p. 59-61, 2014.

SMYTH, J. A. et al. A sequential histopathologic and immunocytochemical study of chicken anemia virus infection at one day of age. **Avian Disease**, v. 37, n. 2, p. 324-38, 1993.

SNOECK, C. J. et al. Epidemiology of chicken anemia virus in Central African Republic and Cameroon. **Virology Journal,** v. 9, p. 189, 2012.

TANIGUCHI, T. et al. Chronological observations on hemato-pathological changes in chicks inoculated with chicken anemia agent. **National Institute of Animal Health quarterly (Tokyo),** v. 23, n. 1, p. 1-12, Spring 1983.

TODD, D. et al. Animal circoviruses. **Advances in Virus Research,** v. 57, p. 1-70, 2001.

TODD, D.; MAWHINNEY, K. A.; MCNULTY, M. S. Detection and differentiation of chicken anemia virus isolates by using the polymerase chain reaction. **Journal of Clinical Microbiology,** v. 30, n. 7, p. 1661-6, 1992.

TORO, H.; EWALD, S.; HOERR, F. J. Serological evidence of chicken infectious anemia virus in the United States at least since 1959. **Avian Disease,** v. 50, n. 1, p. 124-6, 2006.

TORO, H.; RAMIREZ, A. M.; LARENAS, J. Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. **Avian Pathology,** v. 26, n. 3, p. 485-99, 1997.

UNIÃO BRASILEIRA DE AVICULTURA. Relatório Anual 2014, disponível em http://www.ubabef.com.br Acesso em 25 novembro de 2014.

VAN SANTEN, V. L. et al. Pathogenesis of chicken anemia virus: comparison of the oral and the intramuscular routes of infection. **Avian Disease,** v. 48, n. 3, p. 494-504, 2004.

YUASA, N. et al. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. **National Institute of Animal Health quarterly (Tokyo)** v. 23, n. 3, p. 75-7, 1983.

YUASA, N.; TANIGUCHI, T.; YOSHIDA, I. Isolation and some characteristics of an agent inducing anemia in chicks. **Avian Disease,** v.23, p.366–85, 1979.

ZANELLA, J. R. C; Circoviridae, in: Flores, E.F., **Virologia Veterinária**, Santa Maria-RS, Ed. Da UFSM, cap. 13, p. 363-474, 2007.

ZHANG, W. et al. What is for dinner? Viral metagenomics of US store bought beef, pork, and chicken. **Virology,** v. 468-470, p. 303-10, 2014.