UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

Aplicações de Metagenômica Viral em Medicina Veterinária: Análise de Viromas

Autora: Mariana Soares da Silva

Orientador: Prof. Dr. Cláudio Wageck Canal

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Trabalho apresentado como requisito parcial para obtenção do grau de Doutora em Ciências Veterinárias na área de Medicina Veterinária, subárea Medicina Veterinária Preventiva, especialidade Virologia.

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Aplicações de Metagenômica Viral em Medicina Veterinária: Análise de Viromas

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DEDICATÓRIA

Dedico esta tese ao meu marido Bruno, pelo apoio incondicional.

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Agradeço, primeiramente a minha mãe, por ser um exemplo de força e mulher, assim como ao meu pai (*in memoriam*) por ter me incentivado todos os dias a seguir o que realmente me fizesse feliz. À toda minha família, em especial meus irmãos e sobrinhos.

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RESUMO

As novas abordagens metagenômicas virais permitem a detecção imparcial de uma ampla gama de agentes infecciosos de maneira independente da cultura, facilitando o diagnóstico e, consequentemente, o monitoramento de doenças, o que está intimamente atrelado ao conceito "*One World, One Health*". Além disso, abrem possibilidades para análises genéticas comparativas e enriquecimento de bancos de dados genômicos. O gênero Hepacivirus (família Flaviviridae) é um exemplo expressivo de gênero viral que cresceu rapidamente com o advento da metagenômica. Desde 1996, quando o gênero Hepacivirus foi criado, o vírus da hepatite C (HCV) era a única espécie conhecida. No entanto, os hepacivírus (HVs) têm sido detectados em diversos animais domésticos e selvagens, incluindo, equinos, cães, roedores, morcegos, bovinos, tubarões, entre outros. O objetivo geral desta tese foi aprofundar o conhecimento acerca da ecologia viral em diferentes hospedeiros, através da metagenômica na ciência veterinária, destacando diferentes subáreas em que a mesma pode ser empregada. O Capítulo 1 aborda a detecção de um hepacivírus bovino (HNV) através da plataforma de sequenciamento de alto desempenho, Illumina MiSeq. As análises revelaram uma sequência com alta divergência nucleotídica quando comparada aos demais HNVs conhecidos mundialmente. Além disso, baseado na classificação proposta na literatura, trata-se de um provável novo genótipo. Esse estudo, intitulado "*Highly Divergent Cattle Hepacivirus N in Southern Brazil*" foi publicado em 2019, na revista científica *Archives of Virology*. O Capítulo 2 trata-se de dois estudos de metagenômica realizados para análise de viroma. O primeiro intitulado "*New Polyomavirus in Nutria, Myocastor coypus Polyomavirus 1*" no qual obtivemos o sequenciamento de genoma completo de uma nova espécie de PyVs em ratão-do-banhado, pertencente ao gênero *Alphapolyomavirus*, que foi publicado na revista científica Archives of Virology. No segundo estudo, "*Liver Virome of Healthy Pigs Reveals Diverse Small ssDNA Viral Genomes*", foi analisado o viroma de figados de suínos de um abatedouro. Nesse estudo detectamos, pela primeira vez no Brasil, a presença de alguns vírus como *Porcine Circovirus 1* e *Porcine Parvovirus 6* e *7*, além da ausência de vírus potencialmente zoonóticos. A pesquisa foi publicada na revista científica *Infection, Genetics and Evolution* em 2020. Concluindo, a metagenômica viral foi aplicada com sucesso na investigação de três importantes estudos para a ciência veterinária. Os resultados contribuíram com a expansão do conhecimento na área, através da descrição e caracterização de novos e conhecidos vírus, além de enriquecer os bancos de dados genômicos, provendo informação para futuras pesquisas.

ABSTRACT

The viral metagenomic approaches, a culture-independent technique, allow the impartial detection of a wide range of pathogens, improving the diagnosis and, consequently, the monitoring of diseases, which is closely linked to the "One World, One Health" concept. Furthermore, open possibilities for comparative genetic analysis and genomic databases enrichment. Hepacivirus genus (family Flaviviridae) is an expressive example of a viral genus that grew with the advent of metagenomics. Since 1996, when the genus Hepacivirus was created, the hepatitis C virus (HCV) was the only known species. However, hepaciviruses (HVs) have been detected in several domestic and wild animals, including, horses, dogs, rodents, bats, cattle, sharks, among others. The main objective of this thesis was improving the knowledge of different hosts viral ecology through metagenomics in veterinary science, highlighting different sub-areas in which it can be applied. Chapter 1 addresses bovine hepacivirus (HNV) detection through the high-throughput sequencing (HTS), using Illumina MiSeq. The analyzes revealed a sequence with high nucleotide divergence when compared to HNVs worldwide known. In addition, based on the ICTV classification, it is a putative new genotype. This study, entitled "Highly Divergent Cattle Hepacivirus N in Southern Brazil" was published in 2019, in the scientific journal Archives of Virology. Chapter 2 is about two metagenomics studies performed for virome analysis. The first one entitled "New Polyomavirus in Nutria, Myocastor coypus Polyomavirus 1" in which we obtained a new PyVs complete genome species of in nutria, belonging to the genus Alphapolyomavirus, which was published in the scientific journal Archives of Virology. In the second research, "Liver Virome of Healthy Pigs Reveals Diverse Small ssDNA Viral Genomes", the swine liver virome of a slaughterhouse was analyzed. In this study, we detected, for the first time in Brazil, the presence of some viruses such as Porcine Circovirus 1 and Porcine Parvovirus 6 and 7, in addition to the absence of potentially zoonotic viruses. In conclusion, viral metagenomics has been successfully applied in important veterinary science studies. The results contributed to the knowledge expansion in the area, through the description and characterization of new and known viruses, in addition to enriching the genomic databases, providing information for future research.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

aa	Aminoácidos
APPV	Pestivírus Atípico Porcino
C	Core
CoV	Coronavírus
DNA	Ácido desoxirribonucleico
E	Envelope
HAV	Hepacivírus A ou Equino
HAV	Hepacivírus A ou Equino
HCV	Vírus da Hepatite C
HEV	Vírus da Hepatite E
HNV	Hepacivírus N
HVs	Hepacivírus
ICTV	International Committee on Taxonomy of Virus
NS	Não estrutural
ORF	Região aberta de leitura
PCR	Reação em Cadeia da Polimerase
PRRSV	Síndrome Respiratória e Reprodutiva Porcina
PyVs	Poliomavírus
RNA	Ácido ribonucleico
rRNA	RNA ribossomal
SARS	Síndrome Respiratória Aguda Grave

SUMÁRIO

1 INTRODUÇÃO

A metagenômica refere-se ao estudo dos metagenomas, uma análise baseada na sequência genômica coletiva em uma dada amostra (RIESENFELD; SCHLOSS; HANDELSMAN, 2004). Incialmente utilizada em estudos ecológicos, atualmente, a metagenômica é vista como uma importante ferramenta de diagnóstico e de pesquisa. Isso porque a abordagem permite a descoberta de novas espécies de vírus, bactérias e fungos (MOKILI *et al.*, 2013) e pode ser fundamental na identificação de agentes causadores de surtos (HOFFMANN *et al.*, 2012).

O surgimento de novos agentes infecciosos desafiam os esforços de vigilância laboratorial, como visto recentemente na pandemia de SARS-CoV-2, em que a metagenômica viral foi essencial para a rápida caracterização do agente (CARBO *et al.*, 2020). Com a importante característica de ser uma técnica independente de cultivo, fornece à comunidade científica ferramentas para explorar a virosfera, com capacidade para aprofundar os conhecimentos sobre a relevância, diversidade e evolução virais (DELWART, 2007; KWOK *et al.*, 2020).

Para que haja um embasamento na análise de novos agentes, também se faz necessário investigar a comunidade viral em reservatórios e vetores em potencial (BLOMSTRÖM, 2011). Os estudos de viromas abriram possibilidades para análises comparativas da composição genética de muitas amostras e para a detecção de novos vírus (BELÁK *et al.*, 2013). Esses estudos têm sido realizados em humanos (OGILVIE; JONES, 2015) e nas mais diferentes espécies animais, como bovinos (BAUERMANN *et al.*, 2017; MASUDA *et al.*, 2014; SCHLOTTAU *et al.*, 2016), aves (BOROS *et al.*, 2012, 2018; CHEN *et al.*, 2013), roedores (WU *et al.*, 2018), animais selvagens (A DUARTE *et al.*, 2019), suínos (CHEUNG *et al.*, 2014; HUANG *et al.*, 2010; PALINSKI; MITRA; HAUSE, 2016; YU *et al.*, 2014), entre outros.

O gênero *Hepacivirus*, pertencente à família *Flaviviridae* (ICTV) (SMITH *et al.*, 2016), é um exemplo clássico de gênero viral que cresceu rapidamente com o emprego de ferramentas metagenômicas. Desde 1996, quando o gênero *Hepacivirus* foi criado, o HCV era a única espécie conhecida. No entanto, os hepacivírus (HVs) têm sido detectados em diversos animais domésticos e selvagens, incluindo, equinos, cães, roedores, morcegos, bovinos, tubarões, patos, bicho-preguiça, esquilos e lêmures (AMIT *et al.*, 2013; BEXFIELD *et al.*, 2014; CANAL *et al.*, 2017; CANUTI *et al.*, 2019; CHU *et al.*, 2019; LI *et al.*, 2019; LUKASHEV *et al.*, 2013; QUAN *et al.*, 2012; RAMSAY *et al.*, 2015), e, recentemente, até em mosquitos do gênero *Culex* (WILLIAMS *et al.*, 2020).

O hepacivírus bovino, oficialmente chamado de Hepacivírus N (HNV), foi detectado em diversos países, como Alemanha (BAECHLEIN *et al.*, 2015), Ghana (CORMAN *et al.*, 2015), Brasil (CANAL *et al.*, 2017; DA SILVA *et al.*, 2018), China (LU *et al.*, 2018) e Itália (ELIA *et al.*, 2020), e tem sido caracterizado em diferentes genótipos e subtipos (DA SILVA *et al.*, 2018; ELIA *et al.*, 2020; LU *et al.*, 2019).

Utilizando técnicas *state of the art* para a detecção e identificação viral, nessa tese três estudos foram desenvolvidos. Primeiramente (Capítulo 1), um genoma parcial de HNV foi identificado em uma amostra de bovino do estado do Rio Grande do Sul. As análises revelaram uma sequência com alta divergência nucleotídica quando comparada aos demais HNVs conhecidos mundialmente. Além disso, baseado na classificação proposta na literatura (DA SILVA *et al.*, 2018; LU *et al.*, 2019), trata-se de um provável novo genótipo. Esse estudo, intitulado "*Highly Divergent Cattle Hepacivirus N in Southern Brazil*" foi publicado em 2019, na revista científica *Archives of Virology*.

O Capítulo 2 aborda dois estudos de metagenômica viral realizados para análise de viromas em animais de produção. No primeiro estudo, amostras de figados suínos, utilizados para o preparo de embutidos, foram coletadas em um abatedouro localizado em Nova Santa Rita, no

Rio Grande do Sul. No artigo intitulado *"Liver Virome of Healthy Pigs Reveals Diverse Small ssDNA Viral Genomes",* o viroma das amostras foi analisado com o objetivo principal de mapear possíveis agentes zoonóticos que poderiam ser fontes de infecção para os humanos. Nesse estudo, detectamos, pela primeira vez no Brasil, a presença de alguns vírus como Circovírus Suíno 1 (PCV1) e Parvovírus Suíno 6 (PPV6) e 7 (PPV7), além da ausência de vírus potencialmente zoonóticos. A pesquisa foi publicada na revista científica *Infection, Genetics and Evolution,* em 2020. No segundo estudo, intitulado *"New Polyomavirus in Nutria, Myocastor coypus Polyomavirus 1"*, obtivemos o sequenciamento de genoma completo de uma nova espécie de Poliomavírus (PyVs) em ratão-do-banhado, pertencente ao gênero *Alphapolyomavirus,* que foi publicado na revista científica *Archives of Virology.*

O objetivo geral desta tese foi aprofundar o conhecimento acerca da ecologia viral em diferentes hospedeiros, através da metagenômica na ciência veterinária, destacando diferentes subáreas em que a mesma pode ser empregada. Os resultados contribuíram com a expansão do conhecimento na área, através da descrição e caracterização de novos e conhecidos vírus, além de enriquecer os bancos de dados genômicos, provendo informação para futuras pesquisas.

2 REVISÃO BIBLIOGRÁFICA

2.1 Metagenômica

A metagenômica é uma importante ferramenta na descoberta de novos micro-organismos, através da análise da totalidade do material genômico de uma comunidade a partir de amostras biológicas ou ambientais (CASTRIGNANO; NAGASSE-SUGAHARA, 2015). Estima-se que existam em torno de 10^{30} células bacterianas e 10^{31} partículas virais habitando nosso planeta (MUSHEGIAN, 2020). O estudo dessa grande diversidade microbiana torna-se limitado pelos métodos convencionais de detecção, tendo em vista que menos de 1% dos micro-organismos são, de fato, cultiváveis (RIESENFELD; SCHLOSS; HANDELSMAN, 2004). Assim, com o advento das técnicas de metagenômica, independentes de cultivo, os microbiomas de diferentes nichos vêm sendo descobertos (CANI, 2018; PAYNE *et al.*, 2017).

O termo metagenoma foi utilizado pela primeira vez em 1998 (HANDELSMAN *et al.*, 1998) em referência a uma análise do conteúdo gênico de todos os micro-organismos presentes em uma amostra do solo. A partir de então, o termo tem sido usado para nomear o conjunto de genomas obtidos diretamente de um ambiente ou amostra clínica, enquanto que o termo metagenômica refere-se ao estudo desses dados (WOOLEY; GODZIK; FRIEDBERG, 2010). Fornecendo a capacidade de examinar a relação entre estrutura e função gênica em diferentes espécies, esses dados abriram espaço no campo da genômica comparada e da biologia de sistemas (RIESENFELD; SCHLOSS; HANDELSMAN, 2004; WOOLEY; GODZIK; FRIEDBERG, 2010).

A metagenômica mostra-se útil na descoberta de novas espécies e cepas de agentes microbianos (MOKILI *et al.*, 2013), na identificação de causadores de surtos (HOFFMANN *et al.*, 2012) e na investigação de doenças complexas (CHO; BLASER, 2012). Além disso, devido à diminuição dos custos das tecnologias de sequenciamento de alto desempenho (NGS) de DNA

e às ferramentas avançadas de bioinformática, uma rápida evolução na ciência da metagenômica foi desencadeada (KUMAR *et al.*, 2015). Entretanto, embora represente uma poderosa ferramenta para explorar a diversidade microbiana, um dos principais desafios consiste na análise das sequências obtidas (SHARPTON, 2014). Os dados produzidos pelas plataformas de HTS correspondem a imensos conjuntos de sequências curtas de leitura (*reads*), o que dificulta a montagem e anotação dos genomas. Em razão disso, recursos computacionais, assim como o uso de softwares de bioinformática são requeridos para obter a informação de interesse (SCHOLZ; LO; CHAIN, 2012).

2.1.1 Metagenômica Viral

Os vírus são as entidades biológicas mais abundantes no planeta, provavelmente infectando todos os organismos celulares e até mesmo outros vírus (ZHANG *et al.*, 2019). A metagenômica viral refere-se ao estudo do genoma de todos os vírus existentes em amostras ambientais (WINTER *et al.*, 2014) ou biológicas (SHAN *et al.*, 2011) - denominados viromas -, ou ainda na identificação do genoma de um agente viral responsável por uma doença específica ou pelo efeito citopático que causa em cultura de células (HOFFMANN *et al.*, 2012). Com todas as possibilidades que a técnica oferece, os estudos têm ajudado a expandir o conhecimento das interações vírus-hospedeiro, da biologia evolutiva e ainda passaram a auxiliar na vigilância de patógenos, assim como na descoberta de agentes responsáveis por novos surtos (DELWART, 2007; ZHANG *et al.*, 2014).

No entanto, ao contrário dos organismos celulares, os vírus não compartilham um único gene que possa ser alvo de reações baseadas em PCR, como por exemplo, gene ribossomal 16s (rRNA), em bactérias (WANG; QIAN, 2009). Além disso, há muitas famílias virais que compartilham de uma baixa identidade genética (SMITH *et al*., 2016). Por essas razões, torna-se impossível desenvolver ensaios de PCR "*pan-viral*" para detecção de todos os vírus dentro de uma determinada amostra. Ao contrário dos testes de diagnóstico viral que se baseiam no uso de *primers* e/ou anticorpos, a metagenômica viral não requer conhecimento prévio dos agentes virais presentes nas amostras investigadas. A técnica visa fornecer a composição genética completa de populações virais de uma amostra de forma imparcial (DELWART, 2007a; HANDELSMAN, 2005), além de não depender de isolamento viral por cultivo celular.

Como exemplo da grande contribuição da metagenômica, os vírus de RNA são um grupo viral que teve seu conhecimento bastante expandido. Os estudos revelaram notáveis níveis de diversidade entre eles; e os vírus descritos recentemente (pertencentes a novas espécies, gêneros, famílias e ordens) (HARVEY *et al.*, 2018; WANG; HAN, 2018) também ajudaram a preencher lacunas entre famílias já conhecidas, mostrando que a virosfera dos vírus de RNA é diferente do que se pensava com base em agentes cultiváveis ou causadores de doenças (Figura 1) (ZHANG; SHI; HOLMES, 2018).
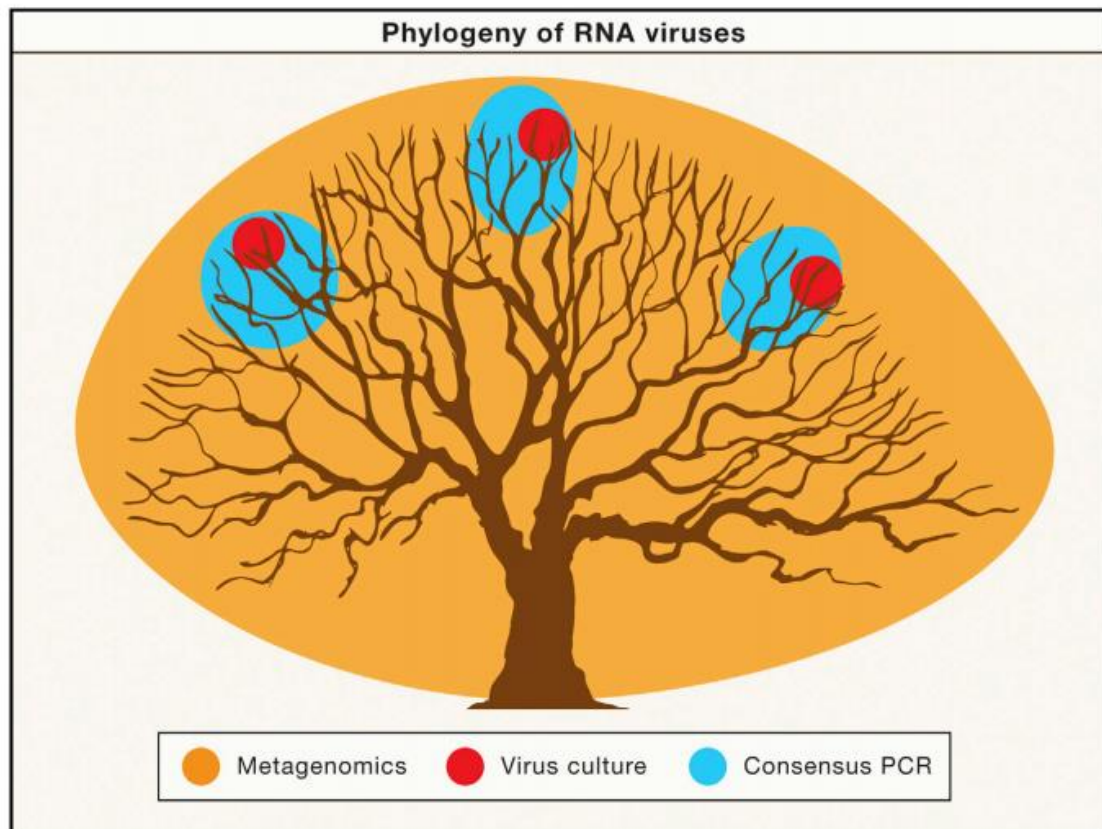

Figura 1 – Abrangência de detecção da metagenômica em relação aos métodos convencionais. A árvore representa a diversidade filogenética dos vírus de RNA. Os círculos coloridos ilustram a extensão de vírus que podem ser descobertos com o uso de três abordagens: cultivo celular, PCR e metagenômica. Fonte: adaptado de (CONCEIÇÃO-NETO *et al.*, 2015).

O estudo de viromas tem gerado dados para um gama extremamente variável de amostras, como amostras ambientais (PRATAMA; VAN ELSAS, 2018; ZEIGLER ALLEN *et al.*, 2017), fezes humanas e de animais (MORENO *et al.*, 2017; SHAN *et al.*, 2011; SIQUEIRA *et al.*, 2018), alimentos de origem animal (CIBULSKI *et al.*, 2021), órgãos (OGILVIE; JONES, 2015), trato respiratório (MITRA *et al.*, 2016; WYLIE, 2017), entre outros. Os viromas realizados em animais domésticos e selvagens sujeitos à mudanças ambientais, condições de superlotação ou em íntima relação com o ser humano são de grande valia para o monitoramento de vírus conhecidos e descoberta de novos agentes (DELWART, 2007a).

Em resumo, a metagenômica viral e as ferramentas para a análise dos dados produzidos, são importantes e refinadas abordagens que permitem a detecção e descrição continuada de novos vírus. Isso acarreta a uma melhor compreensão das doenças novas e "antigas", bem como a um maior conhecimento das funções dos diferentes vírus em humanos e animais (CASTRIGNANO; NAGASSE-SUGAHARA, 2015; MILLER *et al.*, 2013; TEMMAM *et al.*, 2014).

2.1.1.1 *Preparo de Amostras para Estudos de Metagenômica Viral*

A preparação das amostras para os estudos de metagenômica viral exige algumas etapas que objetivam garantir a detecção de ácidos nucleicos virais. Uma dessas etapas é o processamento da amostra para a concentração de partículas virais que inclui filtração, ultracentrifugação e remoção enzimática dos ácidos nucleicos desprovidos de capsídeo (DÁVILA-RAMOS; CASTELÁN-SÁNCHEZ; MARTÍNEZ-ÁVILA, 2019; DELWART, 2007). Devido à alta concentração de DNA requerida para o HTS, outra etapa importante inclui a amplificação randômica dos fragmentos genômicos extraídos da amostra, possibilitando acessar, inclusive, os vírus presentes em menor quantidade. (DÁVILA-RAMOS; CASTELÁN-SÁNCHEZ; MARTÍNEZ-ÁVILA, 2019; KOHL; NITSCHE; KURTH, 2016; WOOLEY;

GODZIK; FRIEDBERG, 2010). Na Figura 2 podemos observar um resumo de fluxograma do preparo de amostras para metagenômica viral.
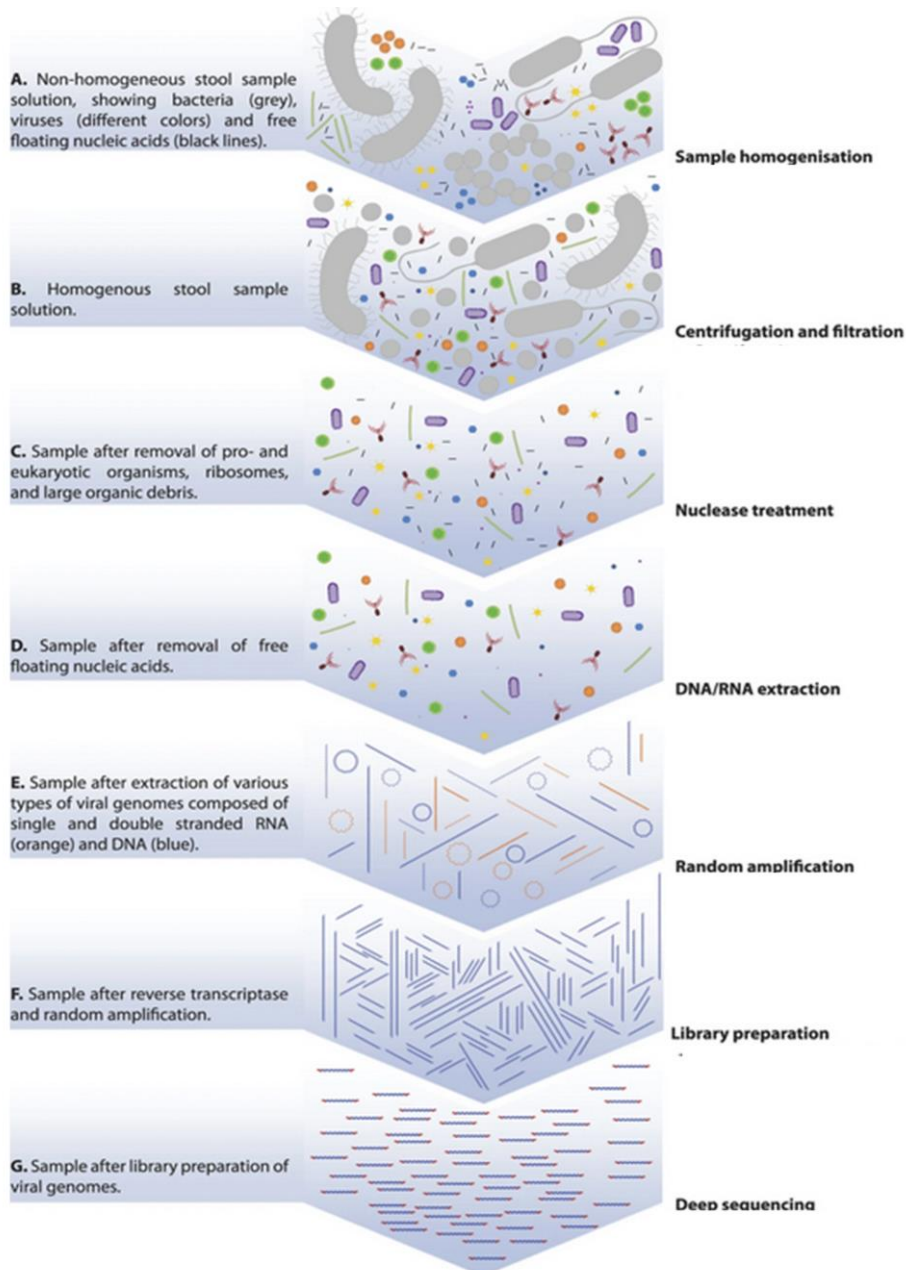

Figura 2 - Esquema proposto por Conceição-Neto *et al*. para preparo de amostras para metagenômica viral.

2.1.2 Metagenômica Viral na Medicina Veterinária

Doenças infecciosas emergentes surgem constantemente na população humana e animal. Uma significativa porcentagem dessas doenças são zoonóticas (em torno de 70%), causadas por vírus de origem animal (WANG; CRAMERI, 2014). Com a expansão geográfica dos seres humanos e o aumento da proximidade com reservatórios de vida selvagem, a probabilidade de novas doenças que podem cruzar a barreira de hospedeiros animais e humanos, aumenta (CIBULSKI; DE LIMA; ROEHE, 2020; JONES *et al.*, 2008). Além disso, os vetores artrópodes também desempenham um papel, muitas vezes necessário, no ciclo e propagação de uma doença, como observado na Doença do Oeste do Nilo e na Doença da Língua Azul (WEAVER; REISEN, 2010).

A metagenômica viral abriu possibilidade para a rápida detecção de agentes em surtos, como na detecção do vírus Schmallenberg, um novo orthobunyavirus na Europa, com grande importância epidemiológica (HOFFMANN *et al.*, 2012). A técnica também levou ao sequenciamento rápido, com ótimo custo-benefício de genomas completos do vírus da gripe aviária (CROVILLE *et al.*, 2012), vírus da peste suína clássica (LEIFER; RUGGLI; BLOME, 2013) e vírus da língua azul (RAO *et al.*, 2013).

Diferentes espécies de vírus de RNA, incluindo o vírus da síndrome respiratória e reprodutiva porcina (PRRSV) e o pestivírus atípico porcino (APPV) têm sido detectados em amostras clínicas de suínos utilizando HTS (SUTTON *et al.*, 2019; TAN; DVORAK, 2019). Outras questões científicas também são esclarecidas com o uso de metagenômica, como estudos de Influenza A que mostraram uma variabilidade genética a nível de *quasiespécie* com implicações na reposta imune (HOPER *et al.*, 2012), resistência a antivirais (WU *et al.*, 2013) e patogenicidade (YOUK *et al.*, 2019).

Novos vírus vêm sendo descobertos nas diferentes espécies animais como bovinos (BAUERMANN *et al.*, 2017; DAUDT *et al.*, 2019; MASUDA *et al.*, 2014; SCHLOTTAU *et*

al., 2016), pequenos ruminantes (PFAFF *et al.*, 2017; REUTER *et al.*, 2012), aves (BOROS *et al.*, 2012, 2018; CHEN *et al.*, 2013), roedores (WU *et al.*, 2018), animais selvagens (A DUARTE *et al.*, 2019), suínos (CHEUNG *et al.*, 2014; HUANG *et al.*, 2010; PALINSKI; MITRA; HAUSE, 2016; YU *et al.*, 2014), entre outros (Tabela 1). A maioria dos estudos de metagenômica animal disponíveis na literatura foram realizados em suínos, o que pode ser explicado pela importância da suinocultura no mundo e por agentes virais emergentes como o vírus da Diarreia Epidêmica Suína (PEDV) (CHEN *et al.*, 2018) e o vírus responsável pela Peste Suína Africana (PSA) (RAO *et al.*, 2013).

Tabela 1 - Exemplos de novos vírus detectados por metagenômica na medicina veterinária.

Espécie Animal	Novo Vírus (metagenômica)	Referência
Bovinos	Astrovírus	SCHLOTTAU *et al.*, 2016
	Rotavírus	MASUDA *et al.*, 2014
	Papillomavírus	BAUERMANN *et al.*, 2017 DAUDT, et al., 2019
Ovinos	Astrovírus	PFAFF *et al.*, 2017
Aves	Coronavírus	CHEN *et al.*, 2013
	Picobirnavírus	PANKOVICS *et al.*, 2018
	Picornavírus	BOROS *et al.*, 2012
Suínos	Astrovírus	YU *et al.*, 2013
	Bocavírus	BLOMSTROM *et al.*, 2009
	Circovírus	CHEUNG *et al.*, 2014
	Parvovírus	HUANG *et al.*, 2010

Sabe-se que produtos cárneos e seus derivados podem ser fonte de infecções entéricas em humanos, através da contaminação da a carne durante o abate ou através da incompleta cocção de alguns alimentos (ZHANG *et al.*, 2014). O vírus da Hepatite E (HEV) em suínos é um exemplo de vírus que pode ser transmitido através do consumo de carne suína malpassada e embutidos (BERTO *et al.*, 2012). Devido a essa preocupação, principalmente em se tratando de saúde pública, a metagenômica torna-se uma excelente ferramenta para monitorar produtos de origem animal (CIBULSKI *et al.*, 2021; ZHANG *et al.*, 2014).

Os estudos metagenômicos aplicados a medicina veterinária estão claramente atrelados ao conceito "*One World, One Health*", que tem sido constantemente enfatizado e discutido (WANG; CRAMERI, 2014). Sendo assim, essa abordagem busca aumentar a comunicação e colaboração entre diferentes disciplinas, como medicina humana, medicina veterinária, saúde pública, microbiologia, genética, entre outros (DELWART, 2007b; KUBACKI; FRAEFEL; BACHOFEN, 2020; TEMMAM *et al.*, 2014).

2.1.3 Bioinformática Aplicada à Metagenômica Viral

A detecção de vírus conhecidos e desconhecidos é hoje realizada rotineiramente através da metagenômica viral. Devido à velocidade de sequenciamento e a diminuição dos custos, as tecnologias de sequenciamento, como HiSeq (Illumina), SOLiD (ABI) e Ion Torrent Proton (Life Technologies), a bioinformática é hoje a parte mais importante, e cada vez mais exigente, na análise metagenômica (BZHALAVA, 2013; ZHANG; SHI; HOLMES, 2018).

Interpretar informações significativas de milhões de novas sequências genômicas apresenta um sério desafio para os bioinformatas. Em micro-organismos cultivados, os dados genômicos vêm de um único clone facilitando a montagem e a anotação das sequências. Em metagenômica, os dados vêm de comunidades microbianas heterogêneas, às vezes contendo mais de 10.000 espécies. Da amostragem à montagem, anotação de genes e previsão de funções,

a bioinformática enfrenta novas demandas na interpretação de dados (WOOLEY; GODZIK; FRIEDBERG, 2010).

As etapas básicas para a análise dos dados obtidos de HTS são avaliação da qualidade das *reads*, montagem, classificação taxonômica e anotação. Uma série de plataformas e softwares computacionais são utilizados visando obter a identificação de milhares de espécies em um tempo razoável (CINGOLANI; SLADEK; BLANCHETTE, 2015). A verificação da qualidade das *reads* é essencial antes de qualquer montagem ou análise. Dependendo da tecnologia de sequenciamento, as taxas de erro variam e devem ser avaliadas. O escore *Phred* (Tabela 2) é utilizado para estimar a probabilidade de erro de cada nucleotídeo, e classifica as *reads* de acordo com a acurácia na inserção das bases (BZHALAVA, 2013). Uma ferramenta empregada para essa análise chama-se FastQC (ANDREWS, 2018).

Tabela 2 - Escore *Phred* para análise de qualidade de sequências.

Escore Phred	Probabilidade de base incorreta	Acurácia
10	1 em 10	90%
20	1 em 100	99%
30	1 em 1000	99.90%
40	1 em 10000	99.99%
50	1 em 100000	99.999%
60	1 em 1000000	99.9999%

A partir da seleção das *reads*, sequencias maiores podem ser montadas (*contigs*) (WOOLEY; GODZIK; FRIEDBERG, 2010). Duas formas são frequentemente empregadas para montagem de metagenomas: montagem guiada por referência e montagem *de novo* (MILLER *et al.*, 2013). Na montagem guiada por referência, os *contigs* são mapeados a sequencias referência, desde que estas, possuam suficiente identidade com as sequências geradas, o que pode dificultar a análise em casos de alta divergência genética. Na montagem *de novo*, patógenos ainda desconhecidos podem ser descobertos. Trata-se de um método para construir genomas a partir de um grande número de fragmentos de DNA curtos ou longos, as *reads* ou os *contigs*, sem nenhum conhecimento *a priori* da sequência ou ordem correta desses fragmentos (LISCHER; SHIMIZU, 2017). Assim, as sequências sobrepostas são mapeadas resultando na construção de uma sequência consenso de um genoma completo ou parcial (WOOLEY; GODZIK; FRIEDBERG, 2010) (Figura 3).
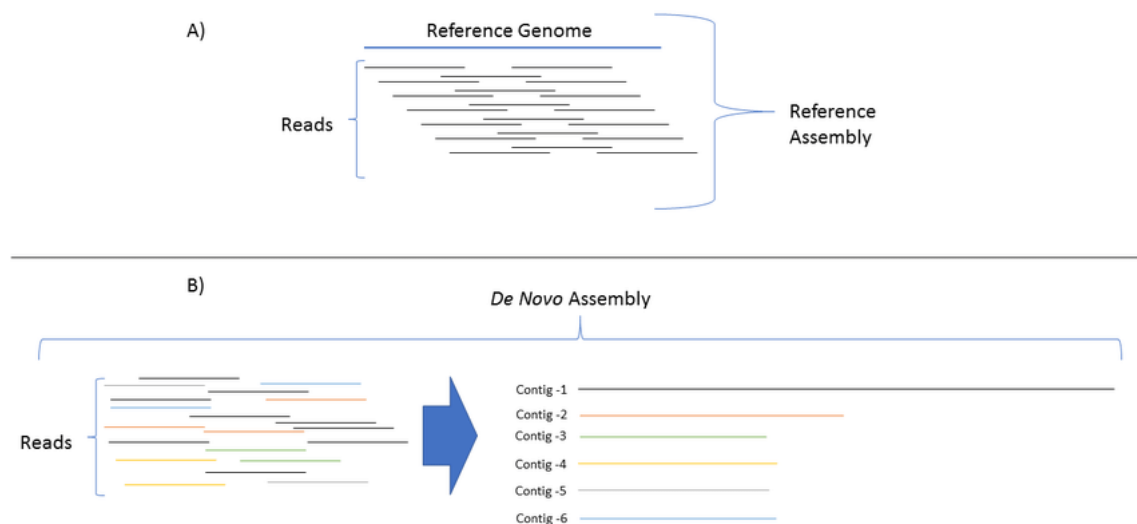
Figura 3 - Comparação entre montagens "*assembly*" utilizando genoma referência versus abordagem "*de novo*". Fonte: (NOUNE, 2012).

Para classificação taxonômica, a comparação das sequências obtidas com os bancos de dados públicos através de ferramentas de alinhamento, como o BLAST, que é a mais comumente empregada (CONESA; GOTZ, 2008; DEREEPER *et al.*, 2010). Assim, as sequências podem ser agrupadas de acordo com a identidade entre elas e em relação a dados já disponíveis (WOOLEY; GODZIK; FRIEDBERG, 2010).

A etapa seguinte consistirá na predição de regiões codificantes, seguida pela anotação gênica, através da identificação das regiões abertas de leitura (ORFs). Esta tarefa é altamente desafiadora tendo em vista que muitas ORFs são parciais e uma grande fração delas não tem homólogos já anotados disponíveis. Uma forma de realizar essas predições é por homologia em bancos de dados como Swiss-Prot e NCBI-nr (ESCOBAR-ZEPEDA; DE LEÓN; SANCHEZ-FLORES, 2015).

2.2 O gênero *Hepacivirus*

2.2.1 Biologia e classificação

O gênero *Hepacivirus* pertence à família *Flaviviridae*, que também inclui os gêneros *Flavivirus, Pestivirus* e *Pegivirus* (SIMMONDS *et al.*, 2017). São vírus pequenos, de aproximadamente 50 nm, envelopados e com genoma RNA fita simples de sentido positivo, com aproximadamente 9,5 kb. Hepacivírus (HVs) diferem de outros membros da família *Flaviviridae* por sua limitada multiplicação em cultivo celular (CHEVALIEZ; PAWLOTSKY, 2006). O gênero *Hepacivirus* cresceu consideravelmente nos últimos anos com o advento das plataformas de sequenciamento de alto desempenho e, atualmente, já foi detectado em diversas espécies animais (Figura 4), e tudo indica que a diversidade desse gênero é muito maior do que a reportada até o momento.

Figura 4. Árvore filogenética representativa do gênero *Hepacivirus*. Fonte: adaptado de (Williams *et al*., 2020).

A classificação atual divide o gênero em 14 espécies (A-N) (Tabela 3). Os mais recentes HVs ou *related*-HVs detectados (de baleias, patos, bichos-preguiça, mosquitos, entre outros) ainda não foram classificados. O HCV, por exemplo, pertence à espécie C e infecta humanos e, experimentalmente, chimpanzés (BILLERBECK *et al.*, 2013; BUKH, 2012), no entanto, o uso dessa espécie animal em pesquisas e experimentos é bastante restrita. HCV é atualmente classificado em oito genótipos (1-8) (BORGIA *et al*., 2018; SMITH *et al*., 2014) que possuem uma considerável heterogeneidade nas sequências genéticas, transmissão e distribuição geográfica. Na América Latina, os genótipos mais comumente detectados são o 1 e 2 (CRISTINA, 2005). Em média, analisando genomas completos, os genótipos diferem em nível de nucleotídeos em até 30%, e 20% em nível de aminoácidos. Dentro dos genótipos, existe uma classificação em subtipos que diferem entre 15-25% em nível de nucleotídeos (SMITH *et al*., 2014). Essa considerável diversidade genética é marcada pela baixa fidelidade da RNA polimerase RNA-dependente associada a altas taxas de replicação *in vivo*. No entanto, todas as variantes exibem propriedades biológicas similares, incluindo hepatotropismo, propensão à persistência e características de patogenicidade (HEDSKOG *et al*., 2015; SMITH *et al*., 2014).

Tabela 3. Classificação atual do gênero *Hepacivirus.*

Espécie	Denominação prévia	Hospedeiro
Hepacivírus A	Hepacivírus canino e equino	equinos e cães (?)
Hepacivírus B	GBV-B	primatas do novo mundo
Hepacivírus C	HCV	humanos
Hepacivírus D	Hepacivírus Guereza	primatas do Velho Mundo
Hepacivírus E	Hepacivírus de roedores	roedores
Hepacivírus F	Hepacivírus de roedores	roedores
Hepacivírus G	Hepacivírus de Rattus norvegicus 1	roedores do Velho Mundo
Hepacivírus H	Hepacivírus de Rattus norvegicus 2	roedores do Velho Mundo
Hepacivírus I	Hepacivírus de roedores	roedores do Velho Mundo
Hepacivírus J	Hepacivírus de roedores	roedores do Velho Mundo
Hepacivírus K	Hepacivírus de morcegos	morcegos do Velho Mundo
Hepacivírus L	Hepacivírus de morcegos	morcegos do Velho Mundo
Hepacivírus M	Hepacivírus de morcegos	morcegos do Velho Mundo
Hepacivírus N	Hepacivírus de bovinos	bovinos

Fonte: adaptado de (SMITH *et al.*, 2016b).

O Hepacivirus N (que infecta os bovinos), ainda que não cause alterações macro ou microscópicas sugestivas de infecção viral, apresenta carga viral hepática significativamente maior do que a encontrada no soro, evidenciando a sua característica hepatotrópica, como a encontrada no HCV (CORMAN *et al.*, 2015). Com relação aos Hepacivírus K, L e M (correspondentes às espécies virais detectadas em morcegos), apenas um estudo foi realizado até o momento, mas, devido à quantidade de espécies e animais testados, ele foi capaz de inferir que os morcegos podem ser os maiores hospedeiros de HVs (QUAN *et al.*, 2012). No entanto, acredita-se que pouco se sabe, até o presente momento, a respeito de suas características genéticas e biológicas.

2.2.2 Estrutura Genômica

Os genomas dos membros da família *Flaviviridae*, incluindo o gênero *Hepacivirus,* compartilham de uma estrutura muito semelhante. São compostos por duas regiões não traduzidas (UTRs) nas regiões 5' e 3' que desempenham importante função na tradução da poliproteína e na replicação do RNA viral. Além disso, a região 5'UTR é a mais conservada do genoma, sendo muito utilizada no diagnóstico, juntamente com a região da proteína NS3 (SMITH *et al.*, 2016). O genoma possui uma única fase aberta de leitura (ORF) que codifica uma poliproteína de aproximadamente 3000 aminoácidos (aa). Essa poliproteína, quando clivada por peptidases e proteases, origina ao menos dez proteínas: proteína estrutural core (C), duas proteínas do envelope (E1 e E2) e proteínas não estruturais relacionadas à montagem da partícula viral (p7 e NS3) e as não estruturais, envolvidas na replicação (NS3, NS4A, NS4B, NS5A e NS5B) (MORADPOUR; PENIN; RICE, 2007) (Figura 5).

Uma estrutura típica presente no genoma de HCV é o IRES tipo IV (*internal ribosomal entry site*) que possui o sítio de ligação miRNA122, característico de hepatrotopismo, tendo em vista que nos vertebrados, o miRNA122 é altamente expresso no tecido hepático (JOPLING *et al.*, 2005). Essa estrutura foi reportada em outras espécies de HVs, como nos equinos/caninos (HAV), bovinos (HNV), roedores (HIV) e bichos-preguiça (ainda não classificado). No entanto, no HCV, essa estrutura apresenta dois sítios de ligação miRNA122, enquanto que na maioria das demais espécies apenas um sítio foi reportado (MOREIRA-SOTO *et al.*, 2020; SMITH *et al.*, 2016).
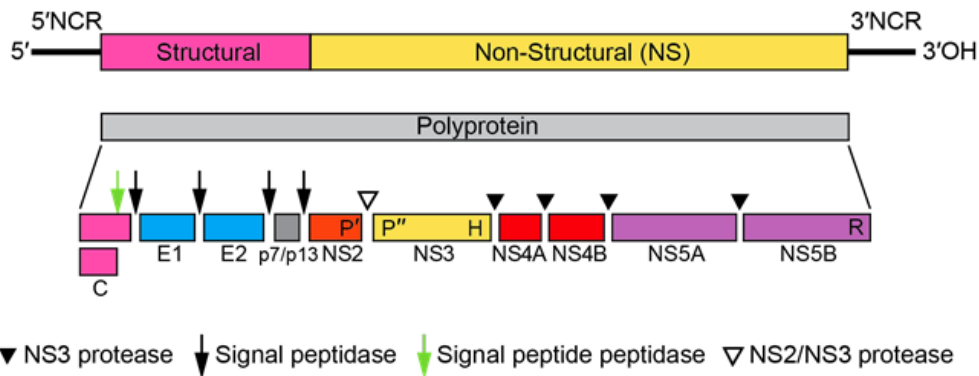
Figura 5 - Organização genômica (acima) e processamento da poliproteína (abaixo). Flanqueada por duas regiões não traduzidas (5'UTR e 3'UTR), uma única ORF codifica uma poliproteína. Essa poliproteína origina 10 proteínas, incluindo as estruturais e as não estruturais. Fonte: adaptado de ICTV *(https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/362/genus-hepacivirus).*

2.2.3 Replicação Viral (HCV)

O ciclo de replicação viral de HCV ainda não é totalmente esclarecido, principalmente devido à falta de um sistema de cultivo celular eficiente (CHEVALIEZ; PAWLOTSKY, 2006). A partícula viral, através da interação das glicoproteínas do envelope, adere-se na superfície celular do hospedeiro. Apesar da existência de muitos estudos com a finalidade de esclarecer os receptores envolvidos, é possível que exista uma série de receptores celulares, como CD81, receptor *scavenger* B tipo I (SR-BI), células dendríticas (DC-SIGN ou CD209), entre outros (MORADPOUR; PENIN; RICE, 2007).

Após a adesão, o vírus é liberado no citoplasma celular por endocitose, como resultado do processo de fusão entre as membranas virais e celulares. No citoplasma, ocorre a descapsidação viral e liberação do RNA genômico. O RNA alcançará os ribossomos da célula do hospedeiro, presentes no retículo endoplasmático rugoso, e fará, inicialmente, a síntese de uma poliproteína precursora com três proteínas estruturais: core e glicoproteínas do envelope E1 e E2, além de seis proteínas não estruturais (NS2, NS3, NS4, NS5A e NS5B). A poliproteína originada será clivada pela ação da protease, que associada a RNA polimerase (codificada pelo gene NS5B) formará o complexo de replicação. As novas fitas serão "empacotadas" pelo core e envelope viral e serão liberadas do retículo endoplasmático rugoso da célula hospedeira,

recomeçando o processo infeccioso (Figura 6) (CHEVALIEZ; PAWLOTSKY, 2006; MORADPOUR; PENIN; RICE, 2007).
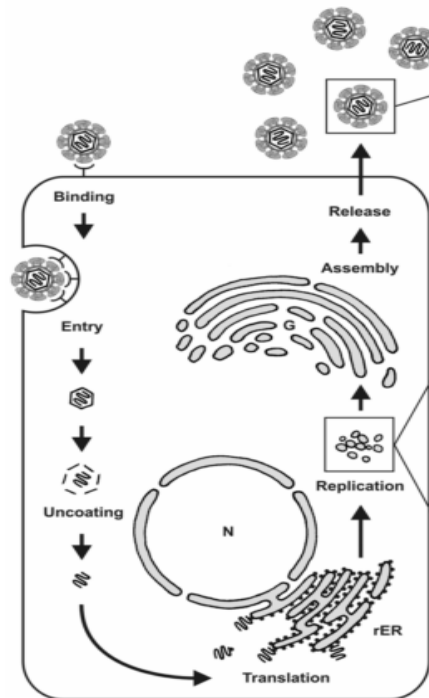
Figura 6 - Ciclo de replicação hipotético de HCV. As partículas de HCV se ligam às células hospedeiras através de uma interação específica entre as glicoproteínas de envelope de HCV e um receptor celular ainda não totalmente esclarecido. As partículas são internalizadas por endocitos. Depois que o genoma viral é libertado do nucleocápsideo e traduzido no retículo endoplasmático rugoso. Após a amplificação do genoma e a expressão das proteínas do HCV, os virions da progênie são montados, e o ciclo infeccioso pode reiniciar. Fonte: adaptado de (CHEVALIEZ; PAWLOTSKY, 2006).

2.2.4 Hepacivírus em Animais

Desde a descoberta do HCV em 1989, pesquisadores propuseram-se a identificar HVs em animais, motivados pela hipótese de que as infecções por HCV poderiam ter uma origem em primatas não humanos (SIMMONDS, 2013). Essa hipótese foi baseada na observação de que áreas endêmicas com alta biodiversidade na África e Ásia eram sobrepostas a populações de macacos do velho mundo. Essa observação é análoga à pandemia de HIV, que possuiu uma origem zoonótica a partir dos chimpanzés (GAO *et al.*, 1999).

No entanto, muitos estudos falharam na detecção de HVs nesses animais (MAKUWA *et al.*, 2003). Ainda assim, muitos vírus de RNA são capazes de cruzar as barreiras entre espécies e se disseminam com facilidade entre animais e o homem. Consequentemente, eles são responsáveis por infecções zoonóticas e tais transmissões cruzadas são responsáveis por algumas das doenças infecciosas mais graves, que ameaçam a saúde milhões de pessoas (SCHEEL *et al.*, 2016; SHARP; RAYNER; HAHN, 2013).

Em 2011, a primeira evidência de HVs em outro hospedeiro emergiu, quando, através da técnica de sequenciamento de alto desempenho, foi identificado um vírus relacionado ao HCV em cães em um surto de doença respiratória nos Estados Unidos. As sequências foram obtidas a partir de amostras de suabe nasal, o que causou certa estranheza, tendo em vista a característica hepatotrópica dos HCV e as análises filogenéticas mostraram aproximadamente 50% de similaridade nucleotídica com o HCV (BEXFIELD *et al.*, 2014). Posteriormente, inúmeras tentativas de identificação de cães infectados ou soropositivos não obtiveram sucesso.

O mesmo grupo de pesquisa que descobriu o hepacivírus canino, realizou um ensaio sorológico para detecção de anticorpos anti-NS3 em uma série de espécies animais e observou altos títulos de anticorpos em 35% dos equinos testados sendo que oito dos animais foram positivos para RNA viral. As sequências de hepacivírus equino apresentaram alta homologia nucleotídica com as de caninos (aproximadamente 99%) (BURBELO *et al.*, 2012). De acordo

com a nova classificação, os HVs equinos (EHV) e caninos (CHV), pertencem à espécie A (*Hepacivirus A*, HAV) (SMITH *et al*., 2016). A alta taxa de similaridade observada sugere duas possibilidades. A primeira, de que a infecção em cães representa um evento recente de transmissão entre as espécies, e a segunda, de que os resultados possam ter sido falsos positivos, devido à alimentação de cães com carne de equinos, ou uso de produtos veterinários com soro equino contaminado, comumente utilizado na produção de vacinas (PYBUS; THÉZÉ, 2016).

Em 2013, a partir do soro de 400 roedores selvagens capturados de quatro espécies de *Rodentia*, foram identificados vários HVs geneticamente distintos (KAPOOR *et al*., 2013). Nesse mesmo ano, um estudo detectou HVs em 1,9% dos camundongos testados (LUKASHEV *et al.*, 2013). Posteriormente, um grupo relatou a descoberta de um novo HVs em amostras de soro obtidas de macacos Colubua (*Colobus guereza*). Foi a primeira descoberta em primatas não humanos, no entanto, a filogenia não ficou como esperada, pois formou-se um clado claramente divergente do HCV (LAUCK *et al.*, 2013).

Recentemente, dois estudos reportaram novos hepacivírus. Um deles detectou 69,7% de amostras positivas em 185 amostras de soro de quatro espécies de patos de cinco províncias da China e, tendo em vista que um mesmo rebanho resultou positivo após 4 meses, o estudo sugeriu uma possível infecção persistente pelo vírus (CHU *et al.*, 2019). O outro estudo, realizado na Austrália, detectou HVs em invertebrados pela primeira vez, em um mosquito *Culex annulirostris*. As análises mostraram que o mosquito havia se alimentado há poucas horas de um pássaro (*Podargus strigoides*), no entanto, não se sabe se esse animal poderia fazer parte do ciclo do vírus (WILLIAMS *et al.*, 2020). Um importante achado também foi descrito em 2020, em que o HCV foi detectado, através de um estudo de metagenômica, em ectoparasitas hepatófagos humanos, percevejos da espécie *Cimex lectularis* na Europa (HARRACA; RYNE; IGNELL, 2020).

Um estudo realizado em 2013 analisou 1.615 amostras de morcegos (soro e tecidos) coletados entre 2007 e 2011, de sete países (Guatemala, Camarões, Nigéria, República do Congo, Quênia, Bangladesh e México), através de sequenciamento de alto desempenho. Nesse estudo, detectaram HVs em amostras de duas espécies de morcegos (*Hipposideros vittatus* e *Otomops martiensseni*), todos frugívoros e coletados no Kenia. Na árvore filogenética realizada no estudo, as sequências apresentaram-se em três diferentes clados, mostrando a alta variabilidade dessas sequências, apesar de serem provenientes de apenas duas espécies de um país (QUAN *et al.*, 2012).

As recentes descobertas de hepacivírus animais oferecem novas oportunidades para ampliar nosso conhecimento sobre interações vírus-hospedeiro dos HVs e poderia levar a descoberta de novos modelos experimentais para HCV. Para estimar o significado desses novos modelos experimentais para o HCV, um entendimento detalhado e a caracterização biológica são cruciais (BILLERBECK *et al.*, 2013).

2.2.4.1 Hepacivírus em Bovinos

O HNV foi identificado pela primeira vez em Ghana e na Alemanha por dois grupos de pesquisa independentes (BAECHLEIN *et al.*, 2015; CORMAN *et al.*, 2015). Muitos estudos têm demonstrado uma distribuição mundial de HNV, incluindo Brasil (CANAL *et al.*, 2017; DA SILVA *et al.*, 2018), China (LU *et al.*, 2018), Estados Unidos (SADEGHI *et al.*, 2017) e Turquia (YEŞILBAĞ *et al.*, 2018). Os *screenings* para detecção do RNA viral têm demonstrado uma prevalência mundial entre 0,6% to 14,8% de bovinos positivos. Assim como o HCV, o HNV é hepatotrópico e pode estabelecer infecções persistentes agudas e crônicas (BAECHLEIN *et al.*, 2015).

No Brasil, em 2017, estudo revelou que o HNV circula no país há pelo menos 20 anos. Utilizando *primers* degenerados para pesquisa de pestivírus em soro de bovinos em 1996, o sequenciamento revelou amostras com baixa identidade com o gênero *Pestivirus*. Com a descrição do novo hepacivírus bovino em 2015, o grupo pode constatar que tratava-se de HNV (CANAL *et al.*, 2017). Após, em 2018, outro estudo brasileiro, demonstrou, através de análises evolutivas, que a origem brasileira do HNV foi concomitante ao aumento da criação de gado no Brasil, que ocorreu por volta de 1970,. Além disso, demonstrou uma origem em comum entre o HNV brasileiro e europeu que data do século XVIII, período marcado pelo ciclo da cana-de-açúcar no Brasil colonial, em que a população bovina aumentou principalmente pela entrada de gado europeu para esse fim (DA SILVA *et al.*, 2018).

Assim como HCV, o HNV possui uma fita de sentido positivo com um genoma de RNA organizado em uma grande ORF, possuindo 8,340 nucleotídeos. Análises detalhadas do genoma revelam que este resulta em uma grande poliproteína clivada por proteases celulares e virais, dando origem a proteínas maduras estruturais (C, E1 e E2) e não estruturais (NS2, NS3, NS4A, NS4B, NS5A e NS5B) (BAECHLEIN *et al.*, 2019). De acordo com os critérios de classificação de genótipo e subtipo de HCV, o HNV tem sido classificado da mesma forma, conforme a

proposta de um estudo (DA SILVA *et al.*, 2018). O último artigo que seguiu essa classificação foi realizado na China e dividiu o HNV em sete subtipos (A-G) (LU *et al.*, 2019) (Figura 7).

As análises filogenéticas baseadas em regiões conservadas do genoma (NS3 e NS5) demonstram que os HNVs possuem grande similaridade entre si, no entanto, apresentam alta distância genética entre outros HVs, sendo mais relacionados aos hepacivírus de roedores (HARTLAGE; CULLEN; KAPOOR, 2016). O curso de infecção natural de HNV nos bovinos revela a existência dos estágios agudo e crônico, e, apesar de não apresentarem sintomatologia clínica, a presença de altos títulos virais no fígado e a predição do sítio de ligação miR-122 confirmam o hepatotropismo do HNV (BAECHLEIN *et al.*, 2015, 2019).
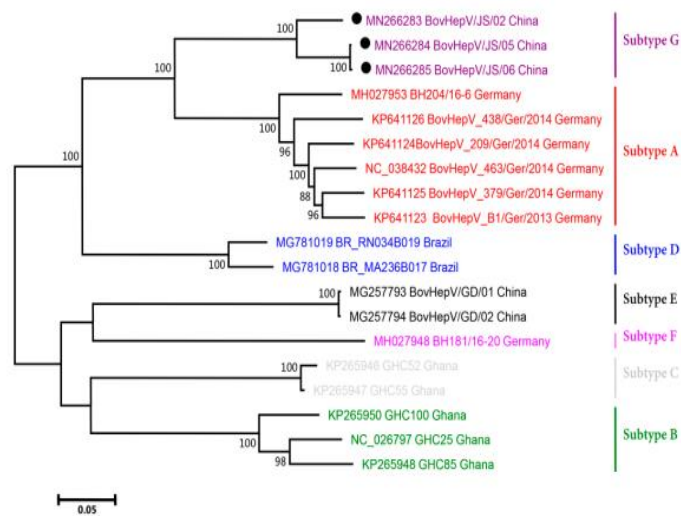

Figura 7 - Árvore filogenética de HNV apresentando a classificação atual de sete subtipos (A-F).
Fonte: adaptado de LU et al., 2019.

2.2.5 Possíveis Cenários Para a Origem Zoonótica do HCV

Os humanos são os únicos hospedeiros naturalmente infectados por HCV, apesar dos chimpanzés serem passíveis de infecções experimentais (YANAGI *et al*., 1997). Como o uso desses animais em ensaios experimentais é limitado, existe uma necessidade de modelos animais para estudos de persistência, patogênese e imunidade de HCV (BUKH, 2012). Ainda não está claro até que ponto os HVs de animais refletem na biologia e patogenia do HCV em seres humanos, mas é bem provável que possam tornar-se úteis na compreensão da doença. Além disso, o aumento do conhecimento deste gênero poderá fornecer indícios sobre a origem de HCV, que até o momento permanece desconhecida.

Um estudo realizou análises filogenéticas com o objetivo de compreender como o HCV se originou em humanos (PYBUS; THÉZÉ, 2016) uma vez que análises prévias da região mais conservada NS3 sugerem uma transmissão de HVs entre diferentes espécies (SCHEEL *et al*., 2016). Apesar da espécie *Hepacivirus A* (HAV), detectada em equinos e caninos, possuir a menor distância genética de HCV, não há evidências para supor que ele tenha se originado de equinos ou cães. Ao invés disso, parece que tanto HCV, como EHV e CHV surgiram da transmissão independente entre espécies, com origens ainda desconhecidas (Figura 8). Essa hipótese foi fortificada a partir de um estudo evolutivo que mostrou que o ancestral comum de todos os genótipos de HCV existiram há pelo menos 3000 anos, enquanto o EHV surgiu há aproximadamente 1100 anos. Esse estudo de estimativas evolutivas temporais parece excluir a teoria de que o HCV tenha se originado de um evento de transmissão de espécie cruzada a partir dos equinos (FORNI *et al.*, 2018).

Uma forte teoria da origem de HCV está relacionada à descoberta de HVs, com uma enorme diversidade genética, em morcegos (QUAN *et al*., 2012) e roedores (AMIT *et al*., 2013). Tendo em vista que os HVs nesses animais são os mais geneticamente divergentes, e, que, possivelmente possuam ancestrais comuns mais remotos, sugere-se que tenham importante

papel na genealogia de HCV (MOREIRA-SOTO *et al*., 2020). Acredita-se na possibilidade de que cada uma das espécies anteriormente identificadas (em equinos, humanos, cães) surgiu através da transmissão bem sucedida entre espécies de HVs de morcegos ou roedores ainda desconhecidos (OLIVER G. PYBUS & REBECCA R. GRAY T, 2013). Outro argumento a favor é de que aproximadamente um quarto dos patógenos humanos emergentes tenham se originado de roedores ou morcegos (VAN BOHEEMEN *et al*., 2012). Além disso, um estudo recente mostrou que cinco sítios do receptor CD81 em morcegos (importantes na interface do vírus HCV com o hospedeiro) sofreram seleção positiva, sugerindo a interferência de HVs na mudança genética de CD81 em morcegos (FORNI *et al*., 2018).

Além da fonte zoonótica desconhecida de HCV, também há incerteza quanto ao número de diferentes hospedeiros que poderiam ter sido infectados antes do vírus ter se adaptado aos seres humanos. Antes do século XX, os diferentes genótipos parecem ter existido em áreas geográficas restritas por pelo menos algumas centenas de anos (YAP *et al*., 1997). Acredita-se que cada genótipo possa ter surgido a partir de fontes zoonóticas distintas em diferentes locais geográficos. No entanto, não há evidências diretas para rejeitar a hipótese alternativa de que o HCV se originou de uma única espécie hospedeira precursora e que seus genótipos divergiram dentro das populações humanas (PYBUS; THÉZÉ, 2016). De fato, a origem clara de HCV ainda não foi identificada, assim, o conhecimento dos eventos moleculares que permitiram a adaptação do HCV aos seres humanos permanece desconhecida. Essa questão pode ser resolvida através da descoberta e do estudo contínuos de novos hepacivírus.

Uma teoria quanto ao possível mecanismo para uma transmissão zoonótica é através de artrópodes, que podem atuar como vetores mecânicos (não replicativos) ou biológicos (replicativos). Pybus e colaboradores (2007) discutiram teorias para a manutenção de HCV endêmico nas populações humanas antes do século XX e exploraram se a transmissão mecânica através de insetos poderia desempenhar um papel importante. O estudo concluiu que esta

hipótese seria mais viável para insetos, do gênero *Tabanidae*, que cortam a pele para se alimentar, transportando maiores volumes de sangue. Os tabanídeos podem atuar como vetores mecânicos e são conhecidos por transmitir o vírus da anemia infecciosa equina (EIAV) (PYBUS *et al*., 2007). A hipótese da transmissão mediada por insetos poderia, em teoria, fornece uma única explicação para a transmissão de HCV em seres humanos e a origem do vírus a partir de um reservatório (PYBUS; THÉZÉ, 2016). Essa hipótese ganhou força atualmente, devido a detecção de hepacivírus em mosquitos *Culex annulirostris* na Austrália. Mas apesar dessa descoberta abrir precedentes para novas teorias de transmissão de HVs entre espécies, o estudo concluiu que o hepacivírus detectado poderia representar um material parcialmente digerido a partir da alimentação do mosquito proveniente de sangue de uma ave (WILLIAMS *et al*., 2020).
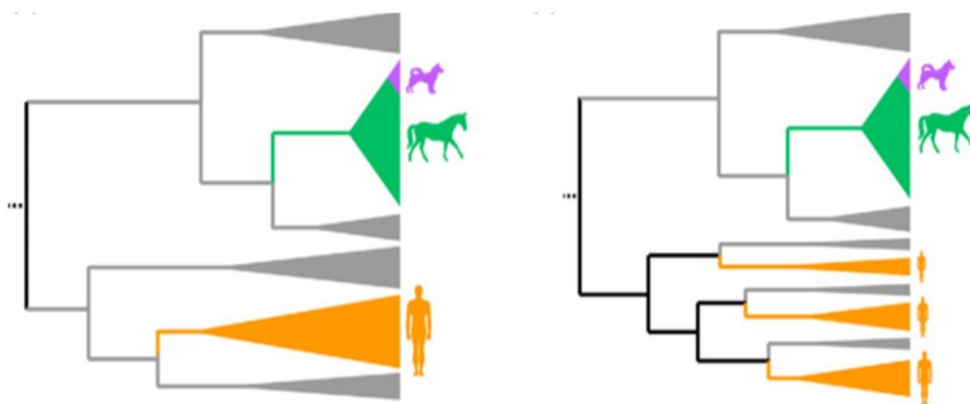
Figura 8 - Possíveis cenários para a origem zoonótica do HCV e da linhagem EHV/CHV. As silhuetas indicam as espécies hospedeiras de cada linhagem viral: cães (roxos), equídeos (verde), humanos (laranja). Em ambos os cenários, o HCV e EHV / CHV são postulados a partir de transmissões independentes de espécies HVs de uma ou mais espécies de origem não identificadas (triângulos cinza). Em (a) esses vírus não identificados são parafiléticos em relação ao HCV, indicando que surgiu de um único evento de transferência ancestral. Em (b) os vírus não identificados se enquadram na diversidade genética do HCV, indicando que possa ter surgido de duas ou mais transmissões separadas de espécies hospedeiras diferentes. Fonte (adaptado): (PYBUS; THÉZÉ, 2016) .

3 Capítulo 1

3.1.1 Artigo científico: "*Highly Divergent Cattle Hepacivirus N in Southern Brazil*"

Artigo publicado em 2019, na revista "*Archives of Virology*", fator de impacto: 2.230.

ANNOTATED SEQUENCE RECORD

Highly divergent cattle hepacivirus N in Southern Brazil

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Abstract

The genus *Hepacivirus* includes 14 species (*Hepacivirus A–N*). In this study, we determined a partial genome sequence of
a highly divergent bovine hepacivirus (hepacivirus N, HNV) isolate from cattle in Southern Brazil. Previously described
HNV isolates have shared 80–99.7% nucleotide sequence identity in the NS3 coding region. However, the sequence deter-
mined in this study had 72.6% to 73.8% nucleotide sequence identity to known HNV NS3 sequences. This high divergence
could be seen in a phylogenetic tree, suggesting that it represents a new genotype of HNV. These data expand our knowledge
concerning the genetic variability and evolution of hepaciviruses.

Human hepatitis C virus (HCV), which belongs to the spe-
cies *Hepacivirus C*, family *Flaviviridae*, genus *Hepacivirus*
[1, 2], is one of the most important human pathogens and
is one of the leading causes of chronic hepatitis, liver cir-
rhosis, and hepatocellular carcinoma [3, 4]. The lack of an
animal model in which HCV infection can be studied in a
straightforward manner and the limited scope of current cell
culture systems has made it difficult to study this important
virus [5].

Hepaciviruses (HVs) contain two untranslated regions
(UTRs), the 5'UTR and 3'UTR, and a single large open
reading frame (ORF) encoding a unique polyprotein that
is cleaved by signal peptidase, NS2/NS3 protease and NS3
protease enzymes into at least 10 mature products: the

structural core protein (C), two envelope proteins (E1 and
E2), nonstructural assembly proteins (p7 and NS2) and other
nonstructural proteins involved in replication (NS3, NS4A,
NS4B, NS5A and NS5B) [2, 3]. The 5'UTR is a highly con-
served region that is widely used to identify flaviviruses [1,
6]. Phylogenetic analysis based on nucleotide sequences of
the NS5B and NS3 genes, which are strongly conserved,
usually yields results similar to those obtained using com-
plete genome sequences [1].

Recently, HVs have been detected in several domestic
and wild animals, including horses [7], rodents [8], bats
[9] and cattle [6, 10–12] These HVs have been assigned to
14 species (*Hepacivirus A-N*) [1]. Hepacivirus N (HNV)
has already been detected in cattle in Germany [13], China
[14], Ghana [12], and Brazil [6, 10], and characterization of
diverse HNV isolates could provide valuable insights into
their biology and evolution [15]. In the present work, we
determined the partial genome sequence of a highly diver-
gent HNV isolate in Southern Brazil, representing a new
genotype of HNV.

In 2016, a serum sample from an Angus bull was pro-
cessed for Illumina MiSeq sequencing [16]. Libraries were
prepared using a Nextera XT DNA Sample Preparation
Kit (Illumina) and sequenced on an Illumina MiSeq plat-
form with a 2 × 250-bp paired-end run (Illumina). A total
of 307,398 high-quality paired-end reads (Q ≥ 30) were
generated. The sequences were trimmed and assembled *de
novo* into contigs, using SPAdes v3.10 (metaSPAdes) [17]
and compared to known sequences in the National Center

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for Biotechnology Information (NCBI) databases using the BLASTn/BLASTx [18].

Illumina MiSeq generated 248 reads that were assembled based on a cattle HV reference sequence (NC_026797), comprising eleven contigs (totaling around 4,000 bp in length; average coverage, 9X). Sanger sequencing was performed with additional primers designed in this study (Supplementary Material) in order to obtain a larger sequence, which was 6,103 bp in length (GenBank Accession Number: MK695669) and included the complete p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B coding regions. Furthermore, a portion of the 5'UTR (138 bp) was amplified using previously described primers [10].

BLASTn analysis showed that the contig shared 72.8% nucleotide sequence identity with a German HNV sequence (GenBank accession number KP641126), and the predicted polyprotein shared 81.4% amino acid sequence identity with a another German HNV sequence (GenBank accession number AKH10571). Alignment and comparison of the entire nucleotide sequence (6,103 bp) with HNV sequences available in the GenBank database showed that it is the most divergent sequence that has been identified so far, with only around 80% identity (Fig. 1).

Phylogenetic reconstructions were performed using the complete NS3 coding region by applying the maximum-likelihood method [19] with the GTR+I+G model, which was selected using the Molecular Evolutionary Genetics Analysis7 (MEGA7) software package [20]. The phylogenetic tree was constructed using representatives of different HV species, and it displayed four well-separated clades, corresponding to hepaciviruses N (cattle), C (human), A (equine) and K, L, and M (bat). The HNV clade was clearly separated into two groups: one including all complete NS3 sequences of HNV isolates from cattle available in the GenBank database and the other with the sequence detected in the present study, supported by a 100% bootstrap value (Fig. 2a). To corroborate the results, a complete NS3 phylogenetic tree was generated using German, Chinese, Ghanaian and Brazilian HNV sequences, showing a separate genetic group within the HNV branch consisting only of the sequence generated in this study (Fig. 2b).

The new isolate showed a high degree of nucleotide and amino acid sequence divergence when compared to known HNV sequences available in the GenBank database. Until now, the nucleotide sequence identity among complete HNV NS3 sequences in the GenBank database has ranged between 80–99.7%. The sequence determined here was only 72.3% to 73.6% identical to the complete NS3 sequences of other HNV isolates. The proposed genotype classification system for HNV is based on the complete polyprotein amino acid p-distance (values of 0.23 to 0.31 distinguish different genotypes) [10]. The sequence generated here had a higher p-distance (0.15-0.16) when compared to the known HNV sequences (0.01-0.07). Although the value did not reach the cutoff point, this sequence might still represent a new genotype, since the analysis was not performed using the complete polyprotein sequence, and there is a clearly separate cluster in the phylogenetic tree.

BLASTn analysis was also performed using the partial 5'UTR sequence (84 bp). This region shared 100% sequence identity with HNV sequences determined 20 years ago [6] in the same region (Southern Brazil) (GenBank accession number KY439908). Unfortunately, only the 5'UTR sequence is available from this older isolate and further analysis cannot be done. The partial 5'UTR sequence was approximately 84% identical to HNV isolates from Germany [13], China [21], Ghana [12] and Northern Brazil [10], which is very low considering that the 5'UTR is extremely conserved. In order to corroborate previous analysis, a phylogenetic tree of the 5'UTR region

Fig. 1 The pairwise nucleotide distance of HNVs based on 6103 bp of the polyprotein region, calculated using the Species Demarcation Tool (SDT)
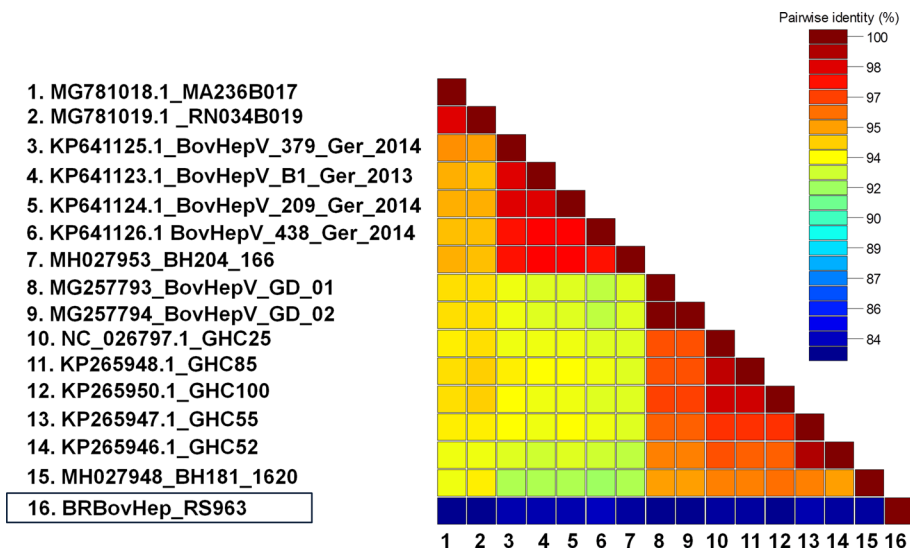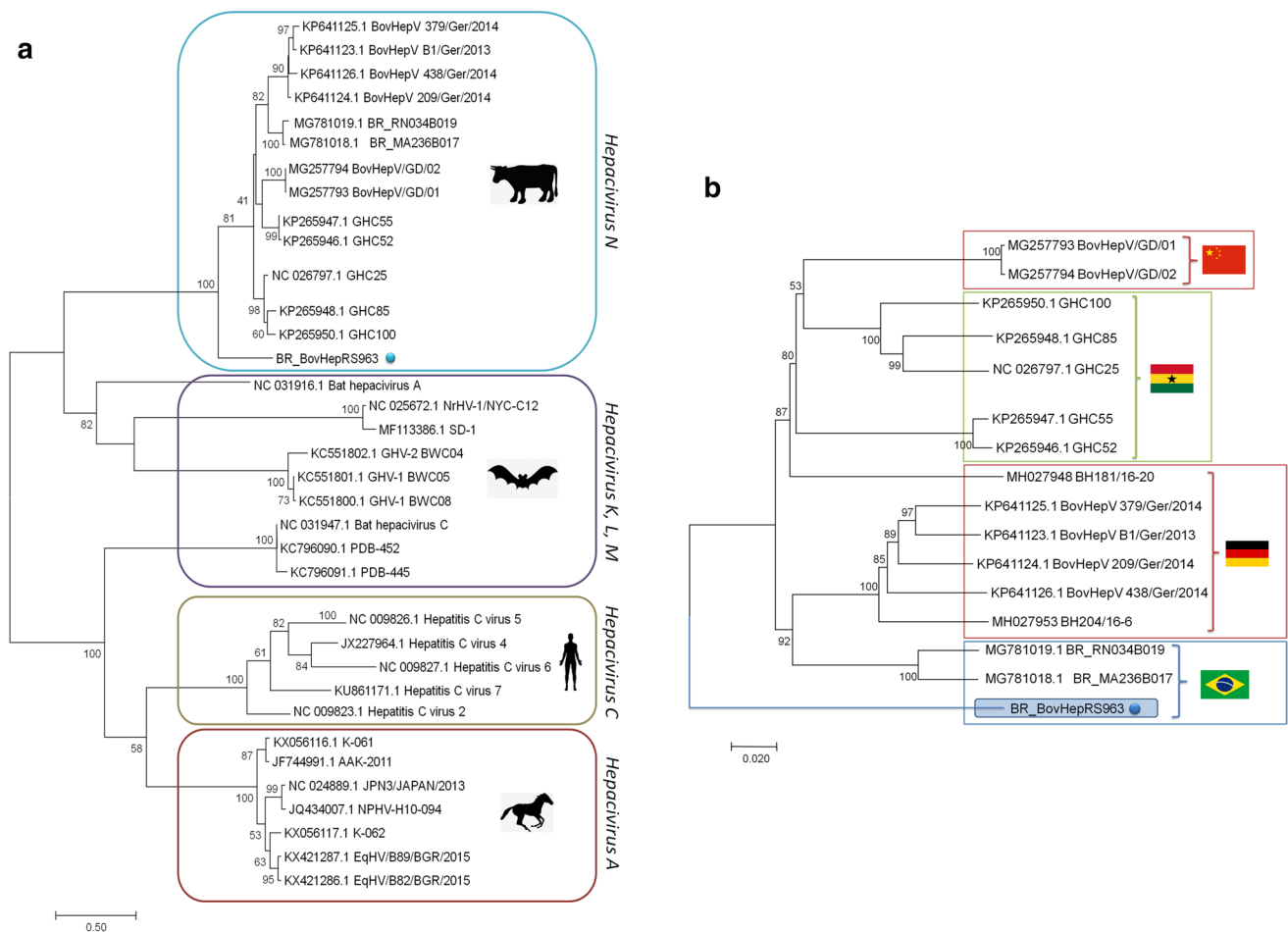
Fig. 2 Phylogenetic trees based on the complete NS3 coding region. MEGA7 was used for phylogeny inference by the maximum-likelihood method. **a)** Phylogenetic tree constructed using sequences from representative members of *Hepacivirus* species (*Hepacivirus A,* *Hepacivirus N, Hepacivirus C* and *Hepacivirus N*). **b)** Phylogenetic tree of HNV sequences. The Brazilian sequence determined in this study is indicated by a blue dot

was constructed using bovine, equine, bat and human hepacivirus sequences. This analysis showed a clearly separated cluster containing the sequence generated in this study and the older HNV sequences from the same Brazilian region [6] (Fig. S1).

Finally, it is important to note that the HNV sequence reported in the present study is highly divergent when compared to worldwide HNV strains. All analysis results suggest that the Southern Brazilian HNVs comprise a highly divergent group. Apparently, the HNV reported here belongs to a putative new genotype of HNV. These results contribute to the database and expand our knowledge concerning the genetic variability of HVs. It is probable that the genetic diversity of HVs is greater than previously imagined. Research on HVs may, in the future, clarify the origin of HCV, and our findings can support future studies on the evolution of HVs.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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3.2   Capítulo 2

3.2.1   Artigo Científico: "*Liver Virome of Healthy Pigs Reveals Diverse Small ssDNA Viral Genome*"

Artigo publicado em 2020, na revista "*Infection, Genetics and Evolution*", fator de impacto:

2.611.
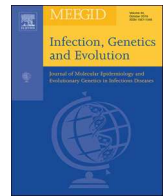
Research paper

Liver virome of healthy pigs reveals diverse small ssDNA viral genomes

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A B S T R A C T

Brazil is a major exporter of pork meat worldwide. Swine liver is a common ingredient in food consumed by humans, thus emphasizing the importance of evaluating the presence of associated pathogens in swine liver. To obtain knowledge, this study aimed to provide insights into the viral communities of livers collected from slaughtered pigs from southern Brazil. The 46 livers were processed and submitted for high-throughput sequencing (HTS). The sequences were most closely related to *Anelloviridae*, *Circoviridae* and *Parvoviridae* families. The present work also describes the first Brazilian PCV1 and the first PPV6 and PPV7 from South America. Virus frequencies revelead 63% of samples positive for TTSuV1, 71% for TTSuVk2, 10.8% for PCV, 13% for PPV and 6% for PBov. This report addresses the diversity of the liver virome of healthy pigs and expands the number of viruses detected, further characterizing their genomes to assist future studies.

1. Introduction

Livestock production in Brazil plays a key role in the demand for nutritious food and food security. The transmission of zoonotic pathogens is an important route for foodborne diseases, including the- contamination of meat during slaughtering and processing and the consumption of raw or undercooked contaminated foods (Koopmans and Duizer, 2004). Pork liver is a common ingredient in food products, such as sausages, figatelli, and pâté, and disease transmission *via* ingestion of contaminated foods is a worldwide concern (Kirk et al., 2015; Koopmans and Duizer, 2004).

High-throughput sequencing allows the detection of viruses that are difficult or impossible to propagate in cell culture and that are not detectable by molecular detection tests since they contain no common genes, such as the ribosomal 16S gene present in bacterial species (Delwart, 2007). This technology has facilitated the "unbiased" detection of known and previously unknown viruses (Goodwin et al., 2016) in the virome because it uses nonspecific primers to detect nucleic acid sequences (Kohl et al., 2016; Virgin, 2014).

The viral investigation in several host species of animals, such as pigs, may be critical to identifying the original host or carriers of viruses that have the potential to cause diseases in farmed animals and humans, such as hepatitis E virus (HEV), which can lead to acute and chronic hepatitis and extrahepatic manifestations in humans (Kumar et al., 2018). In addition, information about the presence of viruses in clinically healthy hosts provides a baseline level for viruses present in these animals in the case an outbreak of disease. Previous works analyzed the swine virome in feces (Amimo et al., 2016; Shan et al., 2011), tonsils (Blomström et al., 2018) and organ pool (including liver, lung and kidney) (Franzo et al., 2018), but no metagenomic analysis with only swine liver samples was performed. Therefore, the present study aimed to evaluate and characterize the virome from 46 swine liver using HTS of healthy animals slaughtered in southern Brazil.

2. Materials and methods

2.1. Samples, viral metagenome and HTS

In 2016, 46 livers were collected from slaughtered swine in a cooperative production family that breeds pigs and owns a small slaughterhouse located in Rio Grande do Sul State, Brazil. HTS was realized in a pool sample (from 46 livers) and conventional PCRs were realized in individual samples to confirm the results and verify the viruses' frequencies. The samples were from the cooperative and other tree different herds.

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All samples were macerated, pooled and centrifuged at 2000 $\times g$ for 30 min, and the supernatant was filtered through a 0.22 μm membrane (Millipore) to remove eukaryotic and bacterial cell-sized particles. Subsequently, samples were ultracentrifuged on a 25% sucrose cushion at 100,000 × g for 3 h at 4 °C in a Sorvall AH629 rotor. The pellet containing the viral particles was incubated for 1.5 h with DNase and RNase (Thermo Fisher Scientific, Waltham, MA, USA) (Thurber et al., 2009). Viral RNA and DNA were isolated using TRIzol™ LS Reagent (Thermo Fisher Scientific) and a standard phenol–chloroform protocol (Sambrook and Russl, 2001), respectively.

Viral DNA was enriched through multiple displacement amplification (MDA), performed with the GenomePlex® Single Cell Whole Genome Amplification (WGA) Kit (Sigma-Aldrich, St. Louis, MO, USA), while the viral RNA was reverse-transcribed and enriched to dsDNA using the TransPlex® Complete Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's recommendations. The DNA products produced from these enrichment protocols (MDA and WTA) were pooled in equimolar amounts and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific). The quality and quantity of the DNA were assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher Scientific) and Qubit™ (Thermo Fisher Scientific), respectively. The viral libraries were further prepared with 1 ng of purified DNA using the Nextera XT DNA Library Preparation Kit and sequenced using an Illumina MiSeq System using an Illumina v2 reagent kit (2 × 150 paired-end reads).

Since the sequencing results detected no RNA virus, we decided to perform another independent analysis processing the enrichment only with TransPlex® Complete Whole Transcriptome Amplification (WTA) Kit (Sigma-Aldrich, St. Louis, MO, USA) to avoid genomes other than RNA. This sample was sequenced individually using Illumina MiSeq System. This procedure was realized in order to confirm the results.

2.2. Bioinformatic analysis

The quality of the sequences generated was evaluated using the FastQC tool. The sequences with bases possessing a Phred quality score < 20 were trimmed with the aid of Geneious software (version 9.0.5). The data were *de novo* assembled on BaseSpace Cloud (Illumina) with the SPAdes genome assembler (version 3.0). Thereafter, the assembled contigs were examined

for similarities with known sequences using BLASTX software within the Blast2GO platform (Conesa and Gotz, 2008) and analyzed using Geneious software. Sequences with *E*-values $\leq 10^{-3}$ were classified as likely to have originated from eukaryotic viruses, bacteria, phages, or unknown sources based on the taxonomic origin of the sequence with the best E-value. Nucleotide and protein comparisons were performed with BLASTN and BLASTP programs (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the sequences that were most closely related to the viral contigs of interest. Representative sequences of viruses belonging to the families *Anelloviridae*, *Parvoviridae* and *Circoviridae* were obtained from GenBank and aligned with the sequences identified in the present study with MUSCLE software (Edgar et al., 2004). Phylogenetic trees were constructed using MEGA6 (Tamura et al., 2011), applying the neighbor-joining method through the p-distance model for the *Anelloviridae* analysis and the maximum likelihood method, Kimura-2 parameter (Kimura, 1980), to the *Circoviridae* and *Parvoviridae* families. All analyses were conducted with 1000 bootstrap replicates.

2.3. PCR and Sanger sequencing

Specific conventional PCR protocols were performed to test for hepatitis E virus (HEV) (Erker et al., 1999) and verify the frequency of torque teno sus virus (TTSuV) (Segalés et al., 2009), porcine circovirus (PCV) (Li et al., 2010), porcine parvovirus (PPV) (Kunzler et al., 2016) and porcine bocavirus (PBov) in each liver (Table 1). Additional primers were designed, and PCRs were performed to obtain the complete genome of porcine parvovirus 7 (Table 1). The PCRs were performed using GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI, USA). PCR amplicons generated with the sets of sequencing primers were purified using the PureLink® PCR Purification Kit (Thermo Fisher Scientific Inc.), and both DNA strands were sequenced with an ABI PRISM 3100 Genetic Analyzer using the 238 BigDye Terminator v.3.1 cycle Sequencing Kit (Applied Biosystems, USA). Overlapping fragments were assembled using Geneious software (Kearse et al., 2012).

3. Results

3.1. Overview of the sequencing data

Liver samples from 46 clinically healthy pigs from a small agroindustry were collected, and viral nucleic acids were deep sequenced.

Table 1
Specific PCR primers used in this study.

Virus species	Primer name	Primer sequence	Reference
Torque teno virus sus 1	TTV1F	5′ - CGGGGTTCAGGAGGCTCAAT-3′	Segales, J. et al.
	TTV1R	5′ - GCCATTCGGAACTGCACTTACT - 3′	
Torque teno virus sus k2	TTV2F	5′- TCATGACAGGGTTCACCGGA - 3′	
	TTV2R	5′ - CGTCTGCGCACTTACTTATATACTCTA - 3′	
Circovirus (Nested)	CV-F1	5′- GGIAYICCICAYYTICARGG - 3′	Li, L. et al.
	CV-R1	5′- AWCCAICCRTARAARTCRTC - 3′	
	CV-F2	5′- GGIAYICCICAYYTICARGGITT - 3′	
	CV-R2	5′- TGYTGYTCRTAICCRTCCCACCA - 3′	
Porcine Parvovirus	P1	5′ - GCACTGAGGGCTACGTTCGTTCTC - 3′	Souza, C.K. et al.
	P2	5′ - TGACCAGGTCCATGAAAAATCTCCC - 3′	
	P5	5′ - CTTAGGTGATTTTCACCGGCCGC - 3′	
	P6	5′ - CTCCTTTGCCTCCCAGATACCCC - 3′	
Hepatitis E virus (Nested)	ORF1con-s1	5′ - CTGGCATYACTACTGCYATTGAGC - 3′	Erker, J. et al.
	ORF1con-a1	5′- CCATCRARRCAG TAAGTGCGGTC - 3′	
	ORF1-cons2	5′- CTGCCYTKGCGAATGCTGTGG - 3′	
	ORF1-cona2	5′- GGCAGWRTACCARCGCTGAACATC - 3′	
Porcine Parvovirus 7	PPV7 43F	5′ - TCGAGGAGGTAGGGGTCC - 3′	Designed in this study
	PPV7 291R	5′- CGTGTCTTCAGGAGCAGC - 3′	
	PPV7 3041F	5′ - ACAGCACCCGAAACGAAC - 3′	
	PPV7 3993 R	5′ - TGAGAAGACACTGGTTTAGCTTC - 3′	
Porcine Bocavirus 1	PBoV1 NS1 F	5′- TGCTGAAACCGCCGTAAAAA-3′	Designed in this study
	PBoV1 NS1 R	5′-CGTGTCTGCTAGTGGTCCTG-3′	
Porcine Bocavirus 1	PBoV3 NS1 F	5′-TCAGTCTGAGATCGGCGAGA-3′	
	PBoV3 NS1 R	5′-GTGAGACTCGTTGTGTCGGT-3′	
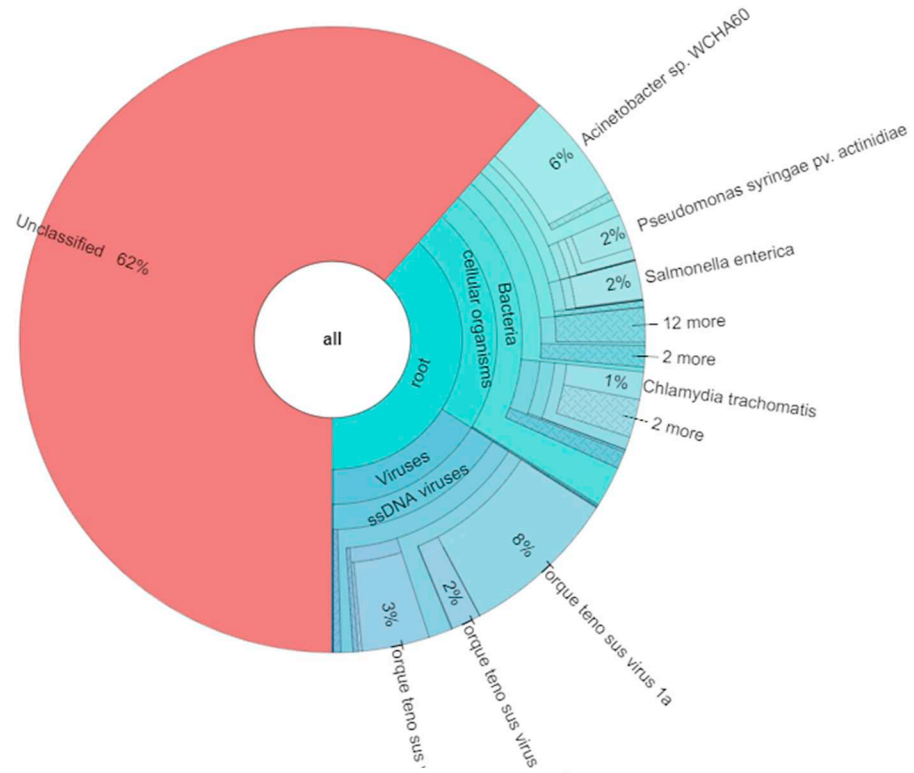
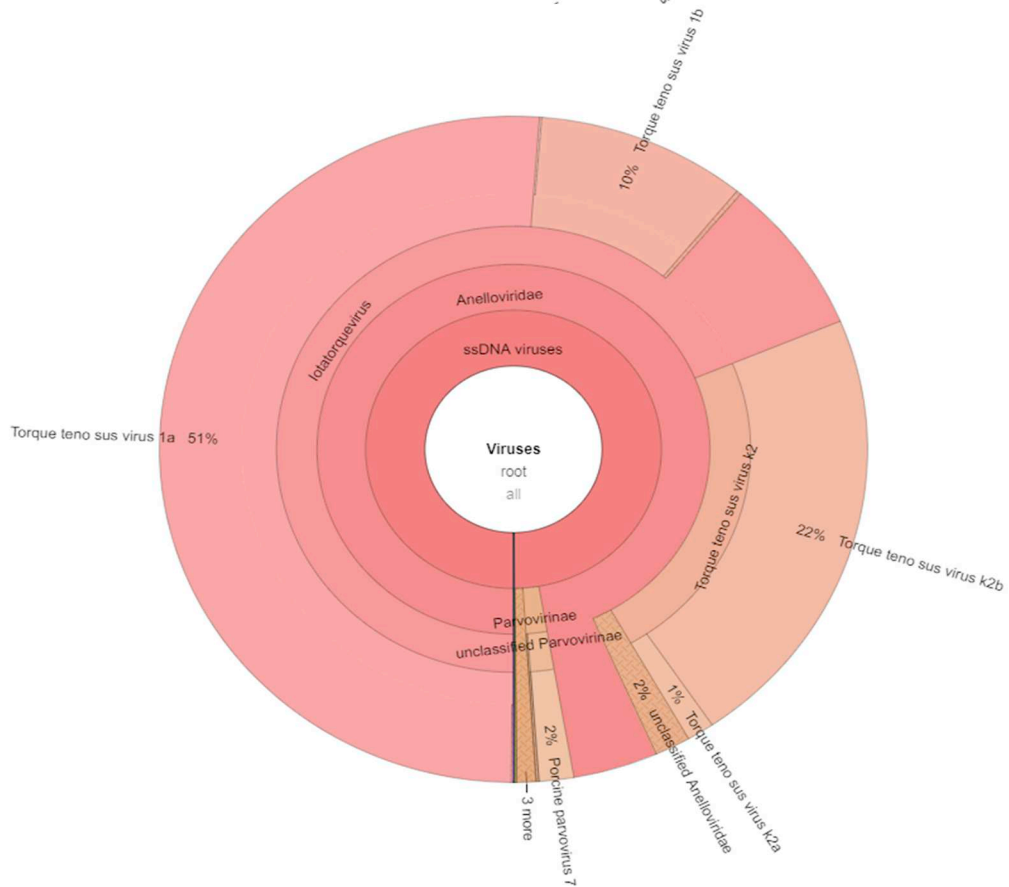**Fig. 1.** Metagenomic graphic results presenting the generated sequence distribution. A. The graphic presents all reads obtained from this analysis, while B. presents the eukaryotic virus representativeness. The analysis was performed using a krona plot within Kaiju. (http://kaiju.binf.ku.dk/server).

HTS generated 267,326 paired-end sequence reads resulting in 3246 *de novo*-assembled sequence contigs (minimum length, 250 nucleotides). The paired-end sequence reads were subjected to the online tool Kaiju (Menzel et al., 2016) (http://kaiju.binf.ku.dk/server) for taxonomic classification (Fig. 1). The overall results showed that 62% of the reads could not be classified, while 27% were related to bacterial and cellular genomes, and 11% corresponded to viral reads. Eukaryotic exogenous virus-related sequences belonging to five viral families with single-stranded DNA (ssDNA) genomes were observed: *Anelloviridae*, *Circoviridae* and *Parvoviridae* (Genus *Bocaparvovirus*, *Copiparvovirus* and unclassified PPV6 and PPV7). Conventional PCRs were performed to identify TTSuVs, PCV, PPV and PBov frequencies and to complete the PPV7 genome. Virus frequencies were evaluated, revealing 63% of samples positive for *Torque teno sus virus 1* (TTSuV1), 71% positive for *Torque teno sus virus k2* (TTSuVk2), 10.8% positive for PCV, 13% positive for PPV and 6% positive for PBov. Additional PCR was realized to test HEV and all samples were negative. Complete results can be observed in Table 2. No RNA viruses were detected even after resubmission of samples for Illumina MiSeq sequencing confirming the absence of RNA viruses in these swine livers.

3.2. Anelloviridae: Torque teno sus virus (TTSuV1 and TTSuVk2)

In the 46 pooled swine liver samples submitted for HTS, the presence of 67 contigs closely related to the *Anelloviridae* family was detected, from which 41 contigs were classified into the genus *Iotatorquevirus* (species *Torque teno sus virus 1*- TTSuV1), and 26 were classified into *Kappatorquevirus* (species *Torque teno sus virus k2*- TTSuVk2) (Table 3). This primary classification was based on BLASTx software within the Blast2GO platform (Conesa and Gotz, 2008) and further analyzed using Geneious software. The contigs ranged between 381 and 2886 nt in length. The specific TTSuV1 and TTVSuk2 PCR protocols (Segalés et al., 2009) targeting the 5'UTR region applied in individual liver samples revealed that 63% (29/46) of samples were positive for TTSuV1, 73% (35/46) were positive for TTSuVk2 and 43% (20/46) were positive for both.

Three positive samples of each conventional PCR were sequenced using the Sanger method. TTSuV1 5'UTR sequences (230 bp in length) shared approximately 96% nucleotide identity between them and 98% identity with a sequence of swine TTSuV1 detected in China (GenBank accession number: JX872391). TTSuVk2 5'UTR sequences (150 bp in

Table 2
Virus frequencies detected in each sample.

Sample	TTV1	TTV2	PCV1	PCV2	PPV4	PPV6	PPV7	PBov1	PBov 3	HEV
1	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
2	negative	**positive**	negative	negative	negative	negative	negative	**positive**	negative	negative
3	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
4	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
5	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
6	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
7	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
8	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
9	negative	negative	negative	negative	negative	negative	negative	negative	**positive**	negative
10	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
11	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
12	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
13	**positive**	**positive**	negative	negative	negative	negative	negative	negative	**positive**	negative
14	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
15	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative
16	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative
17	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
18	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
19	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
20	**positive**	negative	negative	negative	negative	negative	**positive**	negative	negative	negative
21	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
22	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
23	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
24	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
25	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
26	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
27	**positive**	**positive**	negative	**positive**	negative	negative	negative	negative	negative	negative
28	**positive**	**positive**	negative	negative	negative	negative	**positive**	negative	negative	negative
29	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
30	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
31	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
32	**positive**	**positive**	negative	negative	negative	negative	**positive**	negative	negative	negative
33	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
34	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative
35	negative	**positive**	negative	negative	negative	**positive**	negative	negative	negative	negative
36	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
37	**positive**	negative	negative	negative	negative	negative	negative	negative	negative	negative
38	negative	negative	negative	negative	**positive**	negative	negative	negative	negative	negative
39	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
40	**positive**	**positive**	negative	negative	negative	negative	**positive**	negative	negative	negative
41	**positive**	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative
42	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
43	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
44	**positive**	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
45	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative
46	negative	**positive**	negative	negative	negative	negative	negative	negative	negative	negative

Table 3
TTV contigs analysis.

Contig	GB number	Length (bp)	Region	Blastx identity	Query cover	Best-hit Sequence/GenBank number	Country	Genus
TTV_LV04	MN272072	2886	complete genome	98.4%	60%	ctfd011 / AYP28841.1	USA	*Iotatorquevirus*
TTV_LV05	MN272071	2824	complete genome	98.7%	61%	lung3 / ADN28685	China	*Kappatorquevirus*
TTV_LV06	MN272073	2626	complete genome	99.4%	65%	ARA91694 / BR/RS05/2008	Brazil	*Kappatorquevirus*
TTV_LV07	MN272113	1920	complete ORF1	99.9%	99%	TTSuV 1a RS-12_A / AXV43498	Brazil	*Iotatorquevirus*
TTV_LV08	MN272112	1923	complete ORF1	93.59%	91%	PoTTV_VIRES_SC01_C1 / QBA84165.1	China	*Iotatorquevirus*
TTV_LV10	MN272111	1920	complete ORF1	98.09%	99%	PTTV1b-VA / ADD46846.1	USA	*Iotatorquevirus*
TTV_LV11	MN272110	1872	complete ORF1	96.71%	99%	TTV2Gx1/ADN28637.1	China	*Iotatorquevirus*
TTV_LV12	MN272109	1938	complete ORF1	98.98%	99%	TTSuV 1a RS-2 / AXV43470.1	Brazil	*Iotatorquevirus*
TTV_LV13	MN272108	1923	complete ORF1	99.53%	82%	ctch012 / AYP28726.1	USA	*Iotatorquevirus*
TTV_LV14	MN272107	1950	complete ORF1	99.49%	99%	ctcj010 / AYP28896.1	USA	*Iotatorquevirus*
TTV_LV15	MN272106	1920	complete ORF1	98.59%	99%	1p / YP_003587901	Brazil	*Iotatorquevirus*
TTV_LV16	MN272105	1932	complete ORF1	98.63%	99%	TTV1Fj3 / ADN28549.1	China	*Iotatorquevirus*
TTV_LV17	MN272104	1908	complete ORF1	98.43%	99%	FJ/China/2009/TTV1/24 / AFJ75543.1	China	*Iotatorquevirus*
TTV_LV18	MN272103	1947	complete ORF1	99.38%	99%	TTSuV 1a RS-12_B / AXV43502.1	Brazil	*Iotatorquevirus*
TTV_LV19	MN272102	1902	complete ORF1	99.37%	99%	TTsuk2b-gx249 / AXE73125.1	China	*Kappatorquevirus*
TTV_LV20	MN272101	1902	complete ORF1	71.99%	92%	TTSuV 1a RS-4 / AXV43478.1	Brazil	*Iotatorquevirus*
TTV_LV21	MN272100	1923	complete ORF1	99.81%	82%	PoTTV_VIRES_SC01_C3/QBA84167.1	China	*Iotatorquevirus*
TTV_LV22	MN272099	1920	complete ORF1	99.06%	99%	TTV1Bj2–2 / ADN28517.1	China	*Iotatorquevirus*
TTV_LV23	MN272098	1932	complete ORF1	99.53%	99%	TTSuV1-KU1 / ALD62438.1	Japan	*Iotatorquevirus*
TTV_LV25	MN272097	1875	complete ORF1	97.28%	86%	TTSuV2-KU11 / ALD62442.1	Japan	*Kappatorquevirus*
TTV_LV26	MN272096	1887	complete ORF1	98.25%	99%	BR/RS08/2008/ARA91703.1	Brazil	*Kappatorquevirus*
TTV_LV27	MN272095	1947	complete ORF1	98.77%	99%	TTsu1a-gx72 / AXE73115.1	China	*Iotatorquevirus*
TTV_LV28	MN272094	1875	complete ORF1	92.21%	92%	BR/RS03/2008 / ARA91688.1	Brazil	*Kappatorquevirus*
TTV_LV29	MN272093	1920	complete ORF1	98.75%	99%	swSTHY-TT27 / ACR57080.1	Canada	*Iotatorquevirus*
TTV_LV30	MN272092	1950	complete ORF1	97.07%	87%	ctba017 / AYP28833.1	USA	*Iotatorquevirus*
TTV_LV31	MN272091	1947	complete ORF1	97.38%	99%	TTV1Bj2–1 / ADN28513.1	China	*Iotatorquevirus*
TTV_LV32	MN272090	1920	complete ORF1	98.75%	99%	PTTV1b-VA / ADD46846.1	USA	*Iotatorquevirus*
TTV_LV33	MN272089	1815	complete ORF1	99.32%	94%	TTSuV2-KU10 / ALD62450.1	Japan	*Kappatorquevirus*
TTV_LV40	MN272077	1835	partial ORF1 region	98.85%	99%	TTSuV 1a RS-6 / AXV43488.1	Brazil	*Iotatorquevirus*
TTV_LV41	MN272074	1820	partial ORF1 region	98.02%	99%	ctcj014 / AYP28865.1	USA	*Iotatorquevirus*
TTV_LV43	MN272075	1776	partial ORF1 region	91.94%	89%	TTSuV 1a RS-6 / AXV43488.1	Brazil	*Iotatorquevirus*
TTV_LV44	MN272078	1771	partial ORF1 region	95.23%	92%	ctfd011 / AYP28841.1	USA	*Kappatorquevirus*
TTV_LV45	MN272079	1747	partial ORF1 region	95.74%	96%	TTV1Bj2–1 / ADN28513.1	China	*Iotatorquevirus*
TTV_LV49	MN272080	1699	partial ORF1 region	91.52%	97%	BR/RS05/2008 / ARA91694.1	Brazil	*Kappatorquevirus*
TTV_LV52	MN272081	1641	partial ORF1 region	99.08%	99%	TTSuV 1a RS-6 / AXV43488.1	Brazil	*Iotatorquevirus*
TTV_LV57	MN272076	1565	partial ORF1 region	99.12%	87%	TTSuV1-JX3 / ALO77767.1	China	*Iotatorquevirus*
TTV_LV58	MN272082	1557	partial ORF1 region	98.46%	99%	FJ/China/2009/TTV1/24 / AFJ75543.1	China	*Iotatorquevirus*
TTV_LV61	MN272084	1461	partial ORF1 region	97.53%	99%	TTSuV 1a RS-1 / AXV43466.1	Brazil	*Iotatorquevirus*
TTV_LV63	MN272085	1491	partial ORF1 region	96.43%	95%	TTV2Bj7–3 / ADN28597.1	China	*Kappatorquevirus*
TTV_LV66	MN272086	1487	partial ORF1 region	99.19%	99%	TTV2Bj8 / ADN28625.1	China	*Kappatorquevirus*
TTV_LV67	MN272087	1458	partial ORF1 region	99.38%	99%	TTsuk2a-gx163 / AXE73123.1	China	*Kappatorquevirus*
TTV_LV68	MN272114	1437	partial ORF1 region	97.49%	99%	TTV2Bj6–2 / ADN28589.1	China	*Kappatorquevirus*
TTV_LV74	MN272115	1408	partial ORF1 region	99.79%	99%	FJ/China/2009/TTV1/9 / AFJ75540.1	China	*Iotatorquevirus*
TTV_LV77	MN272088	1386	partial ORF1 region	97.83%	99%	38E05 / AFU64562.1	New Zeland	*Kappatorquevirus*
TTV_LV99	MN272116	1127	partial ORF1 region	99.20%	99%	FJ/China/2009/TTV1/24 / AFJ75543.1	China	*Iotatorquevirus*
TTV_LV152	MN272117	921	partial ORF1 region	94.79%	100%	TTSuV 1a RS-1 / AXV43466.1	Brazil	*Iotatorquevirus*
TTV_LV209	MN272118	823	partial ORF1 region	86.13%	99%	TTSuV 1a RS-7 / AXV43490.1	Brazil	*Iotatorquevirus*
TTV_LV299	MN272119	726	partial ORF1 region	97.93%	100%	PoTTV_VIRES_GD02_C12 / QBA84010.1	China	*Kappatorquevirus*
TTV_LV339	MN272120	698	partial ORF1 region	85.78%	99%	BR/RS08/2008 / ARA91703.1	Brazil	*Kappatorquevirus*
TTV_LV443	MN272121	642	partial ORF1 region	95.31%	99%	PoTTV_VIRES_GD02_C2 / QBA84000.1	China	*Kappatorquevirus*
TTV_LV537	MN272122	598	partial ORF1 region	96.84%	95%	PoTTV_VIRES_GX01_C2 / QBA84029.1	China	*Kappatorquevirus*
TTV_LV552	MN520232	592	partial ORF1 region	100.00%	99%	PoTTV_VIRES_SC01_C1 / QBA84165.1	China	*Iotatorquevirus*
TTV_LV660	MN520233	557	partial ORF1 region	93.51%	99%	TTV2Bj7–2 / ADN28577.1	China	*Kappatorquevirus*
TTV_LV664	MN520234	556	partial ORF1 region	99.46%	99%	SC / ADW01383.1	China	*Kappatorquevirus*
TTV_LV959	MN520235	496	partial ORF1 region	95.73%	99%	PoTTV_VIRES_GD02_C16 / QBA84014.1	China	*Iotatorquevirus*
TTV_LV1181	MN520236	462	partial ORF1 region	90.26%	100%	PoTTV_VIRES_GD02_C16 / QBA84014.1	China	*Kappatorquevirus*
TTV_LV1233	MN520237	456	partial ORF1 region	98.01%	99%	PoTTV_VIRES_AH01_C8 / QBA83979.1	China	*Iotatorquevirus*
TTV_L1388	MN520238	442	partial ORF1 region	98.64%	99%	TTV2Jx2 / ADN28657.1	China	*Kappatorquevirus*
TTV_L1427	MN520239	437	partial ORF1 region	97.24%	99%	PoTTV_VIRES_AH01_C8 / QBA83979.1	China	*Kappatorquevirus*
TTV_L1561	MN284870	426	partial ORF1 region	97.84%	97%	TTV2Bj7–2 / ADN28577.1	China	*Kappatorquevirus*
TTV_L1573	MN284871	425	partial ORF1 region	93.55%	87%	PoTTV_VIRES_AH01_C7 / QBA83978.1	China	*Iotatorquevirus*
TTV_L1866	MN284872	404	partial ORF1 region	99.25%	99%	PoTTV_VIRES_SC01_C3 / QBA84167.1	China	*Iotatorquevirus*
TTV_L1870	MN284873	403	partial ORF1 region	95.52%	99%	PoTTV_VIRES_SX02_C3 / QBA84214.1	China	*Iotatorquevirus*
TTV_L1961	MN284874	397	partial ORF1 region	93.18%	98%	PoTTV_VIRES_SC02_C7 / QBA84177.1	China	*Kappatorquevirus*
TTV_2133	MN284875	387	partial ORF1 region	96.09%	99%	PoTTV_VIRES_GD01_C2 / QBA83992.1	China	*Kappatorquevirus*
TTV_L2203	MN284876	384	partial ORF1 region	97.64%	99%	TTSuV 1a RS-4 / AXV43481.1	Brazil	*Iotatorquevirus*
TTV_L2280	MN284877	381	partial ORF1 region	99.21%	100%	PoTTV_VIRES_CQ01_C4 / QBA83986.1	China	*Iotatorquevirus*

length) shared approximately 94% nucleotide identity between them and 90% nucleotide identity with the swine TTSuVk2 sequence detected in Argentina (GenBank accession number: JF451617).

Three full-length TTSuV genomes (TTV_LV04; TTV_LV05; TTV_LV06) and 11 nearly complete genomes – including the complete ORF1 coding region – were generated by HTS. These data allowed genetic analysis according to ICTV, based on the full ORF1 nt sequence. The complete TTSuV genomes recovered (GenBank accession number: MN272071 to MN272073) were 2886, 2824 and 2263 bp in length, with 1133×, 426× and 442× average coverage, respectively. The
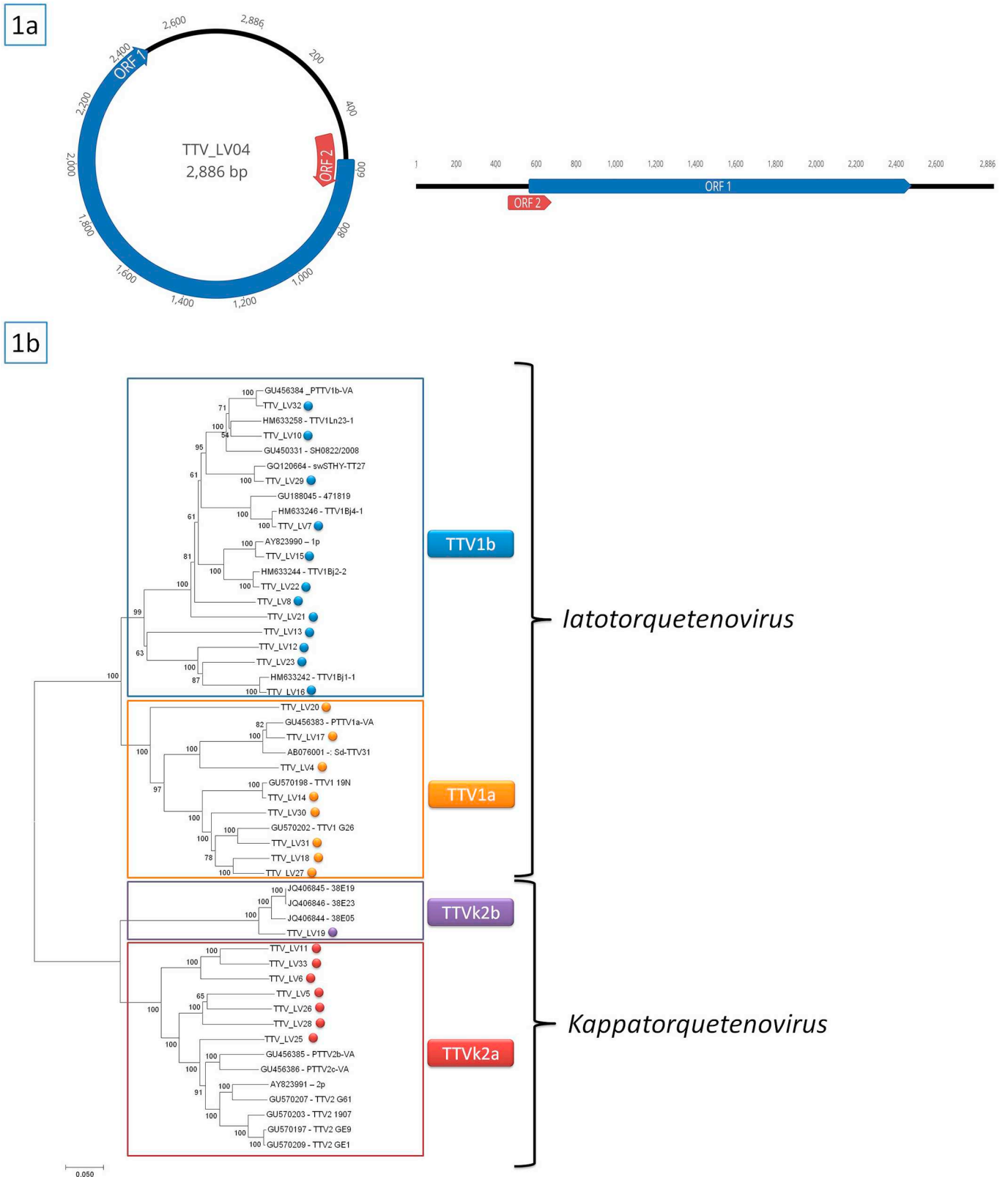
Fig. 2. Genetic characterization of *Torque teno sus virus 1a*. A. Genome organization of the complete sequenced TTV_LV04 genome. B. Complete ORF1 phylogenetic tree based on the neighbor-joining method applied through the p-distance model. Analyses were conducted with 1000 bootstrap replicates. The sequences obtained from this study are indicated by colored dots.

genomes were arranged in a circular form (Fig. 2a), with an overall GC content ranging from 40 to 42%, which is similar to the characteristics of other TTSuVs.

Phylogenetic analysis was performed with 28 ORF1 complete nucleotide contigs sequenced in our study and 23 reference sequences from *Iotatorquevirus* and *Kappatorquevirus*, showing two well-separated clusters corresponding to both genera. Each of these clusters presented two main groups corresponding to TTSuV species (TTSuV1a and

TTSuV1b, TTSuVk2a and TTSuVk2b). Eight sequences were classified as TTSuV1a, which shared 63–90% nucleotide identity; twelve were classified as TTSuV1b, which presented 65–90% identity; seven were classified as TTSuV2a, which shared 68–86% nucleotide identity; and one was classified as TTSuVk2b (Fig. 2b). The TTV_LV04 full-length genome was classified as TTSuV1a, while LV05 and LV06 were both classified as TTSuVk2b.

3.3. Circoviridae: Porcine circovirus (PCV1 and PCV2)

In the liver samples submitted for HTS, the presence of two contigs closely related to the *Circoviridae* family were observed: one was classified as *Porcine circovirus 1* (PCV1) and the other as *Porcine circovirus 2* (PCV2) following the ICTV instructions (Cotmore et al., 2014). To verify the PCV frequency, a specific PCR protocol to analyze samples individually revealed a frequency of 10.8% (5/46) positive livers. All positive sequences were submitted to Sanger sequencing, which confirmed the presence of PCV1 (4/5–80%) and PCV2 (1/5–20%). The four PCV1 sequences (600 bp in length) shared approximately 99% nucleotide identity between them and approximately 98% nucleotide identity with a swine PCV1 strain isolated in Japan (GenBank accession number: KJ808815). The PCV2 sequence (390 bp in length) presented 99% nucleotide identity with a swine PCV2 isolated in Japan (GenBank accession number: LC433844).

HTS analysis revealed a PCV1 full-length contig (PCV1_LV34 – GenBank accession number: MN508363) that was 1759 bp in length, with the first Brazilian PCV1 complete genome described. It was arranged in a circular form, with an overall GC content of 48.7%, which was similar to the characteristics of other PCVs. The average coverage was 110×. The circular genome displayed the archetypal genome organization (Fig. 3a) of PCVs with two major open-reading frames. ORF1 (*rep* gene) is transcribed in a clockwise direction and encodes two viral replication-associated proteins, Rep and Rep', and ORF2 (*cap* gene) is transcribed in an anti-clockwise direction and encodes the immunoprotective capsid protein of the virus (Hamel et al., 1998; Mankertz et al., 1997). The complete PCV1_LV34 genome presented 99% nucleotide identity with the Chinese strain GXdx51 (GenBank accession number: KX827781). The sequence was aligned with 22 PCV1 and one PCV2 representative complete genomes retrieved from GenBank. Phylogenetic reconstruction was inferred and showed two clearly separated groups corresponding to PCV1 and PCV2, and our sequence clustered with the PCV1 sequences, corroborating the results (Fig. 3b).

Currently, PCV2 sequences that share a high level of nucleotide identity are classified into at least five distinct genotypes based on the ORF2 sequence: PCV2a, PCV2b, PCV2c, PCV2d (Xiao et al., 2019), and the newly identified genetic group PCV2e (Davies et al., 2016). The *PCV2 contig (PCV2_LV1411) generated by HTS (1411 nt in length) was mapped to a Chinese PCV2e sequence (MF589524), which shares 97.78% nucleotide identity, corresponding to partial rep and capsid coding regions (Fig. 4a)*. The average coverage was 51×. ORF2 was aligned with 20 representative PCV2 sequences of all subtypes retrieved from GenBank, and the phylogenetic tree showed that the PCV2 sequence detected in this study clustered into the new PCV2e genotype (Fig. 4b).

3.4. Parvoviridae: Ungulate copiparvovirus 2 (PPV4), unclassified PPV6, unclassified PPV7, Ungulate bocaparvovirus 2 (PBoV1) and Ungulate bocaparvovirus 5 (PBoV3)

In the samples submitted for HTS, we detected three contigs closely related to PPV7 (1295, 1037 and 430 nt), one contig closely related to PPV6 (579 nt) (Fig. 5a) and two contigs closely related to PPV4 (471 and 457 nt) (Fig. 5b). To verify the PPV frequency, PCR detection followed by Sanger sequencing was performed on individual samples resulting in 13% (6/46) of them positive for PPV [PPV4 (1/6–17%), PPV6 (1/6–17%) and PPV7 (4/6–66%)].

Both PPV4 contigs presented 99% nucleotide identity with the PPV4

sequence obtained from a wild boar in Romania (unpublished/GenBank accession number: JQ868714). The PPV6 contig presented 100% nucleotide identity with a Chinese strain (unpublished/GenBank accession number: MG760726), and the PPV7 contigs were closely related (approximately 96% identity) to Chinese PPV sequences (GenBank accession numbers: MG543466 and MG543470) (Fig. 5).

The PPV7 contigs generated in this study were 2763 nt in length (63% of the complete genome when mapped to a PPV7 reference sequence). To characterize the sequence generated herein, since this is the first PPV7 detected in South America, specific primers were designed to sequence the complete PPV7 genome (BR_RSPPV7) (Table 1). By overlapping the sequences acquired using the Sanger method with the three contigs obtained by HTS, it was possible to generate the complete BR_RSPPV7 genome; the genome was 3958 bp in length (Fig. 6a) and arranged with an overall GC content of 54.9%, which was similar to the characteristics of other PPVs. The genome was aligned with representative *Parvoviridae* sequences retrieved from GenBank, and a phylogenetic tree was constructed showing 13 clearly separated genera. BR_RSPPV7 clustered with PPV7 sequences within the recently proposed *Chappaparvovirus* genus (Palinski et al., 2016) (Fig. 6b).

We also detected the presence of two contigs closely related to *Bocaparvovirus* genus members in two different species. One contig 427 nt in length (BR_PBoV3) showed 91% nucleotide identity with the porcine bocavirus 3 (*Ungulate bocaparvovirus 5*) sequence IA2F18-1 from Belgium (GenBank accession number: KF025521). A second contig 551 bp in length (BR_PBoV1) presented 99% nucleotide identity with the porcine bocavirus 1 (*Ungulate bocaparvovirus 2*) isolate KU14 from South Korea (GenBank accession number: KJ622366). Specific PCRs performed to identify PBoV1 and PBoV3 revealed a frequency of one sample positive for PBoV1 (1/46–2%) and two samples positive for PBoV3 (2/46–4%) (Fig. 7).

4. Discussion

Monitoring viruses in human and animal species is essential to better understand emerging infectious diseases that are foci under the "one-health" approach (Childs et al., 2009; "Contributing to One World, One Health ₓ A Strategic Framework for Reducing Risks of Infectious Diseases at the Animal – Human – Ecosystems Interface,", 2008; Omingo, 2010). In the present study, we performed high-throughput sequencing to uncover the virome of livers collected from slaughtered pigs. Food products containing pork liver have been repeatedly suspected to be the source of zoonotic agents, mainly hepatitis E virus (Berto et al., 2012; Colson et al., 2010; Szabo et al., 2015). Our results showed the absence of zoonotic viruses in the livers collected from a small slaughterhouse in southern Brazil but revealed a high diversity of ssDNA viruses.

Previous swine virome studies were performed in feces (Amimo et al., 2016; Shan et al., 2011), in which RNA viruses (*Picornaviridae, Astroviridae, Coronaviridae, Caliciviridae* and *Reoviridae* families) and DNA viruses (*Circoviridae* and *Parvoviridae* families) were found, and in tonsils (Blomström et al., 2018), in which RNA (*Picornaviridae, Astroviridae, Caliciviridae, Retroviridae* families) and DNA viruses (*Anelloviridae, Circoviridae, Parvoviridae* and *Herpesvirida* families) were reported. The DNA viruses reported herein showed similar results (viruses from *Anelloviridae, Circoviridae* and *Parvoviridae* families).

Until now, no research has been conducted to investigate the liver virome as a unique sample. Recent metagenomic study was realized in order to compare healthy and Periweaning Failure to Thrive Syndrome (PFTS) pigs, but one of the analysis was performed on an organ pool (including liver, kidney and lung). In this study they found similar DNA viruses as we found (from *Parvoviridae* and *Circoviridae* families). They also reported RNA viruses (Franzo et al., 2018), but once the analysis was performed in a pool, we do not know if these viruses were indeed isolated from the liver. In our study we didn't find RNA virus even when the experiment was repeated, showing that there was probably no RNA
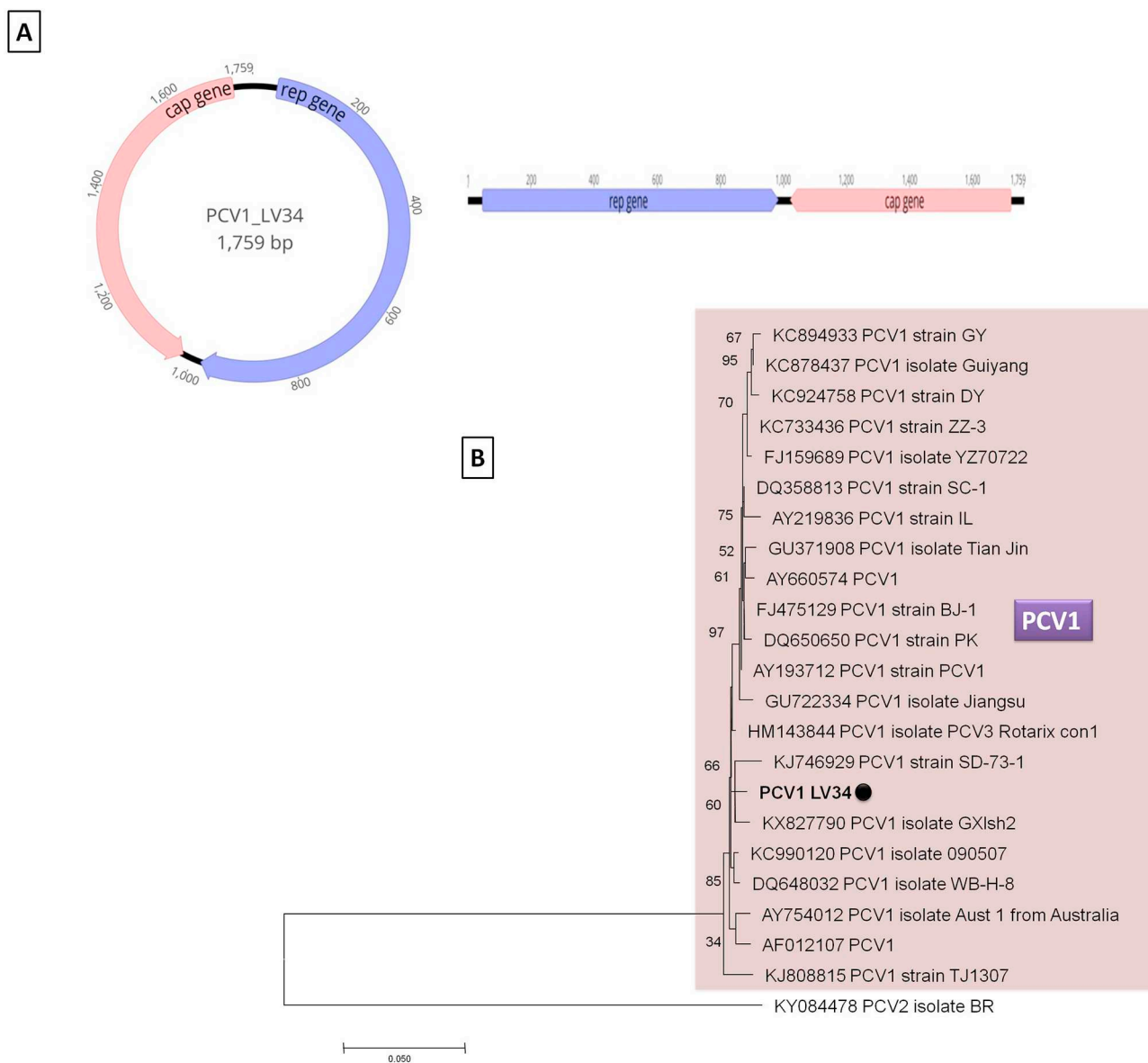
Fig. 3. Genetic characterization of PCV1. A. Genome organization of the complete PCV1_LV34 sequence. B. A complete genome phylogenetic tree was inferred with the maximum likelihood method, Kimura-2 parameter. All analyses were conducted with 1000 bootstrap replicates. The sequence obtained from this study is indicated by the black dot.

virus in the liver at the time of the sample collection.

Our study described the presence of swine viruses that are commonly found in other samples, such as members of *Anelloviridae* and *Circoviridae* families. Moreover, we described the first complete genome of a Brazilian *Porcine circovirus 1* (PCV1), and the first sequences of *Porcine parvovirus 6* (PPV6) and *Porcine parvovirus 7* (PPV7) from South America. The high frequency of DNA viruses herein is in contrast to other metagenomics surveys performed with swine fecal samples, which were dominated by RNA viruses (Shan et al., 2011; Zhang et al., 2014).

Anelloviridae, the family most commonly detected in our study, is a family of single-stranded circular DNA viruses that infects mammals (Okamoto et al., 1998) and comprises 14 genera, including human and animal anelloviruses. Torque teno virus (TTV) was originally isolated in the 1990s from a human patient with posttransfusion hepatitis of unknown etiology (Nishizawa et al., 1997) and since then, has been detected in several apparently healthy animals (Nieto et al., 2011; Manzin et al., 2015; Shan et al., 2011). To date, two genera have been described to infect swine, *Iotatorquevirus* and *Kappatorquevirus*, each comprising

two species, TTSuV1a and -b and TTSuVk2a and -b, respectively. In the present work, TTSuVs were detected in 52% of the swine livers, corroborating previous studies that showed prevalence ranging from 24 to 100% in pigs worldwide (Kekarainen and Segale, 2012; Mckeown et al., 2004), including healthy animals (Kekarainen et al., 2006). Its contribution as a cofactor in postweaning multisystemic wasting syndrome (PMWS) is controversial since some studies suggest that coinfection with PCV2 is related to PMWS (Kekarainen et al., 2006; Rogers et al., 2017), while other studies found no relationship between PMWS and TTSuV (Fumaco et al., 2015; Lee et al., 2010). Our analysis presented a large diversity of TTSuVs in the livers of healthy pigs, showing that the four species are present in southern Brazil. In addition, we characterized three new complete genomes: one classified as TTSuV1a, and two classified as TTSuVk2b.

There are three species of the *Circovirus* genus that infect pigs: PCV1 was first isolated as a PK/15 cell culture contaminant in the 1970s (Tischer et al., 1974) and is widely acknowledged to be nonpathogenic, despite a study that showed that experimental infection of PCV1 could produce lesions in lungs of porcine fetuses and have an impact on
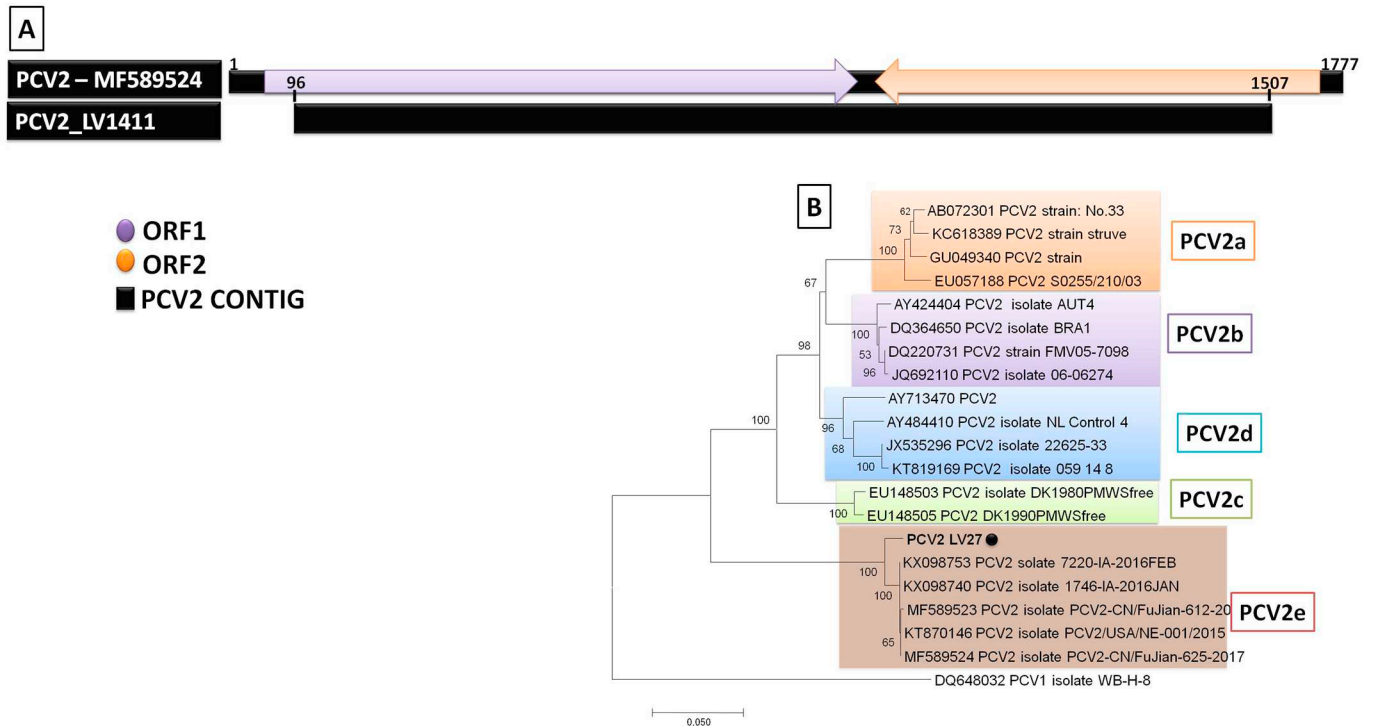
Fig. 4. Genetic characterization of PCV2. A. Schematic image showing the PCV2 contig obtained herein mapped to the Chinese PCV2 sequence retrieved from GenBank (accession number: MF589524). B. Capsid gene phylogenetic tree inferred with the maximum likelihood method, Kimura-2 parameter. All analyses were conducted with 1000 bootstrap replicates. The sequence obtained from this study is indicated by the dot.

porcine alveolar macrophages (Saha et al., 2011). To date, PCV1 has never been described in Brazil, but in the present work, we were able to detect it in four liver samples of healthy pigs and to characterize the first complete genome of a Brazilian PCV1. PCV2, which was detected in only one liver sample, is associated with a series of porcine circovirus-associated diseases, such as the PMWS (Harding et al., 1998), generating worldwide economic losses in pig production (Baekbo et al., 2012). "Porcine circovirus-associated disease" is a collective term that describes a number of conditions associated with PCV2 infections (Allan et al., 2000; Drummond and Rambaut, 2007; Segalés, 2012). Finally, PCV3 is a novel circovirus that was recently described in association with porcine dermatitis and nephropathy syndrome, reproductive failure, and multisystemic inflammation (Ye et al., 2018), but it was not detected in our study.

The *Parvovirinae* subfamily within the *Parvoviridae* family comprises eight genera, five of which include parvoviruses of swine. Porcine parvoviruses are ubiquitous DNA viruses that infect a wide range of animal species. Although some of them can cause severe clinical signs, such as parvovirus B19 in humans, feline panleukopenia virus and canine parvovirus type 2 (currently classified as the same species) in cats and dogs, and PPV1 in pigs, the majority of PPVs have not been associated with any disease (Xiao et al., 2013). The five species of PPVs detected in the present study (PPV4, PPV6, PPV7, PBoV1 and PBoV3)

have not been associated with clinical disease, although they can be present in complex multifactorial diseases. The importance of novel porcine parvoviruses for pig health is poorly understood, and their presence in healthy animals raises the hypothesis that they are commensals (Streck et al., 2015).

In 2010, PPV4 was identified for the first time in the lung lavage of a diseased pig coinfected with PCV2 in China (Cheung et al., 2010). Since then, it has been described in several biological samples of both healthy and sick pigs (Huang et al., 2010; Zhang et al., 2011). It was also detected in Brazilian herds in several biological samples, including semen, lungs, feces, uterus and ovaries, with a frequency of detection of 5.5% (Gava et al., 2015). PPV6 was detected for the first time in aborted pig fetuses in China in 2014, but a retrospective epidemiological study showed that its prevalence was similar in healthy and sick pigs (Ni et al., 2014). Later, it was detected in serum from pigs infected with porcine reproductive and respiratory syndrome virus (PRRS) in the USA and Mexico (Schirtzinger et al., 2015) and in serum samples of pigs in Poland (Cui et al., 2017). PPV7 was first reported in 2016 in rectal swabs of healthy adult pigs in the USA (Palinski et al., 2016) and subsequently in Poland and Korea (Milek et al., 2018; Ouh et al., 2018). In a Chinese study, serum samples from healthy pigs were tested for PPV7 and PCV-2, resulting in a coinfection rate of 17.4% (Wang et al., 2019). In the present study, we detected one (2.2%) positive liver
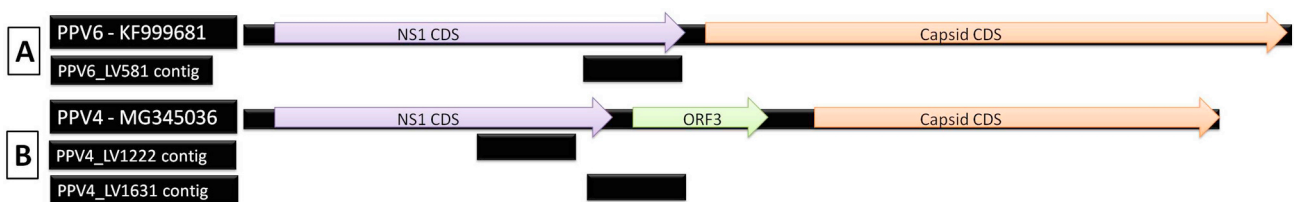

Fig. 5. PPV6 and PPV4 contigs mapped to reference sequences. A) The PPV6 contig (PPV6_LV581 – GenBank accession number: MN557417) was 451 bp in length and mapped no PPV reference sequence (KF999681). B) PPV4 contigs (PPV4_LV1222 and PPV4_LV1613 – GenBank accession numbers: MN557418 and MN557419, respectively) mapped to the PPV4 reference sequence (MG345036) and were 450 and 421 bp in length.
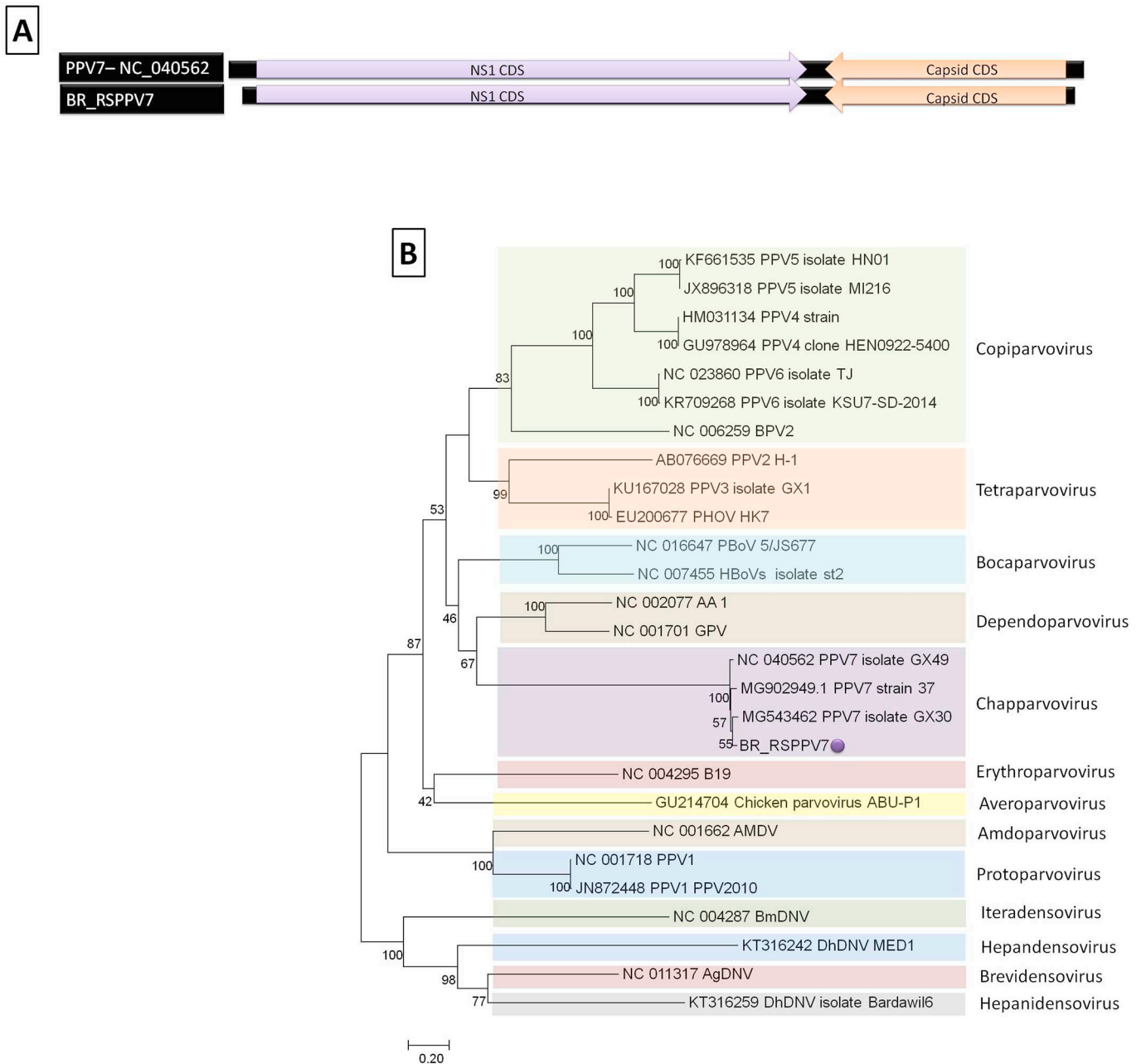
Fig. 6. Genetic characterization of PPV7. A. Schematic image showing the PPV7 sequence obtained herein (BR_RSPPV7 – GenBank accession number: MN515032) and mapped to the reference PPV7 sequence retrieved from GenBank (accession number: NC_040562). B. Complete genome phylogenetic tree constructed with the maximum likelihood method, Kimura-2 parameter. Analyses were conducted with 1000 bootstrap replicates. The sequence obtained from this study is indicated by the purple dot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sample for PPV4, one (2.2%) positive for PPV6 and four (8.7%) positive for PPV7. This is the first detection of PPV6 and PPV7 genomes in South American pigs and the first full-length PPV7 genome characterized.

PBoV1 and PBoV3 are recently discovered bocaparvoviruses that infect pigs. Porcine bocavirus was accidentally discovered in 2009 in Sweden, as it induced PMWS in pigs (Blomstrom et al., 2009). Its importance in pig health and production is yet to be determined. PBoVs are widespread and have been detected in China (Zhang et al., 2011), Ireland (Gunn et al., 2015), Korea (Choi et al., 2013), Uganda (Blomstrom et al., 2013), Cameroon (Ndze et al., 2013) and the United States (Huang et al., 2014). In 2016, a report suggested that PBoV1 was associated with a case of encephalomyelitis in 6-week-old piglets in Germany (Pfankuche et al., 2016). It was also reported that PBoVs and PCV2, which contribute to porcine diarrheal disease, have a high rate of

coinfections in China (Zhai et al., 2010). To the best of our knowledge, this is the first detection of PBoV1 and PBoV3 in South America.

In the present study, we found a high rate of coinfection for all virus species that were tested individually (54%, 25/46). Only tree samples (9%) were negative for all viruses, and 18 (39%) were positive for only one virus species. No samples were coinfected with more than one species of parvovirus or coinfected with parvovirus and PCV1 or PCV2. Viral surveys in animals are important since they can readily document the circulation of known and new viruses, facilitating the detection of emerging viruses and prospective evaluation of their pathogenic and zoonotic potentials. The high level of *Anelloviridae* and *Circovirus* resembles other studies, but the results herein are better in terms of the number of samples examined. The results however confirm that the viruses are endemic in this herd.
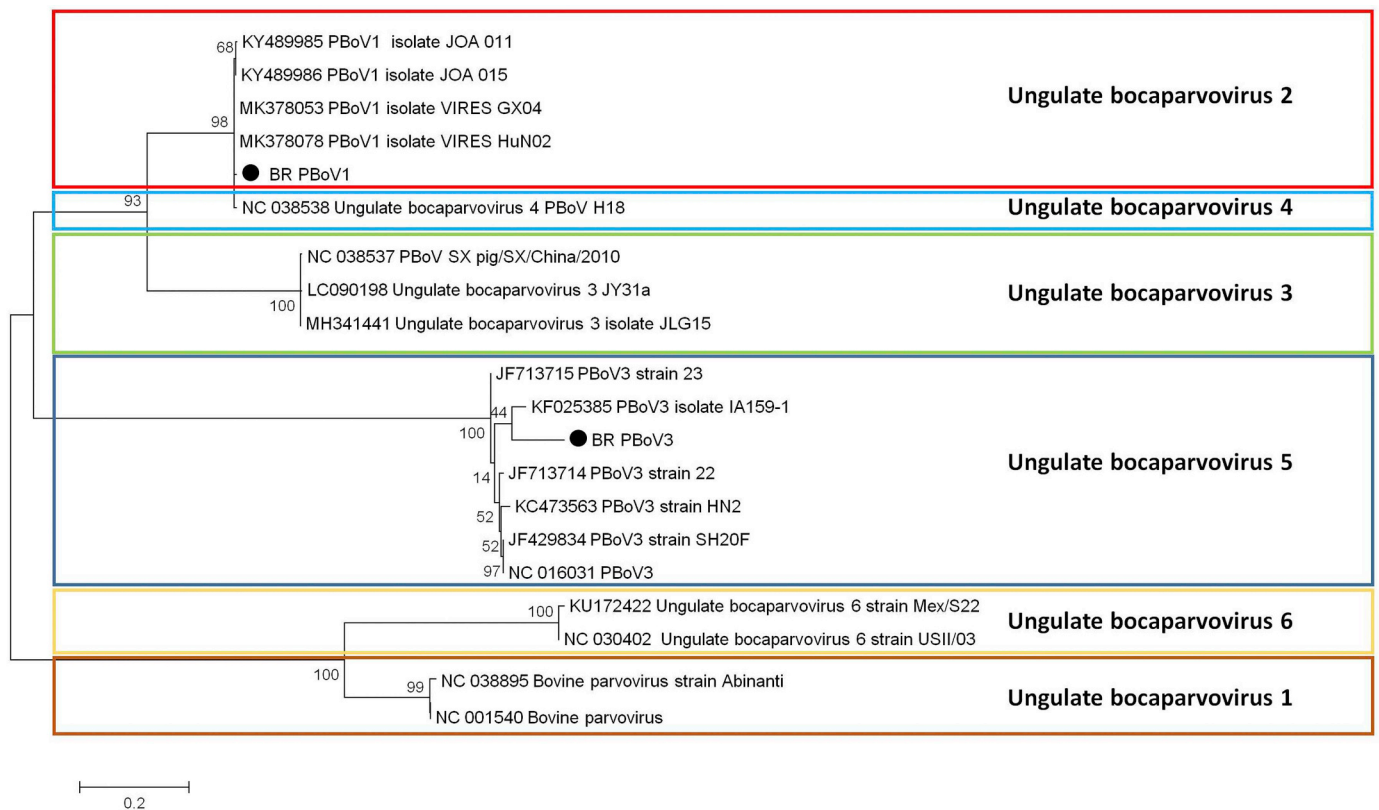
Fig. 7. Genetic characterization of PBov. Phylogenetic tree constructed with the maximum likelihood method based on the Hasegawa-Kishino-Yano model. Analyses were conducted with 1000 bootstrap replicates. The sequence obtained from this study is indicated by the black dot. (BR_PBoV2 - GenBank accession number: MN557420/ BR_PBoV1 - GenBank accession number: MN557421).

5. Conclusion

To our knowledge, this is the first study to investigate the liver virome of healthy pigs. Our findings indicate a highly diverse population of *Anelloviridae, Circoviridae* and *Parvoviridae* members. No zoonotic viruses were detected. HTS enabled the detection of viruses that had not been reported in Brazil until now, such as *Porcine circovirus 1, Porcine parvovirus 6, Porcine parvovirus 7, Porcine bocavirus 1* and *Porcine bocavirus 3*. Coinfections were frequent, similar to results described in previous studies. The results of this study will provide a foundation for future works as they describe virus populations in healthy pig livers, supporting studies in sick animals.

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Ethics approval

All samples were collected according to the project approved by the Universidade Federal do Rio Grande do Sul Ethics Committee (number 28460).

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

Acknowledgments

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3.2.2 Artigo Científico: "*New Polyomavirus Species Identified in Nutria, Myocastor coypus Polyomavirus 1*"

Artigo publicado em 2018, na revista "*Archives of Virology*", fator de impacto: 2.230.

ANNOTATED SEQUENCE RECORD

CrossMark

New polyomavirus species identified in nutria, Myocastor coypus polyomavirus 1

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Abstract
A novel polyomavirus (PyVs) comprising 5,422 bp was identified by high-throughput sequencing (HTS) in pooled organs of nutria (*Myocastor coypus*). The new genome displays the archetypal organization of PyVs, which includes open reading frames for the regulatory proteins small T antigen (sTAg) and large T antigen (LTAg), as well as for the capsid proteins VP1, VP2 and VP3. Based on the International Committee on Taxonomy of Viruses (ICTV) *Polyomaviridae* Study Group criteria, this genome comprises a new PyVs species for the *Alphapolyomavirus* genus and is putatively named "*Myocastor coypus Polyomavirus* 1" . The complete genome sequence of this Myocastor coypus Polyomavirus 1 (McPyV1) isolate is publically available under the GenBank accession no. MH182627.

Annotated Sequence Record

Polyomaviruses (PyVs) are small and non-enveloped viruses, having circular double-stranded DNA genomes with approximately 5,000 base pairs [1]. Taxonomically, PyVs belong to the family *Polyomaviridae* and comprise four genera – the *Alphapolyomavirus*, *Betapolyomavirus*, *Gammapolyomavirus* and *Deltapolyomavirus* [2]. PyVs have been found in many hosts, including birds [3], rodents [4], cattle [5], bats [6], nonhuman primates [7], and humans [8] .

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Genome organization among PyVs is very similar. The proteins are encoded by early and late transcriptional regions separated by a non-coding control region (NCCR), which controls the transcription of the early and late promoters and regulates the initiation of viral DNA synthesis [1]. The early coding region encodes regulatory proteins, known as tumor antigens, including a large T-antigen (LTAg) and a small T-antigen (sTAg), whereas the late coding region encodes the structural proteins VP1, VP2, and VP3 [1]. According to the International Committee on Taxonomy of Viruses (ICTV) *Polyomaviridae* Study Group, the new criteria for species classification are defined through the genetic distance between the LTAg coding regions [2].

In this study, we report the detection and genome characterization of a novel PyV species in nutria (*Myocastor coypus*) using high-throughput sequencing (HTS). The nutria is a semi-aquatic rodent native to South America and has been introduced to every continent, except Antarctica and Australia, for fur and meat production [9]. The animals studied in this research came from a commercial establishment located in Rio Grande do Sul State, authorized by the IBAMA (Brazilian Institute of Environment and Renewable Natural Resources). The project was conducted under protocol number 29415 approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Federal do Rio Grande do Sul.

Samples from liver, kidney, lung, mesenteric lymph node, spleen, and intestine and rectal swabs from three apparently

healthy Brazilian nutria were pooled. The pool sample was macerated, centrifuged at low speed (1,800 x g during 30 min), filtered through a 0.45 µm filter for removal of small debris, and subjected to ultracentrifugation through a 20% sucrose cushion (200,000 x g for 4 h) [6]. The pellet was mixed with nucleases to eliminate non-capsid–protected nucleic acids [10], and viral nucleic acids were isolated using organic extraction protocols.

Viral nucleic acids were enriched using Sigma's® GenomePlex/Transplex kits. DNA fragment libraries were further prepared using a Nextera XT DNA sample preparation kit and sequenced using an Illumina MiSeq System (2 x 150 cycles run). Illumina MiSeq generated a total of 59,376 high-quality paired-end reads (average length of 142.2 nt). The sequences were trimmed and *de novo* assembled into contigs using SPAdes v3.10 (metaSPAdes) [11] and compared to known sequences in the National Center for Biotechnology Information (NCBI) databases using BLASTn/BLASTx. The total contigs with significant similarity in BLASTx/n searches belonged to the host genome, including the complete mitochondrial genome (34 contigs), as well as bacterial (26 contigs), and viral (4 contigs) genomes. Viral contigs were composed of three phages and one Eukarya-related virus (with similarity to the PyVs sequences). The

mitogenome recovered was deposited into GenBank under accession number MH182628. No BLAST hit was found for the 334 contigs (~84%).

The PyV contig recovered was 5,422 bp in length, arranged in a circular form, and with an overall GC content of 44.7%, similar to others PyVs. The average coverage was 6.3x. The circular genome displayed the archetypal genome organization of the PyVs, including coding regions of the regulatory proteins sTAg and LTAg, as well as the capsid proteins VP1, VP2 and VP3 (Fig. 1). These two regions are separated by an NCCR homologous to those of previously described polyomaviruses [12]. The LTAg was generated by alternative splicing of the early mRNA transcript [13]. The intronic region was found to be located between base positions 5,201 to 4,350.

According to the actual ICTV classification criteria, a new polyomavirus species is defined strictly on the following guidelines: i) the complete genome sequence is available in a public database (GenBank accession number MH182627); ii) the genome displays a typical PyV organization (Fig. 1); and iii) there is sufficient information on the natural host (the mitogenome was deposited in GenBank under accession number MH182628). The last criterion (iv) is related to the genetic distance that must be greater than 15% for the

Fig. 1 Schematic diagram showing the genome organization of Myocastor coypus polyomavirus 1. Putative coding regions for VP1, VP2, VP3, small T antigen (sTAg), and large T antigen (LTAg) are marked by arrows
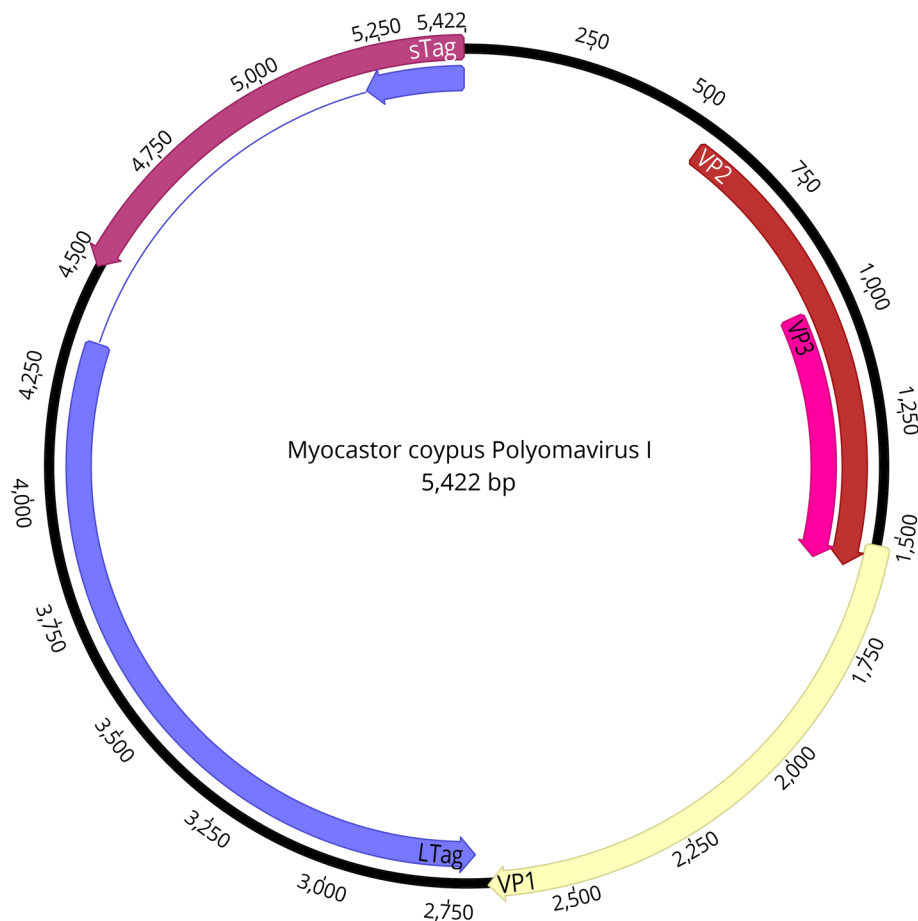
amino acid LTAg region when compared to members of the most related species. Multiple alignment analysis showed nucleotide LTAg genetic distances ranging from 44% to 56% with sequences representative of the most closely related *Alphapolyomavirus* species (species: *Human polyomavirus* 12, *Pan troglodytes polyomavirus* 5, *Acerodon celebensis polyomavirus* 1, and *Ateles paniscus polyomavirus* 1).

In order to finally classify the McPyV1 isolate as representative of a novel *Polyomavirus* species, all PyVs reference sequences were retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and multiple sequence alignments of the LTAg (Supplementary Material 2) were generated using MUSCLE [14]. Phylogenetic reconstructions were performed by applying the Maximum Likelihood on MEGA7 [15] and Bayesian methods on Phylogeny.fr:Mr.Bayes software [16] under the best model of amino acid substitution (LG+F+I+G, as determined on MEGA7) [17]. The maximum likelihood phylogeny was recorded using MEGA7 with 1,000 bootstrap replicates each.

The reconstructed phylogenetic tree of the PyVs ICTV reference sequences showed four major clusters corresponding to the *Alphapolyomavirus-, Betapolyomavirus-, Gammapolyomavirus- and Deltapolyomavirus* genera

(Fig. 2 and Supplementary Material 1). This analysis revealed the isolate representing the new species "*Myocastor coypus polyomavirus 1*" clusters within the *Alphapolyomavirus* genus. Additionally, a Bayesian phylogenetic inference was performed and revealed the same tree topology as the ML analysis (data not shown). McPyV1 was the sister taxa to a clade of virus infecting humans (HPyV12). It was observed that it is more distantly related to polyomaviruses infecting monkeys and bats.

Rodentia comprises the largest order within the class Mammalia, with approximately 40% of documented species [18]. Despite this, only 4% of the characterized PyVs have been detected in rodents. The first PyVs strain from rodents was isolated in 1978 as causing epithelial proliferations in the African multimammate mouse (Mastomy natalensis polyomavirus 1) [19]. PyVs can cause malignant and non-malignant diseases in birds and mammals, including humans, but most mammalian PyVs cause subclinical asymptomatic infections [12]. In this report, a novel genetically distinct nutria PyV was detected with no disease association. These findings will aid our understanding of the expanding genetic diversity of PyVs, mainly in the Order Rodentia.

Fig. 2 Phylogenetic tree based on the complete amino acid LTAg region. MEGA7 was used for phylogeny inference under the maximum likelihood method. The isolate representing the new "*Myocastor coypus polyomavirus* 1" species is indicated with a black dot (●). PyVs ICTV reference sequences were retrieved from the GenBank database. GenBank accession numbers are available for the phylogenetic tree
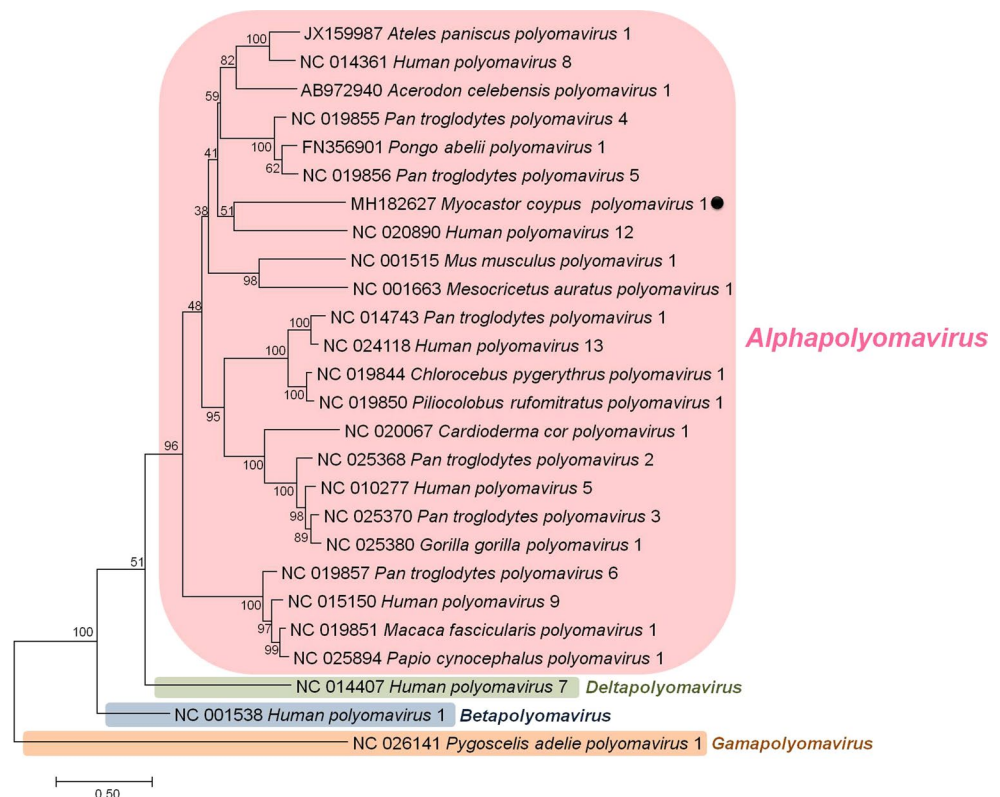
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4 CONCLUSÕES

A metagenômica viral acoplada a HTS, seguida de análises de bioinformática, foi realizada com sucesso nos três estudos de metagenômica viral, aplicada à ciência veterinária, apresentados nessa tese. A descrição e caracterização de novos e conhecidos vírus foram realizadas ampliando o conhecimento da ecologia viral nos hospedeiros estudados. Resumidamente, os três trabalhos que compõem a presente tese de doutorado, levaram as seguintes conclusões descritas abaixo:

- "*Highly Divergent Cattle Hepacivirus N in Southern Brazil*"

    - A sequência de Hepacivírus N descrita no estudo possui uma alta divergência genética quando comparada a sequências de HNV já descritas na literatura.

    - A sequência apresentou uma identidade nucleotídica entre 72,6% a 73.8% com demais sequências de HNV depositadas em bancos de dados. Os resultados sugerem que ela represente um provável novo genótipo.

- "*Liver Virome of Healthy Pigs Reveals Diverse Small ssDNA Viral Genomes*"

    - De acordo com a literatura, até o presente momento, o estudo trata-se do primeiro viroma realizado em fígados suínos.

    - As análises indicaram a presença de uma grande diversidade de vírus ssDNA pertencentes as famílias virais: *Anelloviridae*, *Circoviridae* e *Parvoviridae*.

    - A análise do viroma detectou a presença de vírus até então nunca descritos no Brasil, como PCV1, PPV6 e PPV7.

    - O estudo não detectou a presença de vírus zoonóticos.

- *"New Polyomavirus Species Identified in Nutria, Myocastor coypus Polyomavirus 1"*

- De acordo com a literatura, até o presente momento, trata-se do primeiro estudo de metagenômica viral realizado em uma espécie animal da Família *Echimyidae,* Ordem *Rodentia,* assim como primeira descrição de PyVs na espécie.

- De acordo com os critérios estabelecidos pelo ICTV, a sequência de PyVs descrita no estudo trata-se de uma nova espécie de PyVs pertencente ao gênero *Alphapolyomavirus.*

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