UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

IDENTIFICAÇÃO DE GENOMAS DE UM NOVO CIRCOVÍRUS AVIÁRIO

HELTON FERNANDES DOS SANTOS

Porto Alegre
2012

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Tese submetida ao Programa de Pós-Graduação em Ciências Veterinárias da UFRGS, como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias, área de concentração Microbiologia Veterinária - Virologia.

Orientador: Prof. Dr. Paulo Michel Roehe

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Helton Fernandes dos Santos

IDENTIFICAÇÃO DE GENOMAS DE UM NOVO CIRCOVÍRUS AVIÁRIO

Aprovado em 10 SET 2012

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Prof. Dr. Cláudio Wageck Canal (UFRGS)
Membro da Comissão

Dedico esta tese ao Dr. Frans Rijsewijk (*in memoriam*), pessoa em que levo suas orientações e ensinamentos por toda minha vida.

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“Um sonho sonhado sozinho é um sonho. Um sonho sonhado junto é realidade.”

--Raul Seixas

IDENTIFICAÇÃO DE GENOMAS DE UM NOVO CIRCOVÍRUS AVIÁRIO

RESUMO

A presente tese versa sobre estudos realizados visando a identificação de novos agentes virais em frangos comerciais. No primeiro capítulo, a identificação do girovírus aviário tipo 2 (AGV2) é reportada. Um genoma viral de 2383 nt foi amplificado a partir soros de frangos comerciais por amplificação randômica de DNA. A análise da sequência do produto amplificado, revelou que cerca de 40% das sequencias de nucleotídeos apresentavam similaridade com o genoma do vírus da anemia infecciosa das galinhas (CAV). Dado o baixo grau de identidade com o CAV, o genoma identificado justificou a proposição de um novo tipo de vírus, dentro do gênero *gyrovírus* foi denominado "girovírus aviário tipo 2" (AGV2). O genoma do AGV2 tem organização semelhante a do CAV, com percentagens de similitude de aminoácidos nas regiões codificantes, correspondentes às proteínas virais VP1, VP2 e VP3 do CAV de 38,8%, 40,3% e 32,2%, respectivamente. A fim de analisar a amplitude da disseminação deste agente, foi realizado um estudo para identificar a ocorrência do AGV2 em granjas de frangos de outras regiões. Para atingir esse objetivo, uma PCR específica ao AGV2 foi desenvolvida para amplificar o DNA viral extraído de bulbos de penas da asa e órgãos de frangos. Essa técnica permitiu a detecção do AGV2 em aves de outros locais na Região Sul do Brasil. O DNA viral foi detectado em 90,7% (98/108) das amostras coletadas no estado do Rio Grande do Sul e 60,4% (29/48) das amostras do estado de Santa Catarina. Os mesmo *primers* da PCR foram adaptados para examinar tecidos de cérebro de galinhas da Holanda. Nessas amostras o DNA do AGV2 foi detectado em nove das 21 (42,9%) amostras de tecidos cerebrais em aves com lesões hemorrágicas. Essas descobertas fornecem evidências de que infecções de AGV2 são generalizadas e não se restringem a Região Sul do Brasil. Além disso, esses estudos permitiram a identificação de variantes do DNA genômico do AGV2. Análises filogenéticas demostraram que os genomas examinados poderiam ser divididos em três grupos, com base em diferenças nos genes das ORFs das proteínas VP2 e VP3. Em conclusão, a presente tese abrange estudos da identificação de um vírus aviário, até então desconhecido, denominado girovírus aviário tipo 2, que encontra-se amplamente distribuído. A associação deste agente com enfermidades em aves será tema de estudos que estão sendo realizados.

Palavras-chaves: AGV2, CAV, *Circoviridae*, *Gyrovirus*.

DETECTION OF NEW AVIAN CIRCOVIRUS GENOMES

ABSTRACT
This thesis concerns studies carried out in search for new viral agents in commercial poultry flocks. In the first chapter, the identification of the genome of avian gyrovirus type 2 (AGV2), a new Gyrovirus, is reported. The viral genome the 2383-nucleotide sequence was amplified from sera of commercial broilers by random DNA amplification. Sequence analysis of the amplified product showed that the putative viral sequence had about 40% nucleotide similarity with the genome of its closest relative, chicken anemia virus (CAV). Such low degree of nucleotide similarity justified its classification as a new virus type within the genus, to which the name avian gyrovirus type 2. The amino acid similarity between the predicted viral proteins VP1, VP2 and VP3 of AGV2 and those of CAV was 38.8%, 40.3%, and 32.2%, respectively. In order to examine the amplitude of dissemination of this agent, it became necessary to carry out a search for AGV2 genomes in poultry flocks from other regions. To achieve such objective, an AGV2-specific PCR was designed to amplify viral DNA from nuclei acid extracted from poultry feather shafts. This allowed detection of AGV2 genomes in flocks from other locations in Southern Brazil. Viral DNA was detected in 98/108 (90.7%) of samples collected in the state of Rio Grande do Sul and 29/48 (60.4%) of the samples collected in the state of Santa Catarina. The same PCR primers were adapted to examine brain tissues of chickens from the Netherlands. In such samples, AGV2 DNA was detected in nine out of 21 (42.9%) brain tissue samples from birds with haemorragic lesions. These findings provided evidence that AGV2 infections are widespread and are not restricted to Southern Brazil. In addition, these studies allowed the identification of genomic variants of AGV2. Phylogenetic analyses demonstrated that such genomes could be divided into three clusters. In conclusion, this thesis encompasses studies on the identification of a previously unreported virus, named avian gyrovirus 2, which was later shown to be widely distributed. The relationship between this agent and disease in poultry will be subject of further studies which are being performed in continuation to this work.

Keywords: *AGV2, CAV, Circoviridae, Gyrovirus.*

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1 INTRODUÇÃO

LISTA DE ABREVIATURAS E SÍMBOLOS

ACRMP	Amplificação por Círculo Rolante com Múltiplos *Primers*
AGV2	Girovírus aviário tipo 2
BLAST	*Basic Local Alignment Search Tool*
CAV	Vírus da anemia infecciosa das galinhas
ChiSCV	Vírus circular associado a fezes de chipanzés
EMEM	*Eagles Modified Essential Medium*
FEPAGRO	Fundação Estadual de Pesquisa Agropecuária
HGyV	Girovírus humano
ICBS	Instituto de Ciências Biológicas e da Saúde
ICTV	*International Committe on Taxonomy of Viruses*
IPVDF	Instituto de Pesquisas Veterinárias Desidério Finamor
kb	Mil pares de base
MDCC-MSB-1	Linhagem cellular de limfoblastóides hematopoéticas de galinhas
MPRCA	*Multiply primed rolling-circle amplification*
NCBI	*National Center for Biotechnology Information*
NLS	Sequência de localização nuclear
nm	Nanômetros
nt	Nucleotídeos
ORF	Fase aberta de leitura
pb	Pares de base
PCR	Reação em cadeia da polimerase
PCV1	Circovírus suíno tipo 1
PCV2	Circovírus suíno tipo 2
RodSCV	Vírus circular associado a fezes de roedores
SISPA	*Sequence-Independent Single Primer Amplification*
SPF	Livre de patôgenos específicos
SsHADV	Vírus DNA associado a hipovirulência da *Sclerotinia sclerotiorum*
TLMV	TTV-*like* mini vírus
TTMDV	Torque teno midi-vírus
TTV	Torque teno vírus
UFRGS	Universidade Federal do Rio Grande do Sul
UTR	U*ntranslated region*

SUMÁRIO

1 INTRODUÇÃO

Esta tese compreende estudos visando à identificação do agente viral, girovírus aviário tipo 2 (AGV2) e estudo da sua ocorrência na Região Sul do Brasil e na Holanda.

O girovírus aviário tipo 2 (AGV2) e o vírus da anemia infecciosa das galinhas (CAV) pertencem à família *Circoviridae,* gênero *Gyrovirus* (MURPHY, 1996; RIJSEWIJK et al., 2011). O CAV foi primeiramente isolado e descrito no Japão em 1979 (YUASA et al., 1979). Desde então, o vírus tem sido isolado em quase todo mundo (LUCIO et al. 1990; HOOP, 1992; BUSCAGLIA et al. 1994; TORO et al., 1994; SCHAT, 2003). No Brasil, o primeiro isolamento do CAV foi reportado por BRENTANO et al. (1991), em frangos de corte com atrofia de timo, anemia e pouco ganho de peso, gerando grandes perdas econômicas para a indústria avícola.

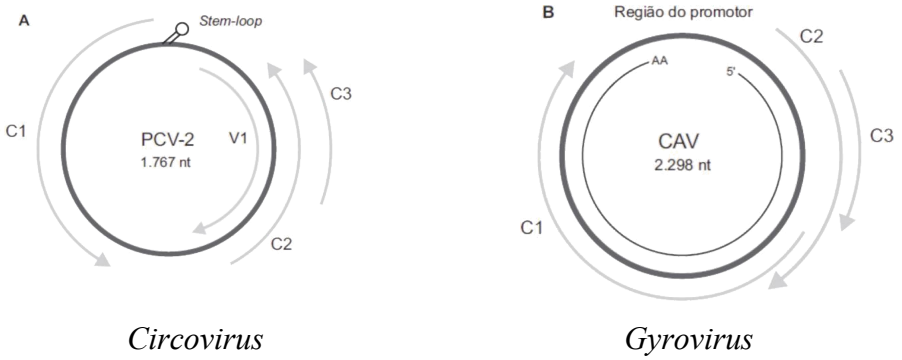
O isolamento *in vitro* do CAV é realizado em cultivos celulares de células MDCC-MSB-1 (MSB1), uma linhagem celular linfoblastóide derivado de tumores da doença de Marek (GORYO et al. 1987). O CAV, no qual, pode ser multiplicado, embora possam ser necessários de 6 a 8 sub-cultivos até o surgimento de efeito citopático (YUASA & YOSHIDA, 1983). Tentativas de isolamento do CAV em cultivos primários de embrião de galinha ou em outros tipos de linhagens celulares tem sido infrutíferos (SCHAT, 2003). Além do isolamento viral apresentar baixa sensibilidade (CARDONA et al. 2000), diferenças genéticas interferem na replicação do vírus (RENSHAW et al. 1996) fato que impossibilita o isolamento de algumas cepas (ISLAM et al. 2002).

O genoma do AGV2 foi descrito pela primeira vez no Brasil em amostras de soro de frangos comerciais, (RIJSEWIJK et al., 2011), em trabalho que é parte dessa tese. Buscando avaliar se a ocorrência desse agente era um fenômeno isolado, particularizado nas regiões de origem das aves examinadas, em cooperação com colegas holandeses, foi realizado um estudo em amostras de cérebros de frangos daquele país (dos SANTOS et al., 2012), confirmando a presença de genomas de AGV2 naquelas amostras. Subsequentemente, outro grupo identificou genomas de AGV2 em amostras de carne de frangos em Hong Kong (CHU et al., 2012). Quase que simultaneamente à publicação do artigo de Rijsewijk et al (2011), foi reportada a identificação do genoma de um outro girovírus que apresenta alta similaridade (96%) com o AGV2 em amostras de pele humana. Este agente foi denominado girovírus humano (HGyV) (SAUVAGE et al., 2011). Após o relato inicial na França, o HGyV foi identificado no Chile em

crianças com diarréia (PHAN et al., 2012). Devido ao alto grau de similaridade entre estes agentes identificados na pele e em fezes humanas com o AGV2, especulou-se que estes agentes poderiam ser fruto de contato e/ou ingestão de carne de frangos contaminados. O papel desses agentes em patologias em humanos permanece indeterminado.

1.1 Etiologia

Os vírus pertencentes à família *Circoviridae* compartilham uma série de características. Todos apresentam um genoma de DNA pequeno, circular, de fita simples. Os *Gyrovirus* apresentam genoma de polaridade negativa, em contraste com os vírus do gênero *Circovirus*, que tem um genoma ambisenso (Figura 1) (NOTEBORN et al., 1991).



Circovirus *Gyrovirus*

Figura 1. Comparação da estrutura genômica dos gêneros *Circovirus* e *Gyrovirus*.

O genoma do AGV2 é constituído de aproximadamente 2383 nucleotídeos (nt) (RIJSEWIJK et al., 2011). O genoma do CAV é constituído por 2298 a 2319 nt (Figura 2) (CLAESSENS et al. 1991; NOTEBORN et al. 1991). O vírion não possui envelope e apresenta simetria icosaédrica, com diâmetro de 25 nm (NOTEBORN et al., 1991). Três proteínas são traduzidas usando um sítio de iniciação alternativo (NOTEBORN et al., 1994a). A VP1 com 51,6 kDa, é uma proteína estrutural do capsídeo (NOTEBORN & KOCH et al., 1995). A VP2, com 24 kDa, além de servir como molde estrutural para a VP1, tem atividade de fosfatase, sendo que provavelmente participa também da indução de apoptose celular (NOTEBORN, 2004). VP1 e VP2 são capazes de induzir a

formação de anticorpos neutralizantes (KOCH et al.,1995). A VP3, uma proteína de 13,6 kDa, também chamada apoptina, induz apoptose celular e, consequentemente, exerce importante função na patogenia do CAV, pois sua ação resulta na depleção de linfócitos T e consequente imunodepressão (NOTEBORN et al., 1994b).

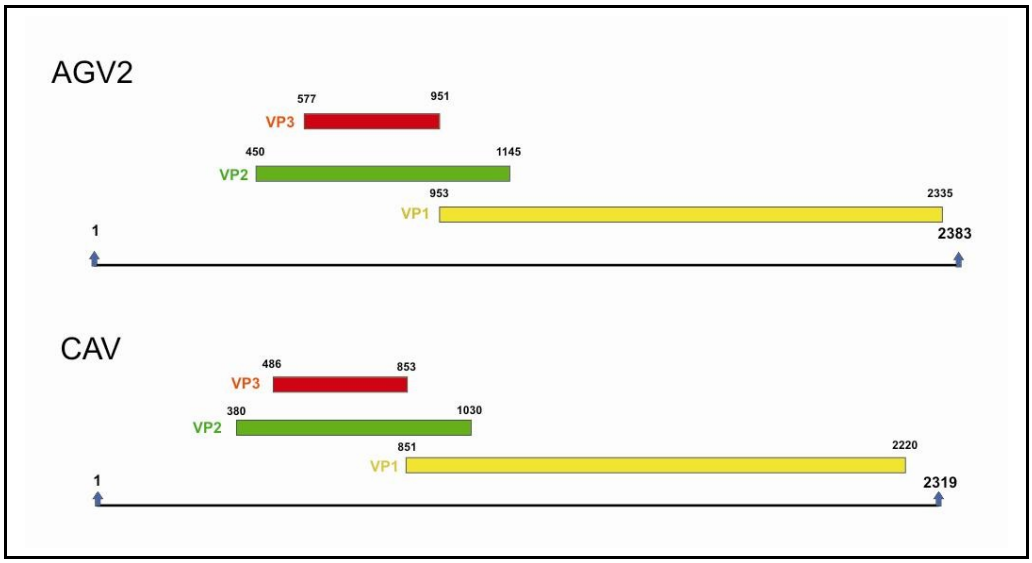


Figura 2: Comparação da estrutura genômica em forma linear do girovírus aviário tipo 2 (AGV2) com o vírus da anemia infecciosa das galinhas (CAV).

O CAV é um vírus altamente resistente a variações de pH, temperaturas elevadas e solventes orgânicos. A fumigação com formol, usada em larga escala em empresas avícolas, não tem a capacidade de inativá-lo completamente, permitindo que o vírus persista por longo tempo nas instalações. Os desinfetantes a base de glutaraldeído (1%) e formol (5%), igualmente usados em instalações avícolas, são capazes de inativar o CAV quando expostos por 24 horas à temperatura ambiente (YUASA & YOSHIDA, 1983).

1.2 Epidemiologia

A infecção por CAV está onipresente em galinhas, podendo ser encontrada na maioria das criações comerciais de aves (SCHAT & VAN SANTEN, 2008). Inicialmente, a propagação vertical foi considerada o mais importante meio de disseminação do vírus (HOOP, 1992; YUASA & YOSHIDA, 1983). No entanto, a propagação da infecção em forma horizontal é crucial para o estabelecimento da infecção em criações comerciais. Essa forma de transmissão ocorre mais

frequentemente pela via fecal-oral. Entretanto, são poucos os estudos sobre essa via de transmissão (JOINER et al., 2005; VAN SANTEN et al., 2004). Recentemente, DAVIDSON e colaboradores (2008) comprovaram que a presença do vírus em penas pode constituir-se em uma das principais formas de veiculação e perpetuação do CAV em criações de aves comerciais.

1.3 Patogenia e sinais clínicos

A infecção clínica do CAV é notada principalmente em pintos de 10 a 14 dias de idade. Frangos com mais de três semanas de idade também são suscetíveis à infecção, desenvolvendo uma doença subclínica (SCHAT, 2003; ADAIR, 2000) Surtos da doença clínica são caracterizados por anemia, atrofia de timo, aplasia de medula óssea e imunodepressão (ADAIR, 2000; YUASA & YOSHIDA, 1983). Matrizes adultas não desenvolvem a anemia infecciosa das galinhas, mas quando infectadas no início ou durante o período de postura, transmitem o vírus verticalmente (YUASA et al., 1980; HOOP, 1992).

Em relação ao AGV2, seu potencial patogênico não foi estudado. Ainda não foi determinado se este agente causa qualquer efeito patogênico em aves. Estudos buscando essas respostas estão em andamento, em continuidade aos trabalhos apresentados nesta tese.

1.4 Diagnóstico

O diagnóstico de infecções pelo CAV pode ser realizado através da inoculação do vírus em pintos SPF de um dia de idade. Nestes, a reprodução das lesões se dará em 14-18 dias após a inoculação (SCHAT, 2003), observando-se anemia, lesões na medula óssea e nos tecidos linfóides, sendo estas as lesões mais evidentes. A mortalidade das aves ocorre entre 12 a 28 dias após a inoculação, que raramente excede 30% (BRENTANO, 2000).

O método de diagnóstico mais usado atualmente para a detecção do CAV e AGV2 é a reação em cadeia da polimerase (PCR). Vários protocolos de PCR para a detecção do DNA tem sido descritos, buscando a otimização do procedimento para diferentes materiais, como tecidos e soro de aves, bulbos de penas e cultivos celulares

(THAM & STANISLAWEK, 1992; TODD et al. 1992; IMAI et al. 1998; CARDONA et al. 2000; YILMAZ et al. 2001; DAVIDSON et al. 2008; dos SANTOS et al., 2012).

Embora a PCR venha sendo amplamente utilizada no diagnóstico de infecções pelo CAV, o desenvolvimento de métodos que não necessitam o conhecimento prévio da sequência tem permitido a investigação de novas variantes virais. Estes métodos tem a vantagem de poder superar eventuais problemas consequentes a variações nas sequências disponíveis. Um desses métodos é a Amplificação por Círculo Rolante com Múltiplos Primers (ACRMP). Esta técnica permite a amplificação de DNAs circulares (DEAN et at., 2001) e lineares (PAEZ et al., 2004) usando oligonucleotídeos randômicos. Outro método semelhante é a SISPA "*Sequence Independent Single Primer Amplification*". Este método tem demonstrado seu potencial na identificação de genomas de novos agentes (Tabela 1) em diversos tipos de espécies (NIEL et al., 2005; BIAGINI et al., 2007).

Em um estudo realizado em amostras de soro de galinhas com quadro clínico de anemia e perda de peso encaminhadas ao IPVDF, amplificado por ACRMP e subsequente digestão parcial com as enzimas de restição (*Bam*HI e *Hind*III), um segmento de DNA que apresentava tamanho semelhante ao do genoma do CAV. Tal genoma foi posteriormente sequenciado e revelou tratar-se de um vírus semelhante ao CAV, porém com um nível de similaridade genômica muito baixo, de cerca de 40%. Este nível de diferenciação justificaria taxonomicamente a criação de uma nova espécie do gênero. Foi então proposta para este agente a denominação de girovírus aviário tipo 2, ou "avian gyrovirus type 2" (AGV2) (RIJSEWIJK et al.,2011). Em semelhança ao CAV, o AGV2 tem um genoma pequeno, composto por uma fita única de DNA circular de 2383 nucleotídeos (RIJSEWIJK et al., 2011).

1.5 Outras considerações

O primeiro capítulo dessa tese tem como objetivo relatar a descoberta do AGV2, propondo sua classificação como uma nova espécie do gênero *Gyrovirus*. O segundo capítulo tem como objetivo reportar a presença de variantes do genoma do AGV2, presentes na Região Sul do Brasil e em aves comerciais da Holanda. Tais estudos aqui apresentados nesta tese, tem a pretensão de servir como base para futuras investigações visando determinar a importância econômica de infecções por esses agentes e o possível impacto destes sobre a cadeia produtiva de aves.

Tabela 1 Grupos de vírus de DNA identificados em tecidos e/ou amostras ambientais através da metagenômica e PCR com primers degenerados para circovírus. (X) - Indica a presença de metagenômico, com semelhanças as proteínas virais ssDNA. (G) - Refere-se a genomas completos com semelhanças significativas para a presença determinada pela metagenômica. (G*) - Refere-se a genomas completos contendo uma proteína com semelhanças significativas a um dado grupo, no entanto, exibem novas formas ou semelhanças com múltiplos grupos. (1) - Genomas semelhantes a circovírus conhecidos (CV), ciclovírus recentemente descobertos (CYV) (ROSARIO et al. 2012).

Tecidos	Anellovírus	Girovírus	Circovírus	Geminivírus	Nanovírus	Parvovírus	Referência
Sangue humano	X						BREITBART & ROHWER, 2005.
Pele humana (HGyV)		G					SAUVAGE et al., 2011.
Trato respiratório humano			X	X	X	X	WILLNER et al., 2009.
Fezes humanas			G (CV; CyV)[1]				LI et al., 2010b.
Fezes de chipanzé (ChiSCV)			X		G*		BLINKOVA et al. 2010.
Pulmão de leão marinho e focas	G						NG, et al., 2011c.
Fibropapiloma de focas		G					NG et al., 2009.
Fezes de morcegos			G (CV; CyV)	X		X	GE et al. 2011; LI, et al., 2010a.
Músculo peitoral de morcego			G (CyV)				LI et al., 2010a.
Fezes de suínos			G*				SHAN et al. 2011.
Fezes de roedores (RodSCV)			G*				PHAN et al., 2011.
Soro de galinhas (AGV2)		G					RIJSEWIJK et al. 2011
Intestino de perus						X	DAY et al., 2010.
Órgãos de peixes			G (CV)				LORINEZ et al., 2011.
Mosquitos	G		G*	G (SsHADV)[1]	G*	X	NG et al., 2011a.
Mariposas				G			NG et al., 2011b.
Abdômen de libélulas			G (CyV)				ROSARIO et al., 2011.
Corais			X	X	X	X	VEGA et al. 2008.

2 OBJETIVOS

2.1 Objetivo geral

- Contribuir para melhoria da sanidade das aves comerciais através da identificação de um novo agente viral (girovírus aviário tipo 2) e suas variantes, com um provável potencial patogênico.

2.2 Objetivos específicos

- Descrever um novo agente (AGV2), semelhante ao CAV, constituindo uma nova espécie do gênero *Gyrovirus*;

- Reportar a ocorrência de variantes do AGV2, bem como estudos sobre a presença desse agente em aves na Região Sul do Brasil e na Holanda;

3 CAPÍTULO 1

3.1 Discovery of a genome of a distant relative of chicken anemia virus in chicken reveals a new species in the genus *Gyrovirus*

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Discovery of a genome of a distant relative of chicken anemia virus in chicken reveals a new species in the genus *Gyrovirus*

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Note: The reported sequence has been submitted to GenBank and has accession number HM590588

Abstract

A 2.4-kb phi29 polymerase amplification product from serum of a diseased chicken was cloned and sequenced. The 2383-nucleotide sequence showed about 40% identity to a representative genome of chicken anemia virus (CAV), the only member of the genus *Gyrovirus*, family *Circoviridae*. The new genome had an organization similar to that of CAV: a putative 50 untranscribed region of about 400 nt followed by three partially overlapping open reading frames encoding VP1, VP2 and VP3 homologs. The amino acid identities between these homologs and those of CAV were 38.8%, 40.3%, and 32.2%, respectively. Based on these limited similarities, it is proposed that the newly identified virus is a member of a new species in the genus *Gyrovirus*. For this new species, the name Avian gyrovirus 2 (AGV2) is proposed.

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Chicken anemia virus (CAV) is the sole described member of the genus *Gyrovirus* of the family *Circoviridae* [18]. It was discovered in Japan in 1979, and it causes anemia and immunosuppression in newly hatched chickens [18, 21]. Based on results of genomic sequencing and expression of open reading frames (ORFs) of CAV, a 50 untranscribed region (5'UTR) [16] and the coding regions for three viral proteins, VP1, VP2 and VP3, were identified [6]. In the middle of 2008, a number of three-week-old chickens in a poultry production unit in the south of Brazil were noticed to display apathy and loss of weight. In an attempt to identify circular DNA viruses that may have contributed to this condition, DNAs from sera of these chickens were subjected to multiply primed rolling-circle amplification (MPRCA) [1]. Here, we report the complete nucleotide sequence of one of the amplified products and show that this sequence is about 40% identical to the genome of CAV. It was inferred that it belongs to a new species in the genus *Gyrovirus*, for which the name Avian gyrovirus 2 (AGV2) is proposed.

On July 14, 2008, four sera were taken from diseased chickens (''Ave-1 to 4'') of a poultry production unit in Rio Grande do Sul, Brazil. Isolation of DNA from chicken sera was performed by phenol extraction as described previously [2]. The DNA

samples (100 ng) were subjected to MPRCA using Phi 29 DNA polymerase (New England Biolabs, Ipswich, MA, USA) [2]. The amplification products were digested with restriction enzymes, separated on a 0.7% agarose gel, and cloned in plasmid pUC18 (New England Biolabs) using standard cloning methods [17]. AGV2 clones were purified, and about 200 ng of each plasmid was used for nucleotide sequence analysis on a multi-channel automatic sequencing device (ACTGene256-Mega Bace, Uppsala, Sweden) using M13 primers (New England Biolabs) or specific AGV2 primers (primer- walking approach). Before sequencing, a GC-rich region was PCR amplified in the presence of 50% 7-deaza-GTP and 50% dGTP (New England Biolabs). The sequences that were obtained were aligned using the sequence assembly program SeqMantmII from DNASTAR Inc., Madison, WI, USA, and analyzed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Sequence comparisons and phylogenetic analysis were performed using the MegAlign 500 program of DNASTAR Inc.

The four obtained Phi 29 amplification products had a unit length of 2.4 kb and contained no *Eco*RI, one *Apa*I site, one *Hind*III site and two *Bam*HI sites. One of the amplification products, ‘‘Ave-3’’, was cloned, subcloned, and sequenced. The nucleotide sequence obtained was 2383 nt long and has been submitted to GenBank with accession number HM590588. Figure 1 shows the schematic representation of the genomic organization of AGV2 and Fig. 2 shows the position of AGV2 in relation to other members of the family *Circoviridae*, including a recently proposed genus named *Cyclovirus* [7].

The 5’ untranscribed region (5’ UTR) of AGV2 is by definition located between the polyadenylation site and the start of transcription. Two canonical polyadenylation signals (AATAAA) were found next to each other between nt 27 and 32 and between nt 39 and 44, where in the homologous position in CAV there is only one AATAAA sequence. The actual polyadenylation takes place some 10 to 30 nt downstream of this signal [3]. Using promoter prediction programs [10, 19], a putative TATA box (GATATAAG) was identified between nt 415 and 422, and a transcription start site was postulated at nt 440, which was located 10 nt upstream of the start of ORF1 (nt 450). The estimated size of the 5’ UTR was thus about 400 nt and was similar in size to the 5’ UTR of CAV [16]. This region contained six almost perfect direct repeat (DR) regions of 22 nt: 5’GTACAGGGGGGTACGTCAt/cCAt/g3’. Five or four (depending on the CAV strain) highly similar versions of these repeats are found in the 5’ UTR of CAV

strains [11]. These repeats are thought to act as enhancers of transcription and may also play a role in viral replication [9].

Using the ORF Finder protocol of the NCBI [13], 14 open reading frames (ORFs) of more than 100 nt were found in the 2383 nt sequence: eight on the sense strand and six on the antisense strand. Only the three largest ORFs showed homology with proteins found in the NCBI database. All three were encoded on the sense strand, but in different reading frames: ORF1 in frame 3 from nt 450 to nt 1145, ORF2 in frame 1 from nt 577 to nt 951 and ORF3 in frame 2 from nt 953 to nt 2335 (Fig. 1). They encode homologs of CAV VP2, VP3 and VP1, respectively.

ORF1 encodes an amino acid sequence of 231 residues, which displayed 40.3% identity with VP2 of CAV. AGV2 VP2 contains a sequence (CX5R, residues [res.] 107-113— the large capitals indicate conserved residues) that is highly conserved in all protein phosphatases (PTPases), including VP2 of CAV. VP2 of CAV is a dual-specificity PTPase that plays a role in viral replication, cytopathology and virulence [15]. Interestingly, the phosphatase motif is part of a larger motif (WX7HX3CX CX5H) that is shared by CAV, torque teno virus (TTV) and TTV-like mini virus (TLMV) [20]. In AGV2, this motif is found in the sequence $W_{LRQCARS}H_{DEI}C_TC_{GRWRS}H$ (res. 95-115). Only the gyroviruses have the PTPase arginine (R) within this motif.

ORF2 encodes an amino acid sequence of 124 residues, which displayed 32.2% identity with VP3 of CAV. CAV VP3, or apoptin, was shown to induce apoptosis of hematopoietic cells, causing the observed anemia in infected chickens and to trigger apoptosis of tumor cells, but not of non-tumor cells [8, 12]. In the N-terminal half of CAV VP3, a short hydrophobic isoleucine-rich stretch is found (CAV res. 33-46) that is required for self-association as well as for binding cellular proteins [4]. This amino acid sequence is well conserved in AGV2 VP3 at res. 38-51. In the C-terminal half of CAV VP3, a bipartite nuclear localization sequence (NLS) was identified. The first part (NLS1) is located at res. 82-88, and the second part (NLS2) at res. 111-121. Between these two domains, a putative nuclear export sequence (NES) was found at res. 97-105. At homologous positions in VP3 of AGV2, NLS-like sequences were found: RRPRR (res. 84-88) and KKLRL (res. 120-124). A NES domain, however, was clearly conserved in AGV2 VP3.

ORF3 encodes an amino acid sequence of 460 residues, which displayed an identity of 38.8% with VP1 of CAV. Like other capsid proteins, it starts with a region that is rich in arginine and lysine residues (27 of the first 60 amino acids). Three

conserved replication motifs have been identified in VP1 of CAV: F_ATLT at res. 313-317, $_{QR}WH_TLV$ at res. 351-357, and Y_ALK at res. 402 405 [14]. At the homologous positions, the following sequences were found in VP1 of AGV2: $F_{AA}L_S$ (res. 325-329), $_{RR}W_{LT}LV$ (res. 363-369) and $_{KAMA}$ (res. 412-415). This comparison suggests that the three motifs are not well conserved and that the third motif is not conserved at all. Another candidate for the third replication motif is a Y_KF_{GT} sequence that is present in VP1 of both CAV and AGV2, located only 7 residues before the Y_ALK/$_{KAMA}$ sequences.

Immediately downstream of the stop codon of VP1 of AGV2, a 73-nt-long GC-rich sequence (joining nt 2324-2383 to nt 1-23) is found that can form two stem-loop structures: 5'-ggggggggggttttgccccccccacaaacccccccccccggggggggatcctcccccccg gacccccg-3'. This sequence shows 60% identity to the corresponding GC-rich region of CAV [11].

This is the first time since the discovery of CAV that a genome of a member of a related species has been discovered. Although there are no proposed rules to demarcate species in the genus *Gyrovirus*, there are recognized criteria used for the genus *Circovirus*: (i) complete nucleotide sequence identity of less than 75% and (ii) capsid protein amino acid sequence identity of less than 70% [5]. The application of these criteria to the data described here (identities of 40% with the CAV genomic sequence and 38.8% with the CAV capsid protein) justify the description of this new virus as a member of a separate species in the genus *Gyrovirus*.

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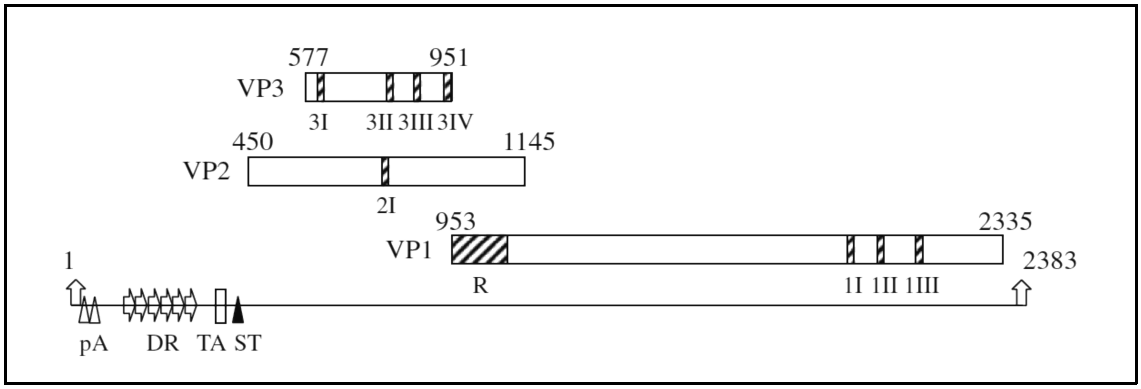

Fig. 1 Schematic representation of the genomic organization of AGV2. The horizontal bars in the upper part represent the sizes and positions of VP3, VP2 and VP1. The numbers above the bars designate their nucleotide positions. The hatched regions within the bars indicate the positions of conserved motifs. Within VP3: 3I = isoleucine stretch, 3II = NLS1, 3III = NES and 3IV = NLS2. Within VP2: 2I = phosphatase motif. Within VP1: R = arginine rich region, 1I, 1II, 1III = rep motifs I, II and III, respectively. The horizontal line in the lower part represents the 2383-nt AGV2 genome. The vertical arrows at the start and end of the genome indicate the positions of the putative stem-loop structures. The open triangles indicate two polyadenylation signals. The six horizontal arrows show the positions of the direct repeats found in the 5' UTR. The open box indicates the position of a putative TATA box, and the closed triangle, the putative transcription start site.

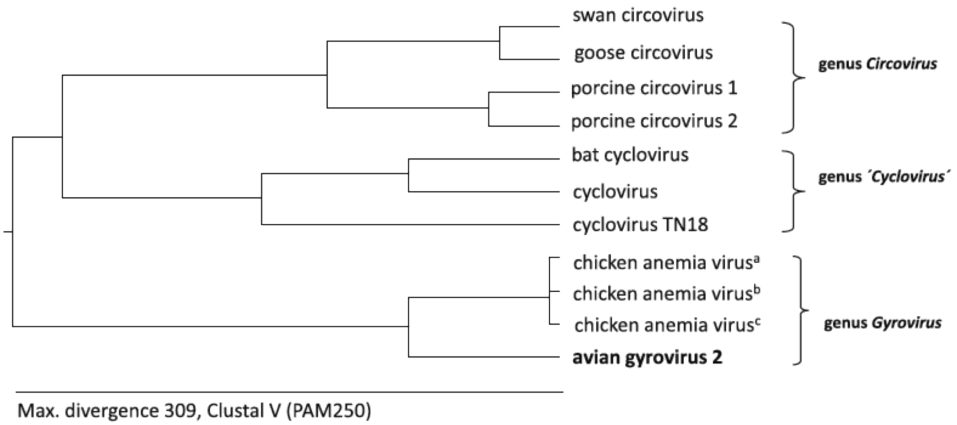

Fig. 2 Phylogenetic tree based on the alignment of amino acid sequences of the VP1 capsid proteins of eight different members of the family Circoviridae and the one described in this study. The avian gyrovirus described in this study is in bold. The VP1 sequences used have the following accession numbers: swan circovirus, ABU48446.1; goose circovirus, AAQ03996.1; PCV1, AAN77864.1 PCV2, ABD42929.1; bat cyclovirus, ADU77000.1; cyclovirus, ADU77014.1; cyclovirusTN18, ADD62480.1; chicken anemia virus[a], AAT85595; chicken anemia virus[b], ADJ18278; chicken anemia virus, AAT85598. The AGV2 sequence has NCBI accession number HM590588.

4 CAPÍTULO 2

4.1 Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands

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Variants of the recently discovered avian gyrovirus 2 are detected in Southern Brazil and The Netherlands

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[1] *In memoriam.*

ABSTRACT

A genome of a virus preliminarily named avian gyrovirus 2 (AGV2), a close relative to chicken anemia virus, was recently discovered in a chicken in the state of Rio Grande do Sul, Southern Brazil. To study the occurrence of AGV2 in Rio Grande do Sul and the neighboring state Santa Catarina, a number of adult chickens (n = 108 and n = 48, respectively) were tested for the presence of AGV2 DNA. An AGV2-specific PCR was developed, optimized and used to analyze DNA extracted from clinical samples. AGV2 DNA was detected in 98/108 (90.7%) of samples collected in the state of Rio Grande do Sul and 29/48 (60.4%) of the samples collected in the state of Santa Catarina. In order to check whether AGV2 DNA would be detected in samples from a geographically distant region, DNA from brain samples of 21 diseased chickens from the Netherlands were tested independently, by the same method. In such specimens, 9/21 (42.9%) brain tissue samples were found to contain AVG2 DNA. Sequence analysis of some of the PCR products demonstrated that the amplified AGV2 sequences could vary up to 15.8% and could preliminarily be divided in three groups. This indicated the occurrence of variants of AGV2, which may reflect differences in geographical origin and/or in biological properties. The data presented here provides evidence that AGV2 seems fairly distributed in chickens in Southern Brazil and that AGV2 also circulates in the Netherlands. Besides, circulating viruses display genetic variants whose significance should be further examined, particularly to determine whether AGV2 would play any role in chicken diseases.

Keywords: avian gyrovirus 2; incidence; variants; poultry industry

4.2 Introduction

Members of the family *Circoviridae* are non-enveloped, icosahedral shaped viruses with diameters between 16 and 26 nm and with small, circular, single-stranded DNA genomes (Todd et al., 1991). The *Circoviridae* is divided in two genera: *Gyrovirus* and *Circovirus* (McNulty et al., 2000). The genus *Gyrovirus* comprises chicken anemia virus (CAV), whereas the genus *Circovirus* contains at present 11

species, including porcine circovirus 1 and 2 (PCV1 and PCV2) (Fauquet et al., 2005; Halami et al., 2008).

CAV is an important pathogen causing economic losses for the poultry industry, particularly by increasing the severity of diseases caused by other pathogens (Todd, 2000; Haridy et al., 2009; Toro et al., 2009). CAV is widely distributed in virtually all countries where commercial poultry are produced (Schat, 2009). CAV is disseminated vertically and horizontally, and the virus may also be present in a latent state in commercial and specific pathogen-free (SPF) chickens (Miller and Schat, 2004). CAV predominantly infects thymus and bone marrow cells, which can result in anemia, bleeding and immunosuppression, leading to secondary infections. CAV-associated disease is more common in young chickens infected vertically. In the absence of maternal antibodies, infection of young chickens – in general less than 2 weeks of age – may result in clinical disease (Schat, 2009). However, CAV disappears from most sites in the chicken as soon as antibodies appear in the serum. An exception is the feather shafts where CAV genomes can be detected long after the acute phase of the disease (Schat, 2009). Infection of chickens older than 3 weeks is usually subclinical, but nevertheless can cause significant losses (Rosenberger and Cloud, 1998).

Recent studies in our laboratory using serum samples from chickens with clinical manifestations like malnutrition and weight loss led to the detection of the genome of a previously unknown virus, tentatively named avian gyrovirus 2 (AGV2). AGV2 has a 2.4 kb circular genome, similar in size and genomic organization to CAV, and shows a nucleotide similarity of about 40% with CAV (Rijsewijk et al., 2011).

The aim of this study was to develop a specific polymerase chain reaction (PCR) to detect AGV2 DNA and to assess the presence of such viral genome in clinical samples of (backyard) poultry collected in Southern Brazil. In addition, the presence of AGV2 DNA in chickens in the Netherlands was studied and sequences of some of the PCR amplicons compared. This study reports preliminary findings on the distribution of this newly discovered virus, whose importance as a pathogen needs to be further examined, as it may have a significant economic impact on the poultry industry.

Materials and methods

4.3.1 Chicken samples

To investigate the presence of AGV2 DNA in backyard chickens, feathers were collected from 104 chickens from 60 flocks situated in 30 districts of the state of Rio Grande do Sul, Brazil, from widely scattered regions within the state. In addition, feathers were collected from 48 backyard chickens from two farms in the neighboring state Santa Catarina. Such flocks were raised for local consumption or marketing. All birds from which feathers were collected were apparently healthy adults and had not been previously vaccinated against any of the most common agents of chicken diseases, including CAV. The proximal 1 cm shaft was collected from the feathers and stored at -20 ºC. In addition to the feathers, organs (thymus, bone marrow and bursa) collected from three adult chickens displaying weight loss and the hemorrhagic brains of one chicken which displayed clinical signs that resembled chicken anemia infection were also used in this study.

To examine whether AGV2 DNA could be detected in a geographically distant region, brain tissues of 21 chickens displaying neurological signs in the Netherlands were collected. The specimens were collected over a 2-year period (2008–2010) from different commercial poultry farms, rearing layers or broilers.

4.3.2 Isolation of DNA

DNA extraction from the feather shafts for AGV2 PCR analysis was performed as previously described (Bello et al., 2001; Davidson et al., 2008) with some modifications, as follows. The proximal shaft fragments of two feathers from each chicken were cut into small pieces of approximately 0.3 cm using sterile surgical blades and were incubated overnight under gentle shaking at 37 ºC in 500 µl lysis buffer (50mM Tris–HCl pH 8, 20 mM EDTA pH 8, 2% SDS and proteinase K at a final concentration of 175 mg/µl). Subsequently, the samples were vortexed and centrifuged at 12,000 xg for 10 min. Supernatants were transferred to new tubes and DNAs were purified with phenol:choloroform:isoamyl alcohol (25:24:1) as described elsewhere (Sambrook and Russell, 2001). Subsequently, 2 volumes of ice-cold 98% ethanol and 50 µl of 2 M NaCl were added and the samples incubated for 30 min. The samples were then centrifuged at 12,000 xg for 15 min and the supernatants discarded. The DNA

pellets were washed with 70% ethanol and resuspended in 50 µl of TE buffer (10mM Tris–HCl, 1 mM EDTA, pH 8). Next, an RNAse digestion was performed (3 µl of 10 mg/µl RNAse for 40 min at 37 ºC). A fraction of each sample (5 µl) was subjected to agarose gel electrophoresis to evaluate the efficiency of extraction. Only samples with at least 10 ng DNA/5 µl were submitted to the subsequent AGV2 PCR. Samples were stored at -20 ºC until used.

DNA extraction from tissues was performed as follows. Tissues were weighed and homogenized in cold Eagle Modified Essential Medium (EMEM) to obtain approximately 10% (w/v) suspensions. The homogenates were centrifuged to remove cell debris and DNA was extracted from 200 µl of the supernatant with a QIAamp DNA Mini kit (Qiagen). The amount of DNA was determined with a nano-drop spectrophotometer. The samples were stored at -20 ºC until analyzed.

4.3.3 AGV2 amplification by PCR

The AGV2 specific primers used in the PCR assay were designed based on the nucleotide sequence of AGV2 variant ‘‘Ave-3’’ (Rijsewijk et al., 2011) using the Primer Select program of sequence analysis software of DNASTAR. The primers (PF: 5’-CGTGTCCGCCAGCAGAAAC-3’ and PR: 5’GGTAGAAGCCAAAGCGTCCAC-3’) amplify a fragment of 345 bp and target a genomic region that codes part of the VP2 and VP3 genes (nt 656 to nt 1001). The PCR reaction contained 0.75 µl 50 mM $MgCl_2$, 2.5 µl 10x PCR buffer 5 pM of each primer, 1.25 U Taq DNA polymerase, 2 µl 10 mM dNTP, 1 µl of the sample DNA and sterilized Milli-Q water to complete a total volume of 25 µl per reaction. Reactions were performed under the following conditions: 7 min at 94 ºC followed by 35 cycles of 30 min at 94 ºC, 30 min at 62 ºC and 30 min at 72 ºC, followed by 7 min at 72 ºC in an Eppendorf Master cycler. Five µl of the amplified DNA were analyzed on a 1% agarose gel. Negative controls were included in every three PCR reactions and one positive control in each set of reactions performed. To avoid contamination, different locations were used to conduct the DNA extractions, to prepare the PCR reaction buffers and to perform PCR analyses.

4.3.4 Sequencing and sequence analysis

Sequencing was carried out in a 3130 Genetic Analyzer with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied biosystems) following the manufacturer's protocol. Each product was sequenced four times in both directions. The quality of DNA sequences was checked and overlapping fragments were assembled using the BioEdit package 7.0.5 (Hall, 1999), Vector NTI 8.0, AlignX and ContigExpress (InforMax, Inc.). The sequences were compared with the corresponding region of AGV2 ''Ave-3'' strain, the first sequence obtained from this virus, which is deposited under the accession number HM590588 at the National Center for Biotechnology Information (NCBI). Assembled sequences with high quality were aligned using Clustal W (Thompson et al., 1994) with default gap penalties. Homology analyses were performed by BLAST (Altschul et al., 1997). Phylogenetic analysis was performed using MEGA 3 (Kumar et al., 2004). Neighbor-Joining (NJ) trees were constructed using Kimura-2 parameters and calculated using pair wise deletion. Bootstrap was resampled as a test of phylogeny using 500 replications (Efron et al., 1996).

4.4 Results

4.4.1 Sensitivity and specificity of the AGV2 PCR

The sensitivity of the AGV2 PCR was determined using tenfold serial dilutions of known quantities of a plasmid (pCR 2.1, Invitrogen) containing the AGV2 genome. The plasmid was diluted in TE and the dilutions used in the PCR ranged from 1012 to 101 DNA molecules. The 345 bp amplification products obtained with 100 molecules of the AGV2 clone were easily visualized on an ethidium bromide-stained agarose gel (data not shown). Therefore, the lower detection limit of this PCR was considered to lie between 100 and 10 molecules of AGV2 DNA. The specificity of the AGV2 PCR was determined by comparing amplification results performed with clones containing the AGV2 and CAV DNA as templates. Tenfold serial dilutions of plasmids containing 10^6, 10^5 or 10^4 of AGV2 or CAV DNA were submitted to the AGV2 PCR. When the CAV

primers were used in the PCR with AGV2 DNA no amplicon was observed. In the reversed situation, when AGV2 primers were used in the presence of CAV DNA, no amplicon was observed either.

4.4.2 Demonstration of AGV2 DNA in feathers and organs of chickens from Brazil

To study the occurrence of AGV2 in chickens of the South of Brazil, samples collected from adult chickens were used in an AGV2 specific PCR. From the 104 feather shafts and the four organs collected from animals in Rio Grande do Sul, 98 (90.7%) revealed the presence of AGV2 DNA by PCR. From the feather samples collected in the state of Santa Catarina, 29/48 (60.4%) were found to contain AGV2 DNA. These results showed that AGV2 DNA is widely distributed in backyard chickens in Southern Brazil.

4.4.3 Demonstration of AGV2 DNA in brains of diseased chickens from The Netherlands

To avoid any chance of contamination, the AGV2 PCR was performed in the partner laboratory in the Netherlands (Central Veterinary Institute, Wageningen UR, Lelystad, the Netherlands), with primers and reagents purchased and prepared independently from the Brazilian laboratories involved in the search. Out of the 21 brain samples from chickens from the Netherlands, 9 (42.9%) were found to contain AGV2 DNA. Therefore, AGV2 DNA can also be detected in chickens in the Netherlands, thus providing evidence that AGV2 is not limited to chickens reared in Brazil and is present at least in these two widely distant countries.

4.4.4 The nucleotide sequences of AGV2 point at the existence of variants

The nucleotide sequences were determined for twenty seven AGV2 amplicons, including 14 samples from Rio Grande do Sul, 8 samples from the state of Santa Catarina and 5 from chickens from the Netherlands (Fig. 1). The obtained sequences revealed that all twenty seven sequences were undoubtedly amplification products from

AGV2 genomes. These sequences were compared to the first sequence originally obtained from the virus, identified here and deposited in NCBI under the number HM590588 (Rijsewijk et al., 2011).

Eighteen out of the 22 AGV2 sequences determined from samples of chickens from Southern Brazil were 100% identical to each other as well as to the corresponding sequence in HM590588 sequence (Figs. 2 and 3). These 18 sequences were obtained from healthy birds. The other 3 Brazilian AGV2 sequences, obtained from chickens displaying apathy and weight loss (samples 068_11, AGV2_N1, AGV2_N5) were clearly different from the AGV2 sequences from healthy animals. These 3 samples displayed 11.5% nucleotide differences (data not shown), in comparison to the other Brazilian AGV2 genomes. These nucleotides differences lead to 5 and 8 amino acid changes, in VP2 and 3, respectively, in relation to the sequences from healthy animals (Figs. 2 and 3). On the other hand, sample SM_BRA obtained from a chicken with brain lesions had 13.1% nucleotide differences (data not shown) in relation to the AGV2 strains from healthy chickens, leading to one (VP2) and 9 (VP3) amino acid changes in relation to the corresponding amino acid sequences obtained from the healthy chickens.

Interestingly, sample SM_BRA was identical to3 out of the 4 sequenced Dutch AGV2 amplicons (named 14_HOL, 15_HOL and 20_HOL). However, the other Dutch sequence, named 8_HOL, showed 8.7% nucleotide differences in relation to the sequence of healthy chickens and 11.6% nucleotide differences in relation to the other four Dutch sequences (data not shown). This sample showed 6 amino acid changes in VP2 and VP3(Figs. 2 and 3) when compared to the corresponding amino acid sequences of healthy animals.

Using all twenty seven sequences of AGV2 PCR products and the corresponding region of the sequence HM590588, a phylogenetic tree was assembled (Fig. 4). The tree displayed three clusters; one formed by most Brazilian sequences, obtained from healthy chickens; another cluster comprised the AGV2 sequences of chickens from the Netherlands plus the Brazilian sequence SM_BRA. The third cluster comprised the AGV2 sequences from three diseased adult chickens from Brazil (Fig. 4).

4.5 Discussion

To study the occurrence of a recently identified virus of chickens, AGV2, in the South Region of Brazil, a number of adult backyard chickens from Rio Grande do Sul and the neigh boring state of Santa Catarina (n = 108 and n = 48, respectively) were tested for the presence of viral DNA using a newly developed PCR. Viral DNA was detected in samples of 98 out of 108 sampled chickens from Rio Grande do Sul and 29 out of 48 samples from Santa Catarina. There is a significant difference when the incidences of AGV2 in these two states are compared. This is probably related to the number of farms where the samples were collected; the samples in Santa Catarina originated from only two farms, while 60 flocks from 30 districts in Rio Grande do Sul were analyzed. In addition, the number of samples collected in Santa Catarina was also reduced when compared to the samples collected in Rio Grande do Sul, which may also have influenced these results. The detection of AGV2 DNA in a large proportion of chickens, collected from farms distributed over two states in Southern Brazil, associated to the occurrence of the first AGV2 (‘‘Ave-3’’ virus), which was identified in commercially reared chickens, indicate that AGV2 virus infections are widely disseminated among chickens in Southern Brazil. Moreover, the additional detection of AGV2 DNA in chickens from the Netherlands provides evidence that AGV2 infections are not limited to Southern Brazil. Indeed although we have only examined samples from two countries, the fact that these two are so geographically apart, plus the intense borderless dissemination of commercial chicken breeds supports the idea that AGV2 is probably distributed worldwide, such as the closely related CAV (Schat, 2009). In addition, these findings suggest that AGV2 infections in chickens are not a recent event, though with the available data it would not be possible to make any guess on a time scale to its origin. In view of its similarity with CAV, it is possible that AGV2 may have been circulating in chickens for a long time already, perhaps masked by, or misinterpreted as, CAV.

In order to identify variants of AGV2 genomes from distinct geographical regions, either with or without history of disease, the nucleotide sequences of twenty seven AGV2 amplicons from Brazil and the Netherlands were determined. The nucleotide sequences of 18 amplicons obtained from feathers of healthy chickens from Rio Grande do Sul and Santa Catarina were identical to each other, and could be

phylogenetically grouped together, along with the sequence HM590588, the first complete AGV2 sequence reported (Rijsewijk et al., 2011). On the other hand, all 3 sequences obtained from organs of diseased adult chickens from Rio Grande do Sul (displaying weight loss) showed 100% identity to each other and were clearly different from the 18 sequences from healthy animals. In the phylogenetic analysis these segregated into a separate cluster. Among the sequences obtained from chickens with history of brain lesions, five originated from chickens from the Netherlands and one originated from a Brazilian chicken. Surprisingly, when these samples were analyzed, three of the sequences obtained from the Netherlands (14_HOL, 15_HOL and 20_HOL) were identical to the Brazilian sequence named SM_BRA, whereas 2 other samples were genetically different from each other and from the other Dutch samples. When the sequence data of these samples were used in the phylogenetic analysis, they also segregated into a distinct cluster. These findings allow us to speculate that there may be pathogenic and non-pathogenic variants of AGV2; moreover, there may be variation in the pathogenic potential of AGV2 strains.

These matters will undoubtedly require further investigation in the near future, as they may have different impact on the poultry industry. Besides the wide distribution of AGV2 DNA in chickens, it is remarkable that some of the determined nucleotide sequences showed quite a number of substitutions. The genomes of CAV isolates are notorious for their limited variability (van Santen et al., 2001). Moreover, in CAV, the nucleotide region coding both the VP2 and VP3 proteins, which corresponds to the AGV2 region targeted by the PCR used here, is even less variable than the rest of the CAV genome (He et al., 2007). An analysis of 36 CAV VP2 and VP3 nucleotide sequences deposited at the NCBI database, comprising sequences from the most geographically varied sources, revealed that 100% of the VP2 sequences and 99% of the VP3 sequences were identical. On the other hand, for AGV2, when the 22 sequences of the VP2/3 region obtained from animals from Southern are compared with each other, up to 15.8% nucleotide differences are detected. This indicates that AGV2 genomes have a higher variability than CAV reported worldwide. This may be useful to facilitate the discrimination among AGV2 genotypes. Although the number of presently available sequences is still low, the differences between AGV2 genomes from the two Brazilian states and the Netherlands and from birds with clinical histories varying from ‘‘healthy’’ to ‘‘with brain lesions’’ suggest two different types of association; first, sequence variation may be related to the geographical origin of viruses. However, this

may not be absolute, since different variants may be detected in birds from a same region, as was the case with some samples from Rio Grande do Sul. Second, sequence variation may be related to differences in pathogenicity of AGV2 strains; this might be supported by the identification of the AGV2 variant SM_BRA, which was found to cluster along with AGV2 sequences obtained from chickens with comparable lesions in Europe.

It is tempting to speculate on the possible associations between AGV2 and disease. Its close relative CAV is pathogenic for young and not for adult chickens, causing severe immunossupression which leads to a number of infectious diseases that typically affect animals that live under intensive farming practices. This perhaps could also be the case for AGV2. Nevertheless, it will be also of interest to examine whether AGV2 exerts any type of interaction with other infectious agents of chicken, e.g., contributing to the development of more severe infections.

On the other hand, AGV2 may be non pathogenic for chickens; moreover, there may be differences in pathogenicity depending on the variants infecting a particular bird or flock (breed or age related, for instance). Besides, chickens may harbor more than one AGV2 variant, though in the present study only one variant could be amplified from each bird sampled; this, however, may be consequent to the methodology employed, where perhaps other variants might have been missed in a particular sample. Experiments will be performed in the near future to examine such possibilities.

4.6 Conclusions

AGV2 seems fairly widespread among chickens in Southern Brazil; it can also be detected in chickens in the Netherlands, suggesting a worldwide distribution of the agent. Nucleotide sequence analysis of the putative VP2- VP3 coding region of AGV2 showed a substantial degree (up to 15.8%) of sequence variability among variants. The occurrence of such variants may be related to differences in geographic distribution as well as to differences in virulence. Futures studies must be conducted to determine whether infection of chickens with AGV2, or its variants, plays some role in the appearance of disease in poultry, alone or in association with other chicken pathogens.

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Isolate	Health status	Country
3_HOL	Brain lesions	The Netherlands
8_HOL		
14_HOL		
15_HOL		
20_HOL		
SM_BRA		Brazil
5SC_BRA	Healthy	
8SC_BRA		
12SC_BRA		
14SC_BRA		
15SC_BRA		
20SC_BRA		
22SC_BRA		
1RS_BRA		
2RS_BRA		
3RS_BRA		
4RS_BRA		
A6RS_BRA		
A12RS_BRA		
B4RS_BRA		
B5RS_BRA		
B7RS_BRA		
B8RS_BRA		
4SC_BRA		
068_11	Weight loss	
AGV2_N1		
AGV2_N5		

Fig. 1. Sequences used in the present study for the amino acid alignment and phylogenetic analysis.

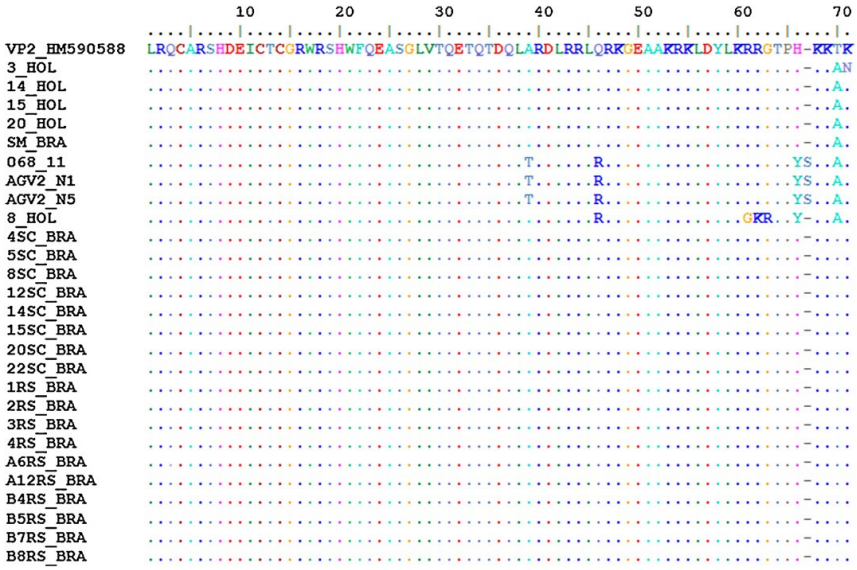

Fig. 2. Comparison of the amino acid sequences of VP2 of AGV2 obtained from different clinical samples of chickens. VP2_HM590588 corresponds to the VP2 region of the first sequence of AGV2 deposited in NCBI, under this accession number. Samples 3_HOL; 8_HOL; 14_HOL; 15_HOL and 20_HOL were obtained from chickens from the Netherlands displaying brain lesions. SM_BRA sample was obtained from a chicken with brain lesions in Brazil. Samples 068_11; AGV2_N1 and AGV2_N5 were obtained from animals displaying apathy and weight loss in Southern Brazil and samples 4SC_BRA to B8RS_BRA were obtained from healthy chickens in Southern Brazil.

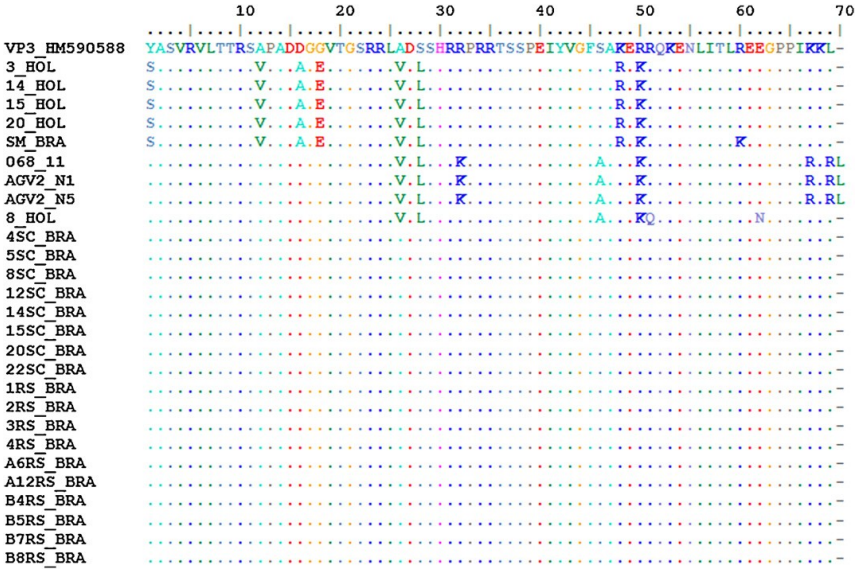

Fig. 3. Comparison of the amino acid sequences of VP3 of AGV2 obtained from different clinical samples of chickens. VP3_HM590588 corresponds to the VP3 region of the first sequence of AGV2 deposited in NCBI, under this accession number. Samples 3_HOL; 8_HOL; 14_HOL; 15_HOL and 20_HOL were obtained from chickens from the Netherlands displaying brain lesions. SM_BRA sample was obtained from a chicken with brain lesions in Brazil. Samples 068_11; AGV2_N1 and AGV2_N5 were obtained from animals displaying apathy and weight loss in Southern Brazil and samples 4SC_BRA to B8RS_BRA were obtained from healthy chickens in Southern Brazil.

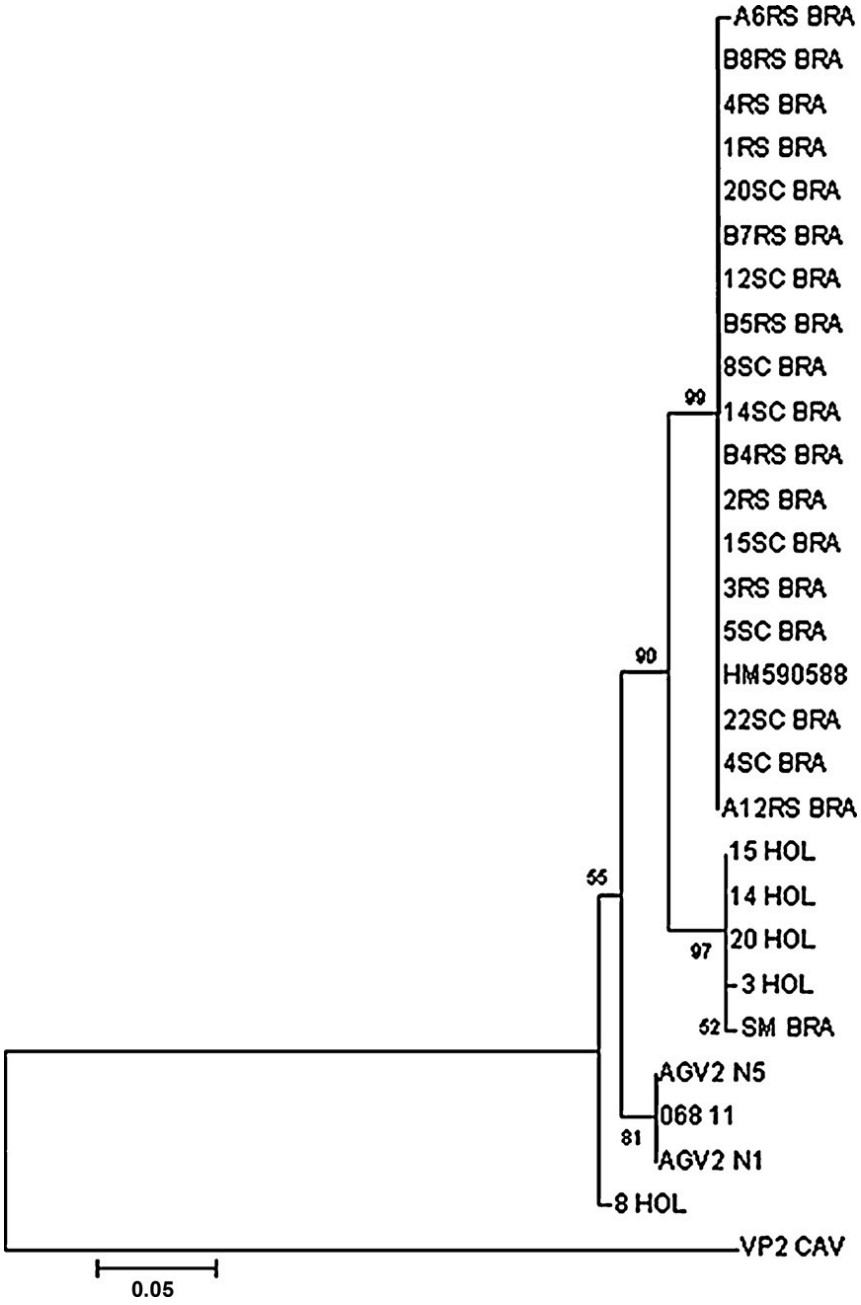

Fig. 4. Phylogenetic analysis of the AGV2 sequences obtained from chickens from Southern Brazil and the Netherlands. The analysis was performed by Neighborn-Joining method, with 500 boostraps and kimura-2 parameter, in MEGA 5.0 software. A sequence of CAV VP2 was used as an outgroup.

6 CONSIDERAÇÕES FINAIS

Neste estudo foi apresentado o relado de um novo agente viral, o girovírus aviário tipo 2, aspectos que visaram contribuir para um melhor entendimento da biologia deste agente para a verificação da presença deste agente em regiões distintas geograficamente.

No primeiro estudo, foi detectado um genoma viral de aproximadamente 2,4 kb, em soros de frangos comerciais. A subsequente digestão parcial com as enzimas de restinção *Bam*HI e *Hind*III, foi possível observar um segmento de DNA que apresentava tamanho semelhante ao do genoma do CAV. Análise das sequências revelou tratar-se de um vírus semelhante ao CAV, porém com um nível de similaridade genômica muito baixo, de cerca de aproximadamente 40%.

O novo genoma apresentou uma organização estrutural semelhante ao do CAV: com uma região não traduzida com cerca de 400 nt seguido de três ORFs parcialmente sobrepostas, que codificam três proteínas VP1, VP2 e VP3. A identidade entre esses aminoácidos frente ao CAV foi de 38,8%, 40,3% e 32,2%, respectivamente. Esta diferenciação justifica taxonomicamente a identificação de uma nova espécie dentro do gênero. Propondo neste capítulo um novo membro para o gênero *Gyrovirus* denominado girovírus aviário tipo 2, ou "avian gyrovirus type 2" (AGV2).

Buscando determinar a ocorrência desse novo agente em regiões geograficamente distintas, fez-se necessário investigar a presença do mesmo em amostras de outras regiões. Com esse objetivo, foi desenvolvida uma PCR específica para detectar o genoma do AGV2 em amostras de aves na região Sul do Brasil e da Holanda. Nesta amostragem, 90,7% (98/108) das aves provenientes do Estado do Rio Grande do Sul e 60,4% (29/48) das aves provenientes de Santa Catarina revelaram estar infectadas com o AGV2. Nas amostras provenientes da Holanda 42,9% (9/21) continham genomas de AGV2.

Vinte e sete amplicons, foram sequenciados, sendo 14 amostras do estado do Rio Grande do Sul, oito de Santa Catarina e cinco da Holanda. As sequências obtidas foram comparadas com a primeira sequência originalmente obtida e depositada no NCBI sob o número HM590588 (RIJSEWIJK et al., 2011). Dezoito sequências, obtidas de frangos saudáveis do sul do Brasil, foram identicas entre si e similares a sequência HM590588. Outras três sequências brasileiras, obtidas a partir de frangos apresentando apatia e

perda de peso eram claramente diferentes das sequencias provenientes de animais saudáveis.

Interesantemente, uma amostra brasileira e quatro amostras holandesas, provenientes de aves com lesões cerebrais, foram similares entre si. No entanto, a outra sequência holandesa, apresentou 8,7% de diferenças nucleotídicas relativamente à sequência de aves saudáveis e de 11,6% diferenças de nucleótidos em relação à outras quatro sequências holandesas. A análise filogenética desvendou que os genomas examinados poderiam ser divididos em três grupos, com base em diferenças de nucleotídeos, sugerindo a presença de variantes para o AGV2.

Embora até o presente momento, não tenha sido possível estabelecer uma associação concreta entre a ocorrência de lesões e infecções por AGV2, os estudos aqui apresentados, servirão como base para futuras investigações buscando determinar correlaçções entre infecções por eses agentes e a ocorrência de sinais patológicos em aves, bem como sua importância e o possível impacto econômico dos mesmos sobre a cadeia produtiva de aves.

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