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

ANÁLISE MOLECULAR DE VÍRUS SUÍNOS ATRAVÉS DE ABORDAGEM METAGENÔMICA

Autora: Caroline Tochetto

PORTO ALEGRE
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ANÁLISE MOLECULAR DE VÍRUS DE SUÍNOS ATRAVÉS DE ABORDAGEM METAGENÔMICA

Autora: Caroline Tochetto

Tese apresentada como requisito parcial para obtenção do grau de Doutora em Ciências Veterinárias na área de Medicina Veterinária Preventiva e Patologia, especialidade Virologia

Orientador: Dr. Paulo Michel Roehe

Coorientadora: Dra. Fabiana Quoos Mayer

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APROVADO POR:

_____

Dr. Paulo Michel Roehe
Orientador e Presidente da Comissão

_____

Dra. Fabiana Quoos Mayer
Coorientadora e Membro da Comissão

_____

Dr. Dennis Maletich Junqueira (videoconferência)
Membro da Comissão

_____

Dr. Matheus Weber (videoconferência)
Membro da Comissão

_____

Dra. Ana Cláudia Franco (videoconferência)
Membro da Comissão

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“É importante ter um fim para uma jornada, mas é a jornada que importa no fim”.

André Buric.

RESUMO

A carne suína é a segunda maior fonte de proteína animal consumida no mundo. O sucesso na profunda expansão da suinocultura deve-se principalmente às melhorias sanitárias implantadas nos sistemas de produção intensiva. Todavia, o aumento da densidade de animais contribuiu para a emergência e reemergência de doenças virais, facilitou a transmissão viral entre os animais e promoveu o surgimento de síndromes de etiologia desconhecida ou multifatoriais, incluindo doenças respiratórias e gastrointestinais. A metagenômica viral aliada ao sequenciamento de alto desempenho (HTS) oferece uma oportunidade para identificação, monitoramento e diagnóstico de infecções virais. Por meio dessa abordagem, é possível caracterizar virtualmente todos os genomas virais presentes em determinada amostra (o viroma). A caracterização do viroma de diferentes tipos de amostras alimenta os bancos de dados com informações para análises genéticas comparativas acelerando o entendimento acerca de cadeias de transmissão, taxa de evolução viral e origem de doenças zoonóticas. Além disso, os vírus possuem papel fundamental na saúde de seus hospedeiros e explorar a diversidade viral natural dos suínos é o primeiro passo para a compreensão de síndromes de etiologia indefinida ou de origem multifatorial. Nesse trabalho, o viroma de suínos domésticos foi explorado por meio da abordagem metagenômica viral aliada ao HTS e ferramentas de bioinformática. O soro de suínos com sinais clínicos de doença respiratória revelou a presença de vários vírus de genoma circular DNA fita simples (ssDNA) codificadores de replicase (CRESS DNA) (Artigo 1), dentre os quais foram classificados nas famílias *Genomoviridae*, *Smacoviridae* (no qual um novo gênero é proposto), *Redondoviridae* e *Nenyaviridae*; outros não puderam ser classificados em nenhuma família reconhecida atualmente. Ainda nesse estudo, foram detectados vírus das famílias *Anelloviridae*, *Parvoviridae* (eucarióticos), *Microviridae* e *Inoviridae* (procarióticos). O viroma do soro de suínos clinicamente saudáveis oriundos de granjas de reprodutores suídeos certificadas (GRSC) também foi explorado e revelou vírus das famílias *Anelloviridae*, *Parvoviridae* e vários CRESS DNA não classificados. Em outro estudo ainda em andamento, o viroma de fezes de suínos com, ou sem diarreia, foi investigado e diversas famílias virais foram detectadas. Por fim, esse trabalho também apresenta a continuação de um estudo anterior que, por meio da metagenômica viral e HTS, identificou circovírus suíno tipo 3 (PCV3) em matrizes com natimortos. Aqui, a relação do PCV3 com a natimortalidade em suínos foi investigada por meio de PCR em tempo real nas amostras individuais de matrizes com ou sem natimortos, revelando que esse vírus está amplamente disseminado entre os suínos (Artigo 2). Os resultados apresentados nessa tese ampliam o conhecimento sobre a comunidade viral

presente em suínos domésticos, enriquecem os bancos de dados virais e fornecem uma base para a comparação da diversidade viral em estudos futuros.

Palavras chave: Metagenômica viral. Virômica. Viroma. *Sus scrofa*.

ABSTRACT

Pork meat is the second largest source of animal protein consumed in the world. The success in the deep expansion of pig farming is mainly due to the sanitary improvements implemented in intensive production systems. However, the increase in animal density contributed to the emergence and reemergence of viral diseases, facilitated viral transmission between animals and promoted the emergence of syndromes of unknown or multifactorial etiology, including respiratory and gastrointestinal diseases. Viral metagenomics combined with high-throughput sequencing (HTS) offers a valuable opportunity for the identification, monitoring and diagnosis of viral infections. Through this approach, it is possible to characterize virtually all viral genomes present in a given sample, the virome. The virome characterization of different types of samples feeds the databases with information for comparative genetic analyzes, accelerating the understanding about transmission chains, viral evolution rate and origin of zoonotic diseases. In addition, viruses play a fundamental role in the health of their hosts and exploring the natural viral diversity of pigs is the first step towards understanding syndromes of undefined etiology or of multifactorial origin. In this work, the domestic swine virome was explored using viral metagenomic approach combined with HTS and bioinformatics tools. Swine serum with clinical signs of respiratory disease revealed the presence of several circular single-stranded DNA (ssDNA) Rep-encoding (CRESS DNA) viruses (Article 1), which were classified into the viral families Genomoviridae, Smacoviridae (by which one new genus is proposed), Redondoviridae and Nenyaviridae; others could not be classified in any currently recognized family. Also, viruses from the families Anelloviridae, Parvoviridae (eukaryotic), Microviridae and Inoviridae (prokaryotic) were detected. The serum virome of clinically healthy pigs from certified swine breeders (GRSC) farms was also explored and revealed viruses from the Anelloviridae, Parvoviridae and several unclassified CRESS DNA families. In another study still in progress, swine feces virome with or without diarrhea was investigated and several viral families were detected. Finally, this work also presents the continuation of a previous study that, using viral metagenomics and HTS, identified porcine circovirus type 3 (PCV3) in sows with stillbirths. Here, the relationship between PCV3 and stillbirth was investigated using real-time PCR in individual samples from sows with or without stillbirths, revealing that this virus is widely disseminated in pigs (Article 2). The results presented in here expand the knowledge about the viral community in domestic swine, enrich the viral databases and provide a baseline for viral diversity comparison in future studies.

Keywords: Viral metagenomics. Viromics. Virome. Sus scrofa.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

aa	Aminoácido
ABPA	Associação Brasileira de Proteína Animal
ASFV	Vírus da peste suína africana *(African Swine Fever)*
cDNA	DNA complementar
CDRS	Complexo das Doenças Respiratórias dos Suínos
CP	Proteína do Capsídeo (*Capsid Protein*)
CRESS DNA	Vírus circulares DNA fita simples codificadores de replicase (*Circular Rep-Encoding Single-Stranded DNA Viruses)*
CSFV	Vírus da peste suína clássica (*Classical swine fever virus)*
DNA	Ácido desoxirribonucleico (*Deoxyribonucleic Acid)*
dNTP	Desoxirribonucleotídeos fosfatados (*Deoxynucleotide Triphosphates)*
DTT	*Dithiothreitol)*
EDTA	Ácido etilenodiamino tetra-acético (*Ethylenediamine Tetraacetic Acid)*
FeCl	Cloreto de ferro
GenBank	Banco de dados público de sequências genéticas do *National Center for Biotechnology Information (NCBI)*
GRSC	Granjas de Reprodutores Suídeos Certificada
HTS	Sequenciamento de alto desempenho (*High-Throughput Sequencing)*
ICTV	Comitê Internacional de Taxonomia Viral *(International Committee on Taxonomy of Viruses)*
kb	Mil pares de base
LASL	*Linker Amplification Shotgun Libraries*
LIR	Região Intergênica Longa (*Long Intergenic Region*)
MDA	Amplificação por Deslocamento Múltiplo (*Multiple Displacement Amplification*)
ML	Máxima Verossimilhança (*Maximum Likelihood*)
mM	Milimolar
mRNA	RNA mensageiro *(messenger RNA)*
NaCl	Cloreto de Sódio
NCBI	Centro Nacional de Informação Biotecnológica *(Nacional Center for Biotechnology Information)*
ng	Nanograma

nm	Nanômetro
nmol	Nanomol
nt	Nucleotídeo
ORF	Fase aberta de leitura *(Open Reading Frame)*
Ori	Origem de replicação (*Origin of Replication*)
PCR	Reação em cadeia da polimerase *(Polimerase Chain Reaction)*
PCV2	Circovírus suíno tipo 2 *(Porcine circovirus type 2)*
PCV3	Circovírus suíno tipo 3 *(Porcine circovirus type 3)*
PDCoV	Deltacoronavírus suíno *(Porcine deltacoronavirus)*
PDNS	Síndrome da dermatite e nefropatia suína *(Porcine Dermatitis and Nephropathy Syndrome)*
PEDV	Vírus da diarreia epidêmica dos suínos *(Porcine Epidemic Diarrhea virus)*
PFTS	Síndrome da falha no desenvolvimento peri-desmame *(Periweaning Failure-to-Thrive Syndrome)*
pH	Potencial hidrogeniônico
PIB	Produto interno bruto
PoCV	*Porcine associated circular DNA virus*
PEG	PoliEtilenoGlicol
PoRV	Redondovírus suíno *(Porcine redondovirus)*
PoM	*Porcine associated DNA molecule*
PPV	Parvovírus suíno *(Porcine parvovirus)*
PRRSV	Vírus da síndrome reprodutiva e respiratória dos suínos *(Porcine Reproductive and Respiratory Syndrome virus)*
PRV	Vírus da pseudoraiva (*Pseudorabies virus*)
PSC	Peste suína clássica *(Classical swine fever)*
Rep	Proteína da replicase (*Replication-associated protein*)
RNA	Ácido ribonucleico *(Ribonucleic acid)*
rpm	Rotações por Minuto
SADS-CoV	Coronavírus da síndrome da diarreia aguda em suínos *(Swine Acute Diarrhea Syndrome-coronavirus)*
SIV	Vírus da influenza suína (*Swine Influenza virus*)
SIVD	Doença vesicular idiopática suína *(Swine Idiopathic Vesicular Disease)*
SPF	Livre de Patógenos Específicos *(Specific Pathogens Free)*
SVA	Senecavírus A

ssDNA	DNA de fita simples (*Single Strand DNA*)
TFF	Filtração por fluxo tangencial
TTSuV	Torque teno vírus suíno *(Torque teno sus virus)*
USDA	Departamento de Agricultura dos Estados Unidos (*U.S. Department of Agriculture*)
µL	Microlitro
µm	Micrômetro

SUMÁRIO

1 INTRODUÇÃO

Os suínos são uma importante fonte de proteína animal, sendo a carne suína responsável por mais de um terço da carne produzida mundialmente (USDA, 2021). Apesar dos avanços sanitários que acompanharam o crescimento e expansão da suinocultura, a intensificação da atividade contribuiu para a facilitação da transmissão de micro-organismos e a emergência de novos vírus e síndromes de etiologia desconhecida (JONES et al., 2013; VANDERWAAL;DEEN, 2018). Devido à proximidade dos suínos com os criadores, esses eventos não afetam somente a saúde dos animais, causando prejuízo econômico, mas também a saúde humana (MORES;ZANELLA, 2015; VANDERWAAL;DEEN, 2018); além disso, a maioria das doenças virais emergentes são de origem zoonótica, como por exemplo, a Síndrome Aguda Respiratória Severa (SARS) (GUAN *et al.*, 2003), a Síndrome Respiratória do Oriente Médio (MERS) (AZHAR *et al.*, 2014), Influenza aviária (LAM *et al.*, 2015), Influenza suína (DAWOOD *et al.*, 2009), Ebola (EBOLA OUTBREAK EPIDEMIOLOGY, 2018) e, recentemente, a COVID-19 (ANDERSEN *et al.*, 2020).

O uso de ferramentas que proporcionam uma visão global dos vírus presentes em determinada amostra oferece uma vantagem crucial para o estudo dos vírus. Tal reconhecimento inspirou um esforço global para identificação e caracterização de novos vírus, o chamado Projeto Viroma Global [GVP] (GEOGHEGAN;HOLMES, 2017; CARROLL *et al.*, 2018). A metagenômica viral tem se mostrado uma abordagem poderosa na identificação de novos vírus, uma vez que não depende de cultivo e permite a detecção de todos os genomas virais presentes em determinada amostra (o viroma) (PEREZ-LOSADA *et al.*, 2020; TAN *et al.*, 2020). Além de ter acelerado significativamente a taxa com que novos vírus são descobertos (DUTILH *et al.*, 2017), a metagenômica viral tem proporcionado inúmeras oportunidades de estudo em diferentes áreas, por exemplo, na vigilância epidemiológica (WU *et al.*, 2016; MOHSIN *et al.*, 2021), diagnóstico de vírus emergentes e reemergentes, investigação do viroma associado a doenças complexas ou síndromes de etiologia desconhecida, entre outras (DUTILH *et al.*, 2017; KWOK *et al.*, 2020; TAN *et al.*, 2020). No entanto, o uso da metagenômica viral como ferramenta de vigilância requer conhecimento sobre o viroma "normal" dos animais (KWOK *et al.*, 2020). Além disso, conhecer o viroma é fundamental para o entendimento do papel dos vírus em indivíduos saudáveis e doentes (BARRIENTOS-SOMARRIBAS *et al.*, 2018). Nesse sentido, o viroma de animais assintomáticos tem sido estudado, revelando uma diversidade viral muito além do esperado, composta majoritariamente por vírus benéficos que constituem membros integrais do holobionte (VIRGIN, 2014; ROOSSINCK, 2015; AMIMO

et al., 2016; BLOMSTROM *et al.*, 2016; KARLSSON *et al.*, 2016; BLOMSTROM *et al.*, 2018; TOCHETTO *et al.*, 2020b; PAIM *et al.*, 2021). Ainda assim, o conhecimento sobre a diversidade viral é escasso (PAEZ-ESPINO *et al.*, 2016; ZHANG *et al.*, 2018). Isso porque grande parte das sequências obtidas são desconhecidas, isto é, não apresentam homologia com nenhuma outra sequência disponível nos bancos de dados de referência (DUTILH, 2014; BARRIENTOS-SOMARRIBAS *et al.*, 2018). Dessa forma, estudos que exploram a composição do viroma em diferentes tipos de amostras contribuem para a expansão dos bancos de dados e, consequentemente, favorecem a caracterização de mais genomas virais (PAEZ-ESPINO *et al.*, 2016; DUTILH *et al.*, 2017). Além disso, esses estudos fornecem informações importantes para o entendimento dos padrões e processos de evolução viral, transmissão entre espécies e interação vírus-hospedeiro (DELWART, 2012; ROUX *et al.*, 2019).

O presente trabalho teve por objetivo explorar o viroma de suínos domésticos provenientes de planteis comerciais do Rio Grande do Sul. O primeiro artigo trata de um estudo prospectivo onde os genomas de vírus DNA recuperados de soro de suínos domésticos com sinais clínicos de doença respiratória foi caracterizado. Para isso, foi utilizada a abordagem metagenômica aliada ao HTS e ferramentas de bioinformática. As análises revelaram uma ampla diversidade viral com destaque para os vírus CRESS DNA, os quais foram descritos em único artigo (Artigo 1) aceito para publicação no periódico *Journal of General Virology.*

O segundo artigo foi desenvolvido após um estudo de metagenômica viral ter revelado, de forma inédita no Brasil, a presença do PCV3 em um *pool* de soro de matrizes suínas que tiveram natimortos (TOCHETTO *et al.*, 2020b). Dessa forma, tornou-se mandatório investigar a presença do PCV3 nas amostras individuais de matrizes suínas com ou sem natimortos a fim de compreender o papel do PCV3 na manifestação dessa falha reprodutiva.

O apêndice A traz de forma sucinta outros achados do estudo do viroma do soro de suínos com sinais clínicos de doença respiratória (Artigo 1), incluindo vírus eucarióticos (*Anelloviridae e Parvoviridae)* e procarióticos (*Microviridae* e *Inoviridae*). Já, o apêndice B apresenta, brevemente, dados de um estudo que investigou o viroma do soro de suínos clinicamente saudáveis oriundos de Granjas de Reprodutores Suídeos Certificada (GRSC) do Rio Grande do Sul. As amostras de soro foram recebidas e armazenadas no Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) para fins de diagnóstico de rotina. Trinta e seis amostras de soro foram agrupadas em um único *pool*, processadas para purificação dos genomas virais e submetidas ao HTS.

Por fim, o apêndice C contém os dados iniciais de um estudo que investigou o viroma de fezes de suínos com (n = 15) ou sem diarreia (n = 15). Os animais foram avaliados

individualmente, totalizando 30 *datasets* de viroma. A análise dos dados desse estudo foi realizada durante Doutorado Sanduíche na Seção de Virologia da *Swedish University of Agricultural Sciences* (SLU) na cidade de Uppsala, Suécia.

As informações apresentadas nos apêndices fazem parte de estudos que foram iniciados no doutorado e que não puderam ser finalizados no tempo proposto. Todavia, pretende-se organizá-los para publicação em revista científica internacional.

2 REVISÃO DE LITERATURA

2.1 Suinocultura e doenças virais associadas

A suinocultura é uma atividade de grande relevância econômica e social, e os suínos representam uma importante fonte de alimento para o mundo. Em 2020, a carne suína correspondeu a aproximadamente 38% da produção mundial de carne (ABPA, 2020). Até 2019 a carne suína liderava o *ranking* mundial de fonte de proteína animal mais consumida (USDA, 2021). Em 2020, passou a ocupar o segundo lugar, ficando atrás apenas da carne de frango; estima-se que cerca de 97 milhões de toneladas de carne suína tenham sido consumidas nesse ano (USDA, 2021). No Brasil, o setor é responsável pela geração de mais de 1 milhão de empregos diretos e indiretos (ABCS, 2020). Em 2020, o produto interno bruto (PIB) da cadeia de produção de suínos foi de 22,2 bilhões de dólares (ABCS, 2020). O Brasil se destaca como quarto maior produtor de carne suína do mundo, ficando atrás apenas da China, União Europeia (UE) e Estados Unidos (EUA) (EMBRAPA, 2020; USDA, 2021). Além disso, o Brasil também é o quarto maior exportador de carne suína, atrás de UE, EUA e Canadá (USDA, 2021). Em 2020, a produção brasileira de carne suína correspondeu a 4,12 milhões de toneladas e aproximadamente 1,18 milhões de toneladas foram exportadas para mais de 70 países (ABPA, 2020). A região Sul do Brasil é a principal produtora e exportadora de carne suína, sendo responsável por cerca de 66% da produção nacional (ABPA, 2020).

A expansão da indústria suinícola e a intensificação da atividade foram acompanhadas pela melhoria do manejo sanitário nas criações que impede, por exemplo, o convívio dos suínos com outras espécies. Por outro lado, o aumento da escala de produção e da densidade de animais criados em confinamento facilita a transmissão de micro-organismos, a emergência de novos patógenos e o desenvolvimento de patologias complexas (DREW, 2011; MORES;ZANELLA, 2015; VANDERWAAL;DEEN, 2018). A movimentação e mistura de leitões de diferentes origens, por exemplo, traz benefícios econômicos e de logística, porém ocorre em detrimento de questões associadas à transmissão de agentes infecciosos (MORES;ZANELLA, 2015). Outro fator importante que contribui para a emergência e disseminação de agentes infecciosos é a globalização da atividade, caracterizada pelo frequente trânsito de animais e produtos derivados como carne e germoplasma (DREW, 2011). Exemplos de patógenos virais que emergiram/reemergiram nos últimos anos incluem o vírus da síndrome reprodutiva e respiratória dos suínos (PRRSV), circovírus suíno tipo 2 (PCV2), senecavírus A (SVA), vírus da influenza suína (SIV), vírus da peste suína africana (ASFV) e alguns coronavírus como o

vírus da diarreia epidêmica dos suínos (PEDV), o coronavírus da síndrome da diarreia aguda em suínos (SADS-CoV) e o deltacoronavírus suíno (PDCoV) (KOLBASOV *et al.*, 2018; GAVA, 2019; JURADO *et al.*, 2019; WANG *et al.*, 2019b; PERFUMO *et al.*, 2020; TAN *et al.*, 2020). No Brasil, as principais enfermidades virais emergentes/reemergentes incluem a influenza suína, circovirose suína (PCV2) e a doença vesicular idiopática suína (SIVD) causada pelo senecavírus A (MORES;ZANELLA, 2015; VANNUCCI *et al.*, 2015). Além disso, algumas dessas doenças como a influenza e a hepatite E podem ser transmitidas dos suínos para os humanos, representando uma preocupação sanitária adicional (MORES;ZANELLA, 2015; SEGALEZ, 2015).

Além dos vírus conhecidos por estarem associados a patologias, os suínos podem albergar uma diversidade de vírus cujo papel patogênico ainda não é conhecido, representando potencial fonte para a emergência de novas doenças. Muitas das enfermidades e/ou condições que acometem os suínos são consideradas multifatoriais e em muitos casos não há um agente etiológico conhecido (SOBESTIANSKY *et al.*, 2007; MORÉS, 2018). Os problemas relacionados à mortalidade nas granjas de suínos são um exemplo, estando associados a vários componentes do sistema produtivo como genética, nutrição, manejo, bem-estar, ambiente e infecção (MORÉS, 2018). Apesar de muitas vezes a mortalidade ser tratada como consequência de um problema não infeccioso (SOBESTIANSKY *et al.*, 2007; MORÉS, 2018), o diagnóstico costuma ser direcionado na busca por agentes específicos. Assim, o uso de ferramentas que permitem a detecção de agentes novos ou insuspeitos pode auxiliar no entendimento de patologias complexas e até mesmo de etiologia indefinida, bem como na vigilância de patógenos emergentes e reemergentes (PALLEN, 2014; KWOK *et al.*, 2020).

O avanço nas tecnologias e o rápido desenvolvimento de ferramentas computacionais oferecem novas oportunidades para o estudo dos vírus. A metagenômica viral, acoplada ao sequenciamento de alto desempenho (HTS), têm revolucionado o entendimento acerca da diversidade viral que compõe os mais diversos nichos (SIMMONDS *et al.*, 2017). Nesse contexto, muitos estudos têm investigado a comunidade viral (viroma) presente em animais doentes e saudáveis a fim de ampliar a compreensão sobre algumas patologias (DELWART, 2012; BLOMSTROM *et al.*, 2016; LIU *et al.*, 2016a; BLOMSTROM *et al.*, 2018; QIN *et al.*, 2018; SAPORITI *et al.*, 2021). Embora nem sempre seja possível inferir uma condição causal entre os vírus detectados e a manifestação clínica dos animais, os estudos de viromas podem ser usados como base para outros direcionados ao entendimento acerca da relação dos novos vírus descobertos e determinadas enfermidades. Esse é o caso, por exemplo, do circovírus suíno tipo 3 (PCV3), descoberto em 2016 em suínos com lesões cardíacas e inflamação

multissistêmica (PHAN *et al.*, 2016), e em porcas com sinais clínicos compatíveis à síndrome de dermatite e nefropatia porcina (PDNS, *porcine dermatitis and nephropathy syndrome*) e problemas reprodutivos crônicos (PALINSKI *et al.*, 2017). Testes específicos de diagnóstico foram realizados para determinar a etiologia dessas manifestações, sem sucesso, até a descoberta do PCV3 por metagenômica (PHAN *et al.*, 2016; PALINSKI *et al.*, 2017). A partir daí, vários estudos têm sido conduzidos com intuito de compreender o papel do PCV3 em diferentes manifestações clínicas, dentre elas, as falhas reprodutivas (KIM *et al.*, 2018; TOCHETTO *et al.*, 2018; ZOU *et al.*, 2018; DEIM *et al.*, 2019; SAPORITI *et al.*, 2020; TOCHETTO *et al.*, 2020a).

Outro exemplo são as doenças respiratórias, as quais ocorrem com frequência nas granjas de suínos e são amplamente distribuídas (TAKEUTI *et al.*, 2018). As doenças respiratórias nos suínos são consideradas multifatoriais uma vez que podem ser influenciadas por fatores infecciosos (vírus, bactérias) e não infecciosos (ambiente, nutrição, manejo, etc.) (OPRIESSNIG *et al.*, 2011; RECH *et al.*, 2018). Mundialmente, os principais vírus associados à pneumonia em suínos são o SIV, o PCV2 e o PRRSV (BOCHEV, 2007; HANSEN *et al.*, 2010; OPRIESSNIG *et al.*, 2011; RUGGERI *et al.*, 2020). No Brasil, o PRRVS não foi detectado até o momento (CIACCI-ZANELLA *et al.*, 2004; RECH *et al.*, 2018; GAVA *et al.*, 2021); o PCV2 tornou-se menos relevante após introdução das vacinas (BARBOSA *et al.*, 2008) e o SIV está amplamente distribuído, sendo o subtipo H1N1/2009 o mais prevalente no Brasil (SCHMIDT *et al.*, 2016; RECH *et al.*, 2018; HAACH *et al.*, 2020). Por causa de seu caráter complexo e multifatorial, a caracterização do conjunto de vírus presentes em animais doentes oferece uma oportunidade para identificação de vírus novos ou insuspeitos e fornece uma base para a investigação do potencial patogênico dos vírus identificados (HAUSE *et al.*, 2016a; QIN *et al.*, 2018; FRANZO *et al.*, 2019).

Além do estudo de vírus de potencial patogênico, outra questão importante diz respeito ao conjunto de vírus simbióticos, isto é, vírus que fazem parte da microbiota natural dos suínos e que contribuem para a homeostase do organismo. Assim, o estudo do viroma representa uma ferramenta para conhecer o conjunto de vírus presente em um organismo e servir como base para o entendimento do seu papel em patologias ou condições complexas.

2.2 Viroma

O microbioma corresponde a todo material genético da microbiota (bactérias, vírus, fungos, protozoários, archaeas) que compõem um determinado nicho (TURNBAUGH *et al.*,

2007). Entre esses micro-organismos, as bactérias e os fungos têm sido os mais estudados. A presença de um marcador universal (como o gene RNA ribossômico [rRNA] 16S nas bactérias e o gene rRNA 18S nos fungos) facilita a detecção desses micro-organismos, um "luxo" indisponível aos investigadores de vírus (MOKILI *et al.*, 2012; VIRGIN, 2014; SULLIVAN, 2015; ZOU *et al.*, 2016). Embora essas ferramentas tenham limitações como a não identificação em nível de espécie, elas servem para conhecer o perfil da microbiota bacteriana ou fúngica de um determinado nicho (KLINDWORTH *et al.*, 2013).

A parte do microbioma que engloba os vírus é denominada viroma; assim o termo viroma pode ser definido como o conjunto de todo material genético viral presente em determinada amostra (VIRGIN, 2014). Os vírus são a entidade biológica mais abundante do planeta Terra e o maior reservatório de diversidade genética (ROSARIO;BREITBART, 2011; GÜEMES *et al.*, 2016). Eles infectam espécies de todos os domínios da árvore da vida (MIETZSCH;AGBANDJE-MCKENNA, 2017), afetam os ciclos biogeoquímicos e a dinâmica dos ecossistemas (SUTTLE, 2007; ROHWER;THURBER, 2009) e são componentes essenciais de todos as formas de vida (ROOSSINCK, 2015;KOONIN *et al.*, 2021). Os vírus também têm um papel importante no metabolismo do hospedeiro, fluxo gênico e estruturação das comunidades microbianas e, por isso, entender a relação dos vírus com seus hospedeiros é fundamental (PAEZ-ESPINO *et al.*, 2016). Apenas nos oceanos, estima-se que há pelo menos 10^{31} partículas virais (as quais majoritariamente são bacteriófagos – vírus que infectam bactérias). Considerando que um vírus possui o tamanho médio de 100 nm, 10^{31} vírions dispostos lado a lado atingiriam 10^{33} nm de distância o que corresponde a 10^{21} km ou aproximadamente 10^{8} anos-luz. Para fins de comparação, a galáxia mais próxima da Via Láctea, Andrômeda, está a $2{,}5.10^{6}$ anos-luz de distância; se todos os vírus dos oceanos fossem colocados lado a lado, eles percorreriam uma distância maior que as 60 galáxias mais próximas à nossa (SUTTLE, 2013). De fato, há mais vírus em um litro de água do oceano do que pessoas no planeta e estima-se que o corpo humano alberga cerca de 10^{13} partículas virais (LIANG;BUSHMAN, 2021).

O viroma dos animais pode ser dividido em quatro grupos: (1) vírus que infectam células eucarióticas (viroma eucariótico); (2) vírus que infectam bactérias (viroma procariótico); (3) vírus que infectam archaeas (viroma das archaeas); e (4) elementos genéticos derivados de vírus no DNA do hospedeiro (profagos, retrovírus endógenos, elementos virais endógenos) (VIRGIN, 2014). Esses quatro componentes estão presentes em todos os animais e representam uma porção importante do microbioma.

A composição do viroma nos hospedeiros é moldada por diversos fatores como idade, dieta, fatores genéticos e imunológicos do hospedeiro e localização geográfica (VIRGIN, 2014; LIANG;BUSHMAN, 2021). Embora o papel do viroma dentro do microbioma não seja completamente entendido, alguns estudos indicam que o viroma pode causar efeitos profundos no microbioma e no próprio hospedeiro eucariótico (ALLEN *et al.*, 2011; KARST, 2016). Estudos indicaram que tanto os vírus patogênicos quanto os vírus comensais podem modular a resposta imunológica do hospedeiro e assim induzir ou prevenir doenças (LIM *et al.*, 2015; NEIL;CADWELL, 2018; VLASOVA *et al.*, 2019). Notoriamente, a interação vírus-hospedeiro é complexa e envolve múltiplos fatores como condições ambientais, estrutura e população viral, status do sistema imunológico, entre outros (KOONIN *et al.*, 2021). Alterações significativas no viroma têm sido observadas em indivíduos com doenças inflamatórias no intestino (NORMAN *et al.*, 2015; CLOONEY *et al.*, 2019), diabetes (ZHAO *et al.*, 2017), hipertensão (HAN *et al.*, 2018), AIDS (MONACO *et al.*, 2016) e câncer (NAKATSU *et al.*, 2018).

2.3 Técnicas para o estudo dos vírus

Os vírus podem ser identificados por uma ampla variedade de técnicas. Por mais de um século essas técnicas se basearam em métodos dependentes de cultivo, como o isolamento viral em cultivo celular e ovos embrionados, seguido de observação de efeito citopático (ECP), imuno-histoquímica e sorologia (MOKILI *et al.*, 2012; HAYES *et al.*, 2017) (BEXFIELD;KELLAM, 2011; LIPKIN;FIRTH, 2013). Com isso, os vírus isolados eram classificados de acordo com suas características morfológicas e sorológicas. Muitos vírus conhecidos atualmente foram identificados primeiramente por essas técnicas; no entanto, esses métodos possuem uma limitação importante: estima-se que mais de 99% dos vírus não podem ser cultivados em laboratório por métodos convencionais (BEXFIELD;KELLAM, 2011; MOKILI *et al.*, 2012; TEMMAM *et al.*, 2014).

As dificuldades associadas ao cultivo *in vitro* foram reduzidas após o desenvolvimento de técnicas independentes de cultivo, tais como a PCR, a microscopia eletrônica e métodos de hibridização (BEXFIELD;KELLAM, 2011; TEMMAM *et al.*, 2014; HAYES *et al.*, 2017). Ainda assim, tais técnicas apresentam como principal desvantagem a necessidade de conhecimento prévio a respeito dos vírus a serem pesquisados ou de vírus semelhantes (DELWART, 2007; TEMMAM *et al.*, 2014; HAYES *et al.*, 2017). Tendo em vista estas limitações, outras abordagens tornaram-se necessárias para a identificação de novos vírus. A metagenômica associada ao HTS é uma delas, sendo o método independente de cultivo de maior

sucesso utilizado para o estudo de comunidades virais, que revolucionou a forma como os vírus são estudados (Figura 1) (GÜEMES *et al.*, 2016; HAYES *et al.*, 2017; SIMMONDS *et al.*, 2017).

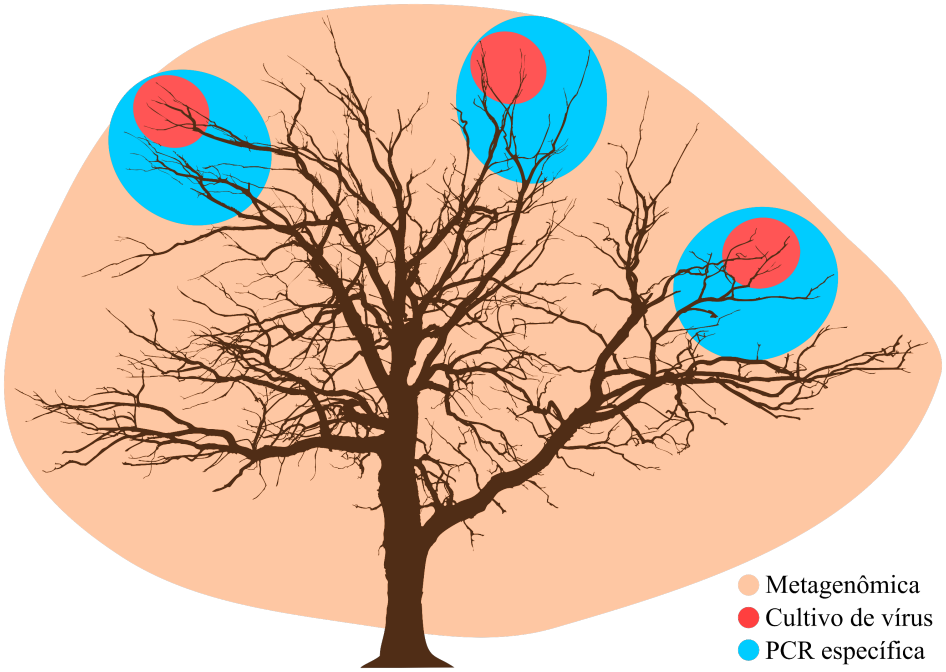

Figura 1 – Maneiras de explorar a virosfera. Árvore representando a diversidade viral. Os círculos coloridos ilustram a extensão dessa diversidade que pode ser descoberta por meio de três abordagens comumente utilizadas: cultivo de vírus/isolamento viral, PCR e metagenômica. Adaptado de Zhang *et al.* (2018).

2.3.1 Metagenômica

A metagenômica consiste no estudo do metagenoma, que, por sua vez, se refere à coleção de genomas de micro-organismos (incluindo bactérias, vírus, fungos, protozoários e archaeas) presentes em determinada amostra (THOMAS *et al.*, 2012; DUTILH, 2014). O termo metagenoma foi cunhado pela primeira vez em 1998 por Handelsman e colaboradores para descrever o conjunto de informações genômicas de todos os micro-organismos (microbioma) presentes em uma amostra de solo, incluindo aqueles não puderam ser isolados (HANDELSMAN *et al.*, 1998).

A metagenômica viral (ou virômica) é uma abordagem independente de cultivo que não requer conhecimento prévio do genoma dos vírus a serem identificados (MOKILI *et al.*, 2012). A técnica foi aplicada pela primeira vez em 2002 para caracterizar comunidades virais marinhas (BREITBART *et al.*, 2002) e, desde então, tem sido utilizada nos mais variados tipos de amostras biológicas e ambientais (MOKILI *et al.*, 2012; HAYES *et al.*, 2017).

Inicialmente, os viromas eram estudados por meio da clonagem dos fragmentos de DNA e sequenciamento pelo método de Sanger (BREITBART *et al.*, 2002; BREITBART *et al.*, 2003). Depois, com o avanço das tecnologias de sequenciamento, isso passou a ser feito de forma mais rápida através do sequenciamento do DNA sem a necessidade de clonagem (SCHOLZ et al., 2012). Atualmente, as plataformas de sequenciamento de alto desempenho (*high-throughput sequencing,* HTS) permitem caracterizar de forma massiva virtualmente todos os genomas virais presentes em determinada amostra e têm transformado significativamente o campo da virologia (KUMAR *et al.*, 2017; ROUX *et al.*, 2019). Em dois anos, o número de genomas virais identificados por metagenômica ultrapassou em cinco vezes o total de genomas sequenciados a partir de vírus isolados (dados referentes ao período de 2016-2018) (ROUX *et al.*, 2019). Apesar disso, o banco de dados de genomas do NCBI (*National Center for Biotechnology Information*) contém atualmente 39.925 vírus com genomas completos conhecidos (acesso em 7 de maio de 2021; filtro aplicado *"Viruses", "Complete", "Exclude partial"*) (NCBI, 2021), o que representa uma porção muito pequena da diversidade viral global (LIANG;BUSHMAN, 2021).

A metagenômica viral envolve basicamente quatro etapas: (i) purificação das partículas virais, (ii) extração de ácido nucleico, (iii) sequenciamento de alto desempenho e (iv) interpretação dos dados (sequências) por meio de análises de bioinformática (Figura 2) (HAYES *et al.*, 2017; QUINCE *et al.*, 2017; ROUX *et al.*, 2021).

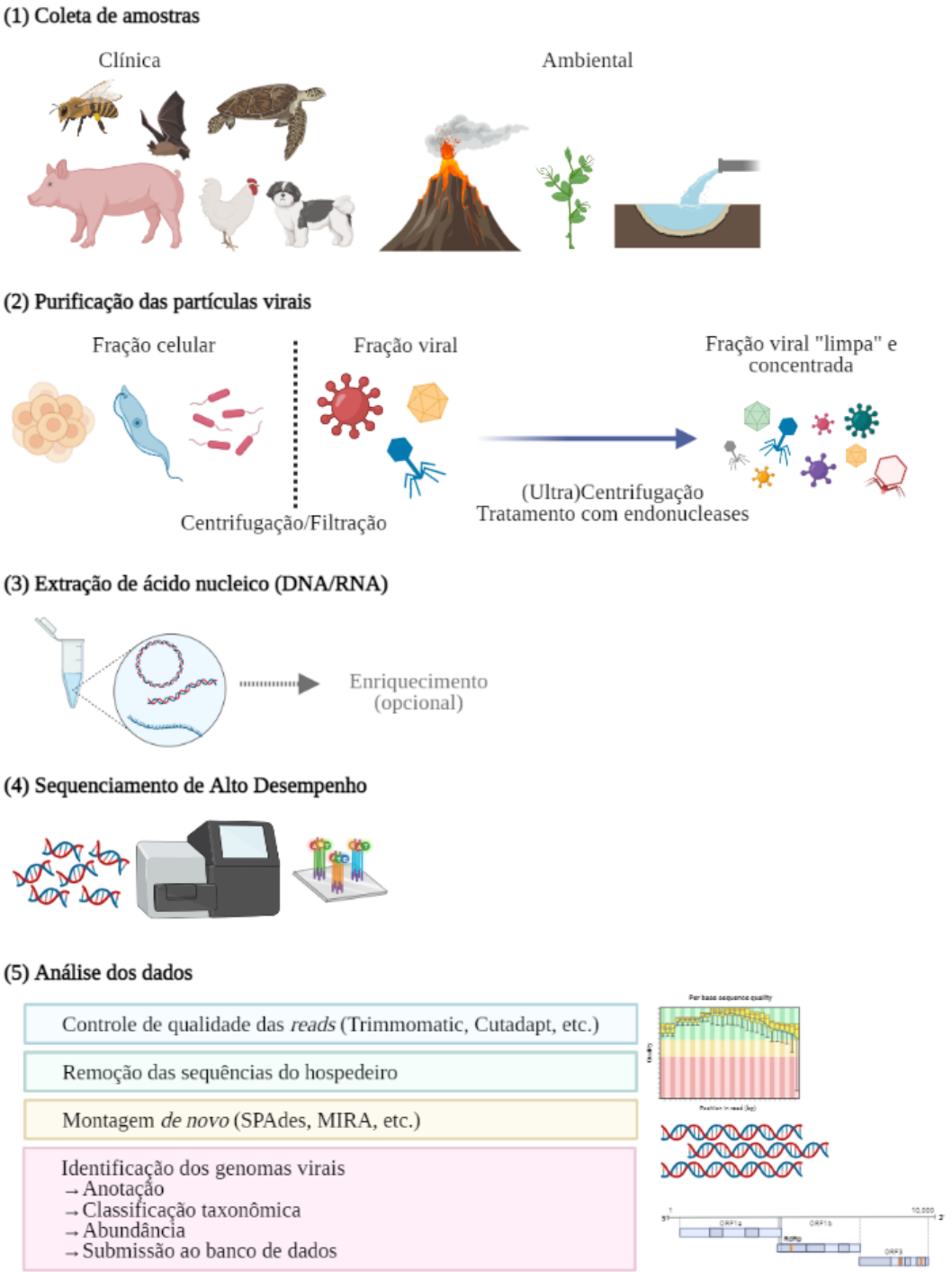

Figura 2 – Exemplificação simplificada das etapas da metagenômica viral.

Vários protocolos têm sido elaborados especificamente para seleção e pesquisa de genomas virais. Independentemente do tipo de amostra, a primeira etapa constitui a purificação das partículas virais realizada frequentemente em três passos: centrifugação, filtração e tratamento com endonucleases (DNase/RNAase) (ROUX *et al.*, 2021). A filtração geralmente é feita através de membranas de 0,22, 0,45 ou 0,8 µm, as quais permitem remover bactérias e debris celulares (LI et al., 2015b; ROUX et al., 2021). Alternativamente, as partículas virais

podem ser concentradas para maior obtenção de material genético viral. Isso pode ser feito de várias maneiras, por exemplo, utilizando cloreto de ferro (FeCl) (JOHN *et al.*, 2015; POULOS *et al.*, 2018), Polietilenoglicol (PEG) (COLOMBET *et al.*, 2007) ou filtração por fluxo tangencial (TFF) (SUTTLE *et al.*, 1991; SACHSENRODER *et al.*, 2012). Em seguida, os vírus são separados de partículas maiores e conteúdo celular por ultracentrifugação (NASUKAWA *et al.*, 2017; GARMAEVA *et al.*, 2019; ROUX *et al.*, 2021). Por fim, as partículas virais obtidas são tratadas com endonucleases (DNase/RNase) para remoção de material genético contaminante proveniente do hospedeiro e outros micro-organismos; dessa forma apenas material genético protegido por capsídeo é mantido para as próximas etapas (ROUX *et al.*, 2013; LI *et al.*, 2015; ROUX *et al.*, 2021).

A extração de DNA/RNA viral pode ser feita por métodos físicos ou químicos (LI *et al.*, 2015; QUINCE *et al.*, 2017). A obtenção de material genético suficiente para o HTS é um passo crítico nesse processo. Por isso, muitas vezes a extração de ácido nucleico é seguida da etapa de enriquecimento, onde o material genético viral é amplificado de forma não específica para aumentar a quantidade para o sequenciamento (LI *et al.*, 2015; SANTIAGO-RODRIGUEZ;HOLLISTER, 2020). Três protocolos de amplificação randômica são amplamente utilizados: amplificação por deslocamento múltiplo (*multiple displacement amplification*, MDA) (DEAN *et al.*, 2002; KRABERGER *et al.*, 2019; KHALIFEH *et al.*, 2021), amplificação de iniciador único independente de sequência (*sequence-independent single-primer amplification*, SISPA) (REYES;KIM, 1991; DJIKENG *et al.*, 2008) e *linker amplification shotgun libraries* (LASL) (BREITBART *et al.*, 2002; PARRAS-MOLTO *et al.*, 2018). No entanto, o enriquecimento introduz alguns vieses que devem ser avaliados de acordo com o objetivo do estudo, como a amplificação preferencial de genomas circulares *ssDNA* e a sub-amplificação de genomas ricos em conteúdo GC (QUINCE *et al.*, 2017; PARRAS-MOLTO *et al.*, 2018). Consequentemente, a abundância relativa dos vírus identificados em estudos que utilizam a etapa de enriquecimento deve ser estimada cuidadosamente (PARRAS-MOLTO *et al.*, 2018; ROUX *et al.*, 2021). Por outro lado, a ausência de enriquecimento prejudica a identificação de vírus raros ou presentes em menor abundância (ROUX *et al.*, 2019).

Por fim, os genomas virais são submetidos ao sequenciamento. Atualmente, várias plataformas de HTS estão disponíveis no mercado (*Illumina, Ion Torrent, Oxford Nanopore*), cada qual com suas vantagens e desvantagens (PEREZ-LOSADA *et al.*, 2020), sendo a *Illumina* predominante entre os estudos de metagenômica viral (QUINCE *et al.*, 2017; KWOK *et al.*, 2020). Os dados obtidos no sequenciamento são verificados e analisados com o auxílio de ferramentas de bioinformática. Primeiramente, as *reads* (sequências curtas de DNA

provenientes das plataformas de HTS) são filtradas por qualidade e os adaptadores removidos (*trimagem*) (PEREZ-LOSADA *et al.*, 2020). Quando aplicável, as *reads* correspondentes ao genoma do hospedeiro podem ser removidas (WILLNER;HUGENHOLTZ, 2013). Depois, as *reads* são montadas em sequências maiores formando os chamados *contigs.* Isso pode ser feito basicamente de duas maneiras: (i) montagem *de novo* (SPAdes, MIRA, MEGAHIT, etc.), e (ii) montagem por referência, onde as reads são alinhadas com um genoma de referência (BBMap, Bowtie2, Geneious, etc.). Os *contigs* são então comparados com bancos de dados virais, anotados e classificados taxonomicamente (KHAN MIRZAEI *et al.*, 2021).

Alternativamente, os genomas virais podem ser analisados diretamente a partir de dados de metagenoma, o qual inclui dados de vírus e outros micro-organismos (QUINCE *et al.*, 2017; ROUX *et al.*, 2021). A desvantagem desse tipo de abordagem é que o a proporção de material genético viral dentro dos metagenomas é muito inferior à do hospedeiro e outros micro-organismos, limitando a identificação dos vírus presentes (ROSE *et al.*, 2016).

Enquanto o HTS abriu diversas possibilidades de estudos, a análise da grande quantidade de dados gerados tornou-se o principal desafio para os pesquisadores (HAYES *et al.*, 2017). Isso porque a maior parte das sequências obtidas nos estudos de viroma (60-95%) compõe a chamada "matéria escura" (do inglês, *dark matter*), isto é, não possuem homologia com nenhuma outra sequência disponível nos bancos de dados (ROUX *et al.*, 2015; KRISHNAMURTHY;WANG, 2017; TISZA *et al.*, 2020). Além disso, alguns genomas virais, ainda que completos, muitas vezes contêm as chamadas ORFans – ORFs com poucas ou nenhuma sequência homóloga nos bancos de dados (FISCHER;EISENBERG, 1999; YIN;FISCHER, 2008). Tendo em vista que as principais ferramentas utilizadas para anotação de novas sequências se baseiam principalmente na comparação com sequências homólogas nos bancos de dados, estudos exploratórios sobre o viroma de diferentes tipos de amostras, ampliam o banco de dados de sequências virais e, consequentemente, facilitam a caracterização de novos genomas virais, o que contribui para o entendimento da evolução viral, cadeias de transmissão e origem de doenças virais emergentes (DELWART, 2012; KO *et al.*, 2020). As aplicações da metagenômica viral estão exemplificadas simplificadamente na Figura 3.

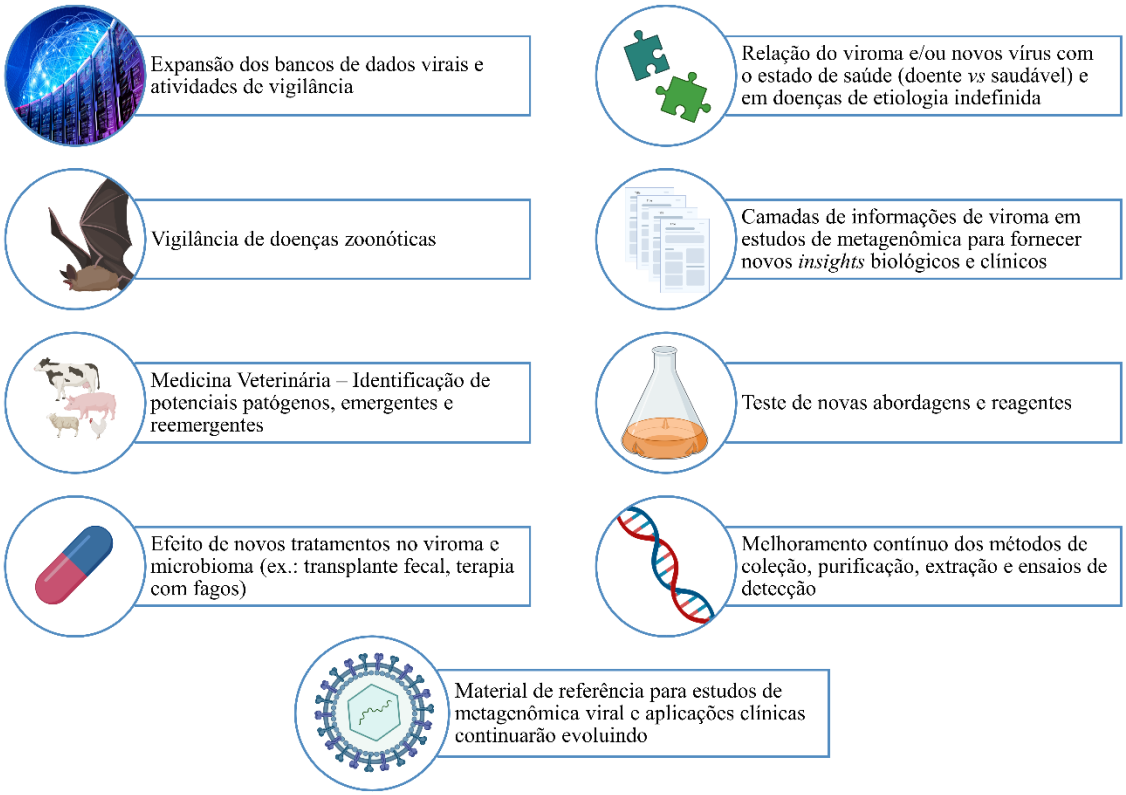

Figura 3 – Potenciais aplicações da metagenômica viral hoje e em estudos futuros. Adaptado de SANTIAGO-RODRIGUEZ and HOLLISTER (2020).

2.3.2 Viroma em suínos

Em suínos, vários estudos de metagenômica têm revelado a diversidade viral presente em diferentes tipos de amostras (Figura 4). Aqui, para fins de simplificação, as famílias virais detectadas nesses estudos foram agrupadas com o sistema relacionado. Desta forma, os vírus identificados em amostras de fezes, suabe retal, intestino ou fluido oral foram listados em “trato gastrointestinal” (Figura 4).

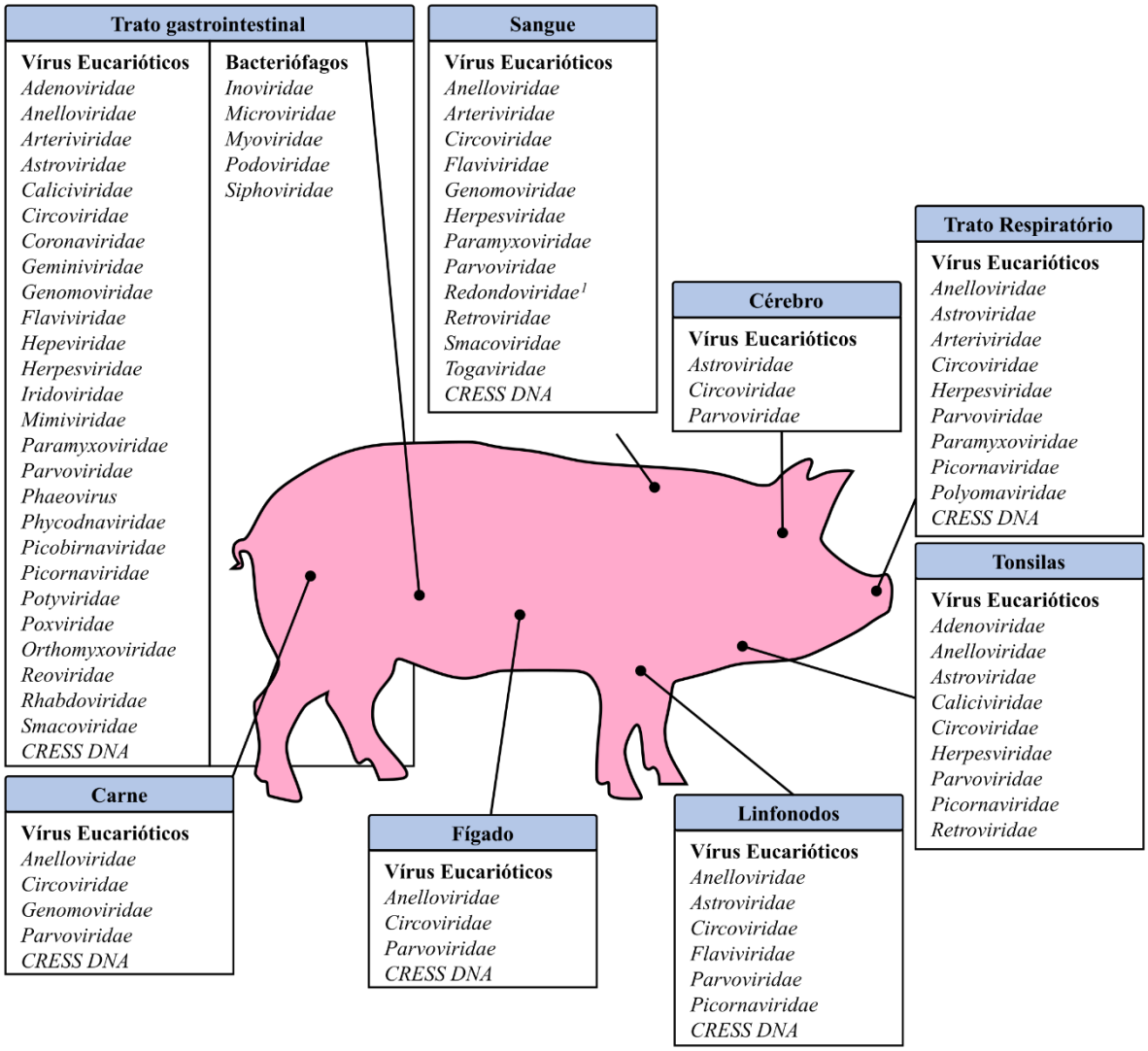

Figura 4 – Vírus identificados em suínos utilizando HTS. Lista das principais famílias virais reportadas de acordo com literatura (BLOMSTROM *et al.*, 2009; SHAN *et al.*, 2011; LAGER *et al.*, 2012; MASEMBE *et al.*, 2012; SACHSENRODER *et al.*, 2012; CHEUNG *et al.*, 2013; SACHSENRODER *et al.*, 2014; ZHANG *et al.*, 2014a; ZHANG *et al.*, 2014b; HAUSE *et al.*, 2015a; AMIMO *et al.*, 2016; BLOMSTROM *et al.*, 2016; HAUSE *et al.*, 2016a; KARLSSON *et al.*, 2016; PALINSKI *et al.*, 2016; STEEL *et al.*, 2016; THEUNS *et al.*, 2016; ARRUDA *et al.*, 2017; PALINSKI *et al.*, 2017; BLOMSTROM *et al.*, 2018; CHEN *et al.*, 2018; QIN *et al.*, 2018; TOCHETTO *et al.*, 2018; FRANZO *et al.*, 2019; GRIERSON *et al.*, 2019; DA SILVA *et al.*, 2020; TOCHETTO *et al.*, 2020b; BARRON-RODRIGUEZ *et al.*, 2021; CIBULSKI *et al.*, 2021; PAIM *et al.*, 2021; SCHUELE *et al.*, 2021). CRESS DNA são vírus de genoma circular DNA fita simples (*ssDNA*) codificadores de replicase (*circular Rep-encoding ssDNA*).

Semelhante aos humanos (LIANG;BUSHMAN, 2021), o trato gastrointestinal dos suínos parece ser o sítio de maior diversidade/abundância viral. A maior parte dos estudos em suínos tem explorado a diversidade viral de fezes de animais saudáveis (SACHSENRODER *et al.*, 2012; YU *et al.*, 2013a; SACHSENRODER *et al.*, 2014; AMIMO *et al.*, 2016; STEEL *et al.*, 2016; THEUNS *et al.*, 2016; BARRON-RODRIGUEZ *et al.*, 2021; NAGAI *et al.*, 2021), com diarreia (CHEUNG *et al.*, 2013; CHEUNG *et al.*, 2014; CHEUNG *et al.*, 2015; CONCEICAO-NETO *et al.*, 2017b; ZHOU *et al.*, 2021) ou são estudos comparativos que

avaliaram o viroma de suínos saudáveis e de suínos com diarreia (SHAN *et al.*, 2011; LAGER *et al.*, 2012; ZHANG *et al.*, 2014a; OBA *et al.*, 2018; TSUCHIAKA *et al.*, 2018). Adicionalmente, outros estudos caracterizaram o viroma do intestino de leitões saudáveis ou com diarreia (KARLSSON *et al.*, 2016), de suínos com síndrome da falha no desenvolvimento peri-desmame (PFTS) (FRANZO *et al.*, 2019) e de suínos saudáveis (WANG *et al.*, 2019a).

No fígado de suínos foram detectados vírus pequenos de genoma ssDNA, como aqueles pertencentes às famílias *Anelloviridae, Circoviridae* e *Parvoviridae* (DA SILVA *et al.*, 2020). Já nos linfonodos, diversas famílias virais foram detectadas em animais com síndrome multissistêmica do definhamento dos suínos (PMWS) e também em animais saudáveis (BLOMSTROM et al., 2009; BLOMSTROM et al., 2016).

No que se refere ao trato respiratório, estudos de viroma a partir de amostras de suabe nasal e tecido pulmonar de suínos saudáveis (SCHUELE *et al.*, 2021; SHI *et al.*, 2021) ou com doença respiratória (PADMANABHAN;HAUSE, 2016; QIN *et al.*, 2018) revelaram majoritariamente vírus eucarióticos ssDNA. Ainda, um estudo explorou o viroma da tonsila de suínos com pneumonia e suínos livres de patógenos específicos (SPF) mostrando grande diversidade viral em ambos os grupos de animais (BLOMSTROM *et al.*, 2018) (Figura 4).

No sistema nervoso central, estudos exploratórios identificaram a presença de vírus das famílias *Circoviridae* e *Parvoviridae* em amostras de encéfalo de suínos com PFTS (FRANZO et al., 2019), e da família *Astroviridae* em suínos com encefalite (ARRUDA *et al.*, 2017). Em carne suína comercializada foram detectados genomas de vírus das famílias *Anelloviridae, Circoviridae, Genomoviridae, Parvoviridae* e outros vírus circulares ssDNA (ZHANG *et al.*, 2014b; CIBULSKI *et al.*, 2021). Por fim, várias famílias virais têm sido identificadas no soro de suínos aparentemente saudáveis ou doentes (Figura 4) (MASEMBE *et al.*, 2012; QIN *et al.*, 2018; TOCHETTO *et al.*, 2020b; PAIM *et al.*, 2021). Para fins de comparação, estudos de metagenômica em amostras de sangue humano têm revelado uma diversidade viral ainda maior, revelando a presença de vírus eucarióticos das famílias *Anelloviridae*, *Flaviviridae, Herpesviridae*, *Marseilleviridae*, *Mimiviridae, Picornaviridae*, *Poxviridae* e também vírus procarióticos das famílias *Inoviridae, Microviridae, Myoviridae, Podoviridae, Phycodnaviridae e Siphoviridae* (RASCOVAN et al., 2016; LIANG;BUSHMAN, 2021). Embora não esteja claro se os bacteriófagos encontrados são derivados de contaminação ambiental, um estudo recente demonstrou que eles podem ser transportados através do epitélio intestinal por transcitose, atingindo a corrente sanguínea (NGUYEN *et al.*, 2017).

A metagenômica viral também tem sido utilizada para identificação e caracterização genética de novos vírus como o PCV3 (PHAN *et al.*, 2016; PALINSKI *et al.*, 2017;

TOCHETTO *et al.*, 2018), parvovírus suíno tipo 6 (PPV6) (SCHIRTZINGER *et al.*, 2015), PPV7 (PALINSKI *et al.*, 2016), pestivírus suíno atípico (APPV) (HAUSE *et al.*, 2015a), posavírus (HAUSE *et al.*, 2015b), ortopneumovírus suíno (HAUSE *et al.*, 2016b), teschovírus suíno (OBA *et al.*, 2018), astrovírus (YU *et al.*, 2013a), bocavírus (BLOMSTROM *et al.*, 2009), bufavírus (LIU *et al.*, 2016a) e vários vírus de genoma circular ssDNA codificadores de replicase, os chamados vírus *CRESS DNA* (*circular Rep-encoding single-stranded DNA*) (SHAN *et al.*, 2011; CHEUNG *et al.*, 2013; SIKORSKI *et al.*, 2013; CHEUNG *et al.*, 2014; CHEUNG *et al.*, 2015; STEEL *et al.*, 2016; TOCHETTO *et al.*, 2020b). Além disso, a presença de vírus insuspeitos foi reportada em diversos trabalhos como o *ndumu virus* (NDUV) (MASEMBE *et al.*, 2012), bastrovírus (BastV) (BAUERMANN *et al.*, 2019; NAGAI *et al.*, 2021), *Sus scrofa* poliomavírus 2 (HAUSE *et al.*, 2018), astrovírus suíno tipo 3 (PoAstV-3) (ARRUDA *et al.*, 2017), pestivírus suíno (ARRUDA *et al.*, 2016), entre outros.

A maioria dos estudos comparativos não identificaram diferenças entre o viroma de suínos saudáveis e doentes (SHAN *et al.*, 2011; ZHANG *et al.*, 2014a; BLOMSTROM *et al.*, 2016; KARLSSON *et al.*, 2016; LIU *et al.*, 2016a; NIIRA *et al.*, 2016; BLOMSTROM *et al.*, 2018; MASUDA *et al.*, 2018; OBA *et al.*, 2018; QIN *et al.*, 2018; TSUCHIAKA *et al.*, 2018; TOCHETTO *et al.*, 2020b), mas mostraram uma ampla diversidade viral, independente do status sanitário dos animais (KWOK *et al.*, 2020). Por outro lado, a composição viral parece ser individual e variável de acordo com a idade dos animais (SACHSENRODER *et al.*, 2014). Todavia, cabe destacar que estudos em humanos têm mostrado que o contexto em geral (i.e., hospedeiro, presença de outros agentes infecciosos, microbiota, dieta, imunidade, etc.) é o principal determinante na forma como os vírus se "manifestam" dentro do organismo, que pode ser de forma deletéria, neutra ou benéfica (CADWELL, 2015; LIANG;BUSHMAN, 2021).

A caracterização do viroma é crucial para o entendimento do papel dos vírus como parte da microbiota em indivíduos saudáveis e doentes (ROHWER;YOULE, 2011; ROSE *et al.*, 2016). Além disso, para que as ferramentas de sequenciamento de alto desempenho sejam utilizadas como uma potencial forma de vigilância é necessário conhecer e entender qual a diversidade viral natural no hospedeiro (KWOK *et al.*, 2020). Além disso, embora haja um número crescente de estudos de viroma em suínos, a diversidade viral detectada nestes estudos evidencia que ainda há muito a ser explorado (ROOSSINCK, 2015; TISZA *et al.*, 2020). Desta forma, estudos explorando o viroma de animais saudáveis e doentes, bem como aqueles que focam em avaliar os vírus ainda não explorados, são necessários.

Assim, o presente estudo objetivou caracterizar o viroma de suínos com diferentes condições clínicas, incluindo animais com sinais clínicos de doença respiratória e animais com

e sem diarreia. Ainda, como seguimento de um estudo de metagenômica em matrizes com natimortos no qual o PCV3 foi identificado, este trabalho também objetivou avaliar se há correlação entre a presença de PCV3 no soro das matrizes com natimortalidade.

3 ARTIGO CIENTÍFICO 1

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A variety of highly divergent eukaryotic single-stranded DNA viruses in sera of pigs

1.1 Author names

Caroline Tochetto[1] - ORCiD 0000-0002-9804-6175

Samuel Paulo Cibulski[2] – ORCiD 0000-0003-0503-8692

Ana Paula Muterle Varela[1] - ORCiD 0000-0003-3390-8691

Cristine Cerva[3] – ORCiD 0000-0003-1509-4452

Diane Alves de Lima[4] – ORCiD 0000-0003-3282-5675

Thais Fumaco Teixeira[3] – ORCiD 0000-0003-0927-9856

Fabiana Quoos Mayer[3] – ORCiD 0000-0002-9324-8536

Paulo Michel Roehe[1] - ORCiD 0000-0002-2370-7661

1.2 Affiliation

[1]Laboratório de Virologia, Departamento de Microbiologia Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, Rio Grande do Sul, Brazil.

[2]Centro de Biotecnologia – CBiotec, Laboratório de Biotecnologia Celular e Molecular, Universidade Federal da Paraíba – UFPB, João Pessoa, Paraíba, Brazil.

[3]Centro de Pesquisa em Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Departamento de Diagnóstico e Pesquisa Agropecuária, Secretaria de Agricultura, Pecuária e Desenvolvimento Rural, Eldorado do Sul, Rio Grande do Sul, Brazil.

[4]Laboratório de Microbiologia do Centro Clínico Veterinário, Centro Universitário da Serra Gaúcha – FSG, Caxias do Sul, Rio Grande do Sul, Brazil.

1.3 Corresponding author

Fabiana Quoos Mayer - bimmayer@gmail.com

1.4 Keyword

CRESS DNA; metagenomics; virome; swine; high-throughput sequencing.

1.5 Repositories

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2. Abstract

Over the last decade, viral metagenomics has been established as a non-targeted approach for identifying viruses in stock animals, including pigs. This has led to the identification of a vast diversity of small circular single-stranded (ss) DNA viruses. The present study focuses on the investigation of eukaryotic circular Rep-encoding ssDNA (CRESS DNA) viral genomes present in serum of commercially reared pigs from Southern Brazil. Several CRESS DNA viral genomes were detected, including representatives of the families *Smacoviridae* (n = 5), *Genomoviridae* (n = 3), *Redondoviridae* (n = 1), *Nenyaviridae* (n=1) and other yet unclassified genomes (n = 9), plus a circular DNA molecule, which most likely belong to *Cressdnaviricota* phylum. A novel genus within the *Smacoviridae* family*,* tentatively named "*Suismacovirus*", comprising 21 potential new species, is proposed. Although the reported genomes were recovered from pigs with clinical signs of respiratory disease, further studies should examine their potential role as pathogens. Nonetheless, these findings highlight the diversity of circular ssDNA viruses in serum of domestic pigs, expand the knowledge on CRESS DNA viruses' genetic diversity and distribution and contribute to the global picture of virome of commercially reared pigs.

3. Introduction

Pigs are one of the most important food-producing animals, accounting for approximately 38% of all meat production worldwide (USDA, 2021). Since they provide a crucial source of food and are in close contact with farmers, viruses causing clinical or subclinical infections can influence food production and human health. Viral metagenomics can provide insights into the composition of viral communities by allowing simultaneous analyses of thousands of genome sequences in a particular sample; such methodology has led to a massive expansion in the knowledge on the diversity of the virosphere (ZHANG *et al.*, 2018).

In this scenario, circular Rep-encoding single-stranded (CRESS) DNA viruses have drawn attention because of their ubiquitous nature. CRESS DNA viral genomes have been identified in a wide range of organisms, including vertebrates (FONTENELE *et al.*, 2019; LIMA *et al.*, 2019; CIBULSKI *et al.*, 2021; KHALIFEH *et al.*, 2021), invertebrates (BREITBART *et al.*, 2015; ROSARIO *et al.*, 2018; KRABERGER *et al.*, 2019a; KRABERGER *et al.*, 2019b; ROSARIO *et al.*, 2019; KUBACKI *et al.*, 2020), plants (RICHET *et al.*, 2019) and also environmental samples (ZAWAR-REZA *et al.*, 2014; KRABERGER *et al.*, 2015; MALKI *et al.*, 2020). A large portion of these viruses was assigned to the recently established phylum *Cressdnaviricota*, which is currently divided into two classes, *Arfviricetes* and *Repensiviricetes* (KRUPOVIC *et al.*, 2020), and seven established eukaryotic CRESS DNA virus families: *Bacilladnaviridae, Circoviridae, Geminiviridae, Genomoviridae, Nanoviridae/Alphasatellitidae, Redondoviridae* and *Smacoviridae* (KRUPOVIC *et al.*, 2020). Moreover, six well-supported clades of unclassified CRESS DNA viruses, named CRESSV1 to CRESSV6, have been recognized (KAZLAUSKAS *et al.*, 2018; KRUPOVIC *et al.*, 2020). However, a large number of viral genomes within the *Cressdnaviricota* phylum do not fall into any of the currently established families/clades (KAZLAUSKAS *et al.*, 2018). In addition, novel CRESS DNA virus families have been proposed, although not officially recognized by the

International Committee on Taxonomy of Viruses (ICTV). That is the case of the families *Nenyaviridae* and *Naryaviridae*, which comprise viruses that infect *Entamoeba* spp. (KINSELLA *et al.*, 2020).

CRESS DNA viral genomes typically possess an origin of replication (*ori*) and encode a replication-associated protein (Rep) and a capsid protein (CP) (ROSARIO *et al.*, 2012) Differently from CP, the Rep usually has conserved domains and shows significant similarity across different groups of CRESS DNA viruses. Although intragenic recombination can occur at the level of functional domains (KAZLAUSKAS *et al.*, 2018), the Rep sequence has been used as the basis for phylogenetic analysis of such viruses (KOONIN *et al.*, 2020; KRUPOVIC *et al.*, 2020).

In pigs, CRESS DNA viruses have been frequently detected in feces (SIKORSKI *et al.*, 2013; CHEUNG *et al.*, 2014; KIM *et al.*, 2014; CHEUNG *et al.*, 2015; STEEL *et al.*, 2016). However, studies focusing on the investigation of viral genomes in serum of these animals are scarce (QIN *et al.*, 2018; TOCHETTO *et al.*, 2020b; HE *et al.*, 2021; PAIM *et al.*, 2021). Additionally, the characterization of novel viral genomes enhances viral databases information increasing the power of future studies to identify more viruses (HAYES *et al.*, 2017; TISZA *et al.*, 2020). The present work reports a variety of highly divergent CRESS DNA viral genomes identified in serum of domestic pigs presenting clinical signs of respiratory disease from commercial pig farms in Southern Brazil.

4. Methods

4.1 Ethics statement

This study was approved by the Ethics Committee on Animal Use from the *Universidade Federal do Rio Grande do Sul* (CEUA-UFRGS), protocol number 31670.

4.2 Sample processing

Swine samples used in this study were collected as part of routine care of swine herds. Serum samples of 41 pigs from 4 commercial swine farms within the state of Rio Grande do Sul, Southern Brazil, were received at the diagnostic laboratory. Clinical signs of respiratory disease were the only information provided by veterinarians who submitted the samples. Such signs were not detailed in the diagnostic requisitions, although usually referring to coughing and sneezing, either at rest or when forced to move.

The samples were filtered through 0.22 μm filters (Millex-GV, PVDF, Millex®) and centrifuged on a sucrose cushion (25%) at ~150,000 x *g* for 4 h at 4 ºC (in a Sorvall AH629 rotor). The obtained pellets were resuspended in 400 μL of ultrapure water and stored at -80 ºC until further processing.

4.3 Viral DNA isolation, enrichment and sequencing

Resuspended pellets were pooled, treated with 2.5 μL DNase (2 U/μL, Turbo DNase Kit, Ambion), 5 μL RNase A (20 mg/mL, Invitrogen) and incubated for 2 h at 37 °C to reduce the concentration of non-encapsidated nucleic acids. The reaction was stopped according to the manufacturer's protocols, by adding EDTA at 15 mM followed by incubation at 75 °C for 10 minutes. Viral DNA was extracted using a standard phenol-chloroform protocol (SAMBROOK;RUSSELL, 2006). Quality and quantity of the extracted DNA was checked by microvolume spectrophotometry (Nanodrop, ThermoScientific, USA) and fluorometry (Qubit, Invitrogen, USA), respectively. The DNA was then enriched by multiple displacement amplification (MDA) using ϕ29 DNA polymerase (New England Biolabs, Canada) (DEAN *et al.*, 2002) and purified with AMPure XP magnetic beads (Beckman Coulter, USA). A negative control (ultrapure water) was included in this step. The quality and quantity of the enriched DNA was verified as above and further checked by agarose gel electrophoresis (0.8%; w/v)

stained with ethidium bromide. Sequencing libraries were constructed using the Nextera DNA sample preparation kit (Illumina®, USA) according to the manufacturers' recommendations. Libraries were sequenced using Illumina Miseq® instrument with the Miseq v2 300 kit (2 x 150 paired-end).

4.4 Quality control, pre-processing and assembly of reads

The quality of the paired-end reads obtained was evaluated with FastQC (v.0.11.7) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were curated with Trimmomatic (v.0.36) to remove adaptors, the first 15 nt of the reads, low-quality reads (base-calling quality dropped below 15 on a 4 bp sliding window) and reads shorter than 36 nt (BOLGER *et al.*, 2014). Paired-end trimmed reads were merged with BBMerge (default parameters) (BUSHNELL *et al.*, 2017). Merged, unmerged and unpaired trimmed reads were *de novo* assembled into contigs using SPAdes v.3.14.0 (BANKEVICH *et al.*, 2012) with multiple *k-mers* of 21, 33, 55, 77, 99, 127 and the following options: "*--sc --merged* (merged reads) *--12* (unmerged reads) *-s* (unpaired reads)". Assembled contigs were taxonomically assigned using diamond (v.0.9.31) with *blastx* option using the default settings (*e-value* cutoff 0.001 and one top hit) (BUCHFINK *et al.*, 2015) and NCBI *nr* database from July 2019 (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The contigs were identified as circular based on identical 5' and 3' ends (ROUX *et al.*, 2014).

4.5 Genome annotation

Putative ORFs with more than 200 amino acids were identified within the complete genomes obtained and annotated using Geneious R8.1.9 software (Biomatters, New Zealand). The potential origin of replication (*ori*) for each genome was identified by locating the canonical nonanucleotide motif NANTATTAC (ROSARIO *et al.*, 2012), or equivalent

151 sequences (VARSANI;KRUPOVIC, 2018). Predicted stem-loop structures were confirmed
152 using Geneious R8.1.3 with default parameters. Splicing sites were predicted with the aid of
153 NetGene2 Server (http://www.cbs.dtu.dk/services/NetGene2/) and checked by comparison
154 with reference genomes and the presence of Rep motifs. Genome coverage was obtained by
155 mapping trimmed reads to reference using low-sensitivity/fastest mode (Geneious R8.1.9). The
156 annotated genomes (n = 19) were deposited in GenBank with accession numbers MW601947
157 to MW601958, MW620871 to MW620875, and MW620877 to MW620878.
158
159 *4.6 Sequence analysis*
160 BLASTp comparisons were performed with all Rep and CP protein sequences obtained
161 here, in search for closely related sequences at GenBank. Rep amino acid sequences were
162 inspected for the presence of the N-terminal HUH (His-hydrophobe-His) endonuclease domain
163 and the C-terminal superfamily 3 (SF3) helicase domain. The HUH endonuclease domain
164 includes three conserved motifs important for rolling circle replication (RCR) denoted as motifs
165 I, II, and III (ILYINA;KOONIN, 1992; CHANDLER *et al.*, 2013). The C-terminal SF3 helicase
166 domain comprises four conserved motifs known as Walker A, Walker B, motif C
167 (GORBALENYA *et al.*, 1990; ROSARIO *et al.*, 2012) and arginine finger (KAZLAUSKAS *et*
168 *al.*, 2017).
169
170 *4.7 Multiple sequence alignments and phylogenetic analysis*
171 To evaluate the relatedness of the novel CRESS DNA viral genomes identified here to
172 previously reported CRESS DNA viral genomes, phylogenetic trees from Rep amino acid
173 sequences were constructed with the sequences described here and their best BLASTp matches
174 in GenBank. These included representatives of the seven families of the *Cressdnaviricota*
175 phylum (*Bacilladnaviridae, Circoviridae, Geminiviridae, Genomoviridae,*

176 *Nanoviridae/Alphasatellitidae, Redondoviridae* and *Smacoviridae*), plus the six groups of
177 unclassified CRESS DNA viruses (CRESSV1-6) (KAZLAUSKAS *et al.*, 2018; KRUPOVIC
178 *et al.*, 2020). The Rep sequences from representatives of *Geminiviridae*,
179 *Nanoviridae/Alphasatellitidae* and CRESSV1-CRESSV6 were obtained from KAZLAUSKAS
180 *et al.* (2018). CRESSV1-CRESSV6 recombinant sequences were excluded. The Rep sequences
181 from *Circoviridae* were obtained from ROSARIO *et al.* (2017).

182 Sequences were aligned using MAFFT v.7.471 (KATOH;STANDLEY, 2013)
183 optimized to accurate local alignment (options "*L-INS-I -leavegappyregion -ep 0.123";* Figs.
184 1, 4 and 6) and optimized to global pairwise alignment (options "*G-INS-I -leavegappyregion -*
185 *ep 0.123*"; Figs. 2 and 5). Alignments were trimmed using TrimAl v.1.2 (CAPELLA-
186 GUTIERREZ *et al.*, 2009) with a gap threshold of 0.2 (Figs. 1, 4 and 6) and manually inspected
187 for the presence of conserved RCR and SF3 helicase motifs.

188 Phylogenetic trees were generated with IQTREE v.1.6.12 (NGUYEN *et al.*, 2015) using
189 the standard automatic best-fit model selection ("rtREV + F + G4", Figs 1, 4 and 6; "LG + F +
190 I + G4", Figs 2 and 5) and Bayesian-like transformation of aLRT (aBayes) (ANISIMOVA *et*
191 *al.*, 2011) for local branch support. Final trees were visualized and edited with FigTree v.1.4.4
192 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v.1.0.1 (https://inkscape.org/).
193 Distribution of pairwise identities were calculated using the Sequence Demarcation Tool (SDT)
194 v.1.2 (MUHIRE *et al.*, 2014). Sequence logos were created using WebLogo 3 server
195 (http://weblogo.threeplusone.com/) (CROOKS *et al.*, 2004). Sequence alignments used in this
196 study are available from the authors upon request.

197

198 **5. Results and Discussion**

199 *5.1 Recovery and characterization of novel CRESS DNA viral genomes*

In this study, eighteen novel CRESS DNA viral genomes and one circular DNA molecule were recovered from pigs' serum samples and are summarized in Table 1. Phylogenetic analyses were performed based on the Rep amino acid sequences of these genomes, with their best matches of BLASTp and those of representative CRESS DNA viruses. In the resultant phylogenetic tree (Fig.1), the CRESS DNA viral genomes detected here are widely distributed among the *Cressdnaviricota* phylum, clustering along with representatives of the families S*macoviridae* (n = 5), *Redondoviridae* (n = 1), *Genomoviridae* (n = 3), *Nenyaviridae* (n = 1) and unclassified CRESS DNA viruses (n = 10) (Fig. 1). The genomes reported here are presented in more detail below, along with relevant details of each taxa.

5.1.1 Smacoviridae

The family *Smacoviridae* currently comprises six genera*: Bovismacovirus, Cosmacovirus, Dragsmacovirus, Drosmacovirus, Huchismacovirus,* and *Porprismacovirus,* encompassing 43 species (VARSANI;KRUPOVIC, 2018). Smacoviruses' genomes range from 2.3 to 2.9 kb and contain two ORFs bidirectionally organized encoding the Rep and CP proteins. These ORFs are separated by two intergenic regions, one of which contains a conserved nonanucleotide motif considered the origin of replication (*ori*) (VARSANI;KRUPOVIC, 2018).

In this study, five smacoviruses' genomes were recovered from sera and are referred to as porcine smacovirus (PoSmaV) 1-5 (Table 1). The genomes displayed different genomic organizations; PoSmaV 1-3 exhibited an ambisense genome (Type IV) with a canonical nonanucleotide motif TAGTATTAC on the non-Rep encoding strand, while PoSmaV 4-5 displayed a unisense genome (Type V) with the nonanucleotide motif TACTAGTAC on the Rep encoding strand. A putative *ori* marked by a predicted stem-loop structure was detected in all smacoviruses' genomes (Fig. 2a). Besides the ORFs for Rep and CP, a third ORF that

encodes a conserved hypothetical protein was identified. In addition, conserved RCR and SF3 helicase motifs were found in all Rep protein sequences identified here (Supplementary Table S1).

Phylogenetic analysis revealed PoSmaV 1-3 clustered with *Porprismacovirus* (Fig. 2b). With basis on the 77% genome-wide identity criterion for species demarcation (Varsani and Krupovic, 2018), PoSmaV-2 was assigned to the *Porcine associated porprismacovirus 2* species, whereas PoSmaV-1 and 3 were assigned to the *Porcine associated porprismacovirus 3* (Supplementary Material S1).

5.1.1.1 A new genus within the Smacoviridae is proposed – "Suismacovirus"

PoSmaV-4 and 5 formed a well-supported clade along with other unclassified smacoviruses which did not fit into any established genera (Fig. 2b). Both of them shared the highest genome-wide identity with CRESS DNA viruses recovered from rectal swabs of pigs. PoSmaV-4 genome shared ~84% nucleotide identity with MH111088 and PoSmaV-5 shared ~78% nucleotide identity with MH111105 (Supplementary Material S2). The common features observed among the genomes of this clade included an unisense genome architecture and a putative *ori* marked by a conserved nonanucleotide ([T/G]A[C/T][T/C][A/G][G/A]T[A/T]C) at the apex of a predicted stem-loop structure located next to the 5' terminal of the *rep* gene. Moreover, the Rep RCR and SF3 helicase motifs were conserved among those (Fig. 3a). In addition, all sequences within this clade had more than 54% Rep pairwise identity, yet less than 42% with members of other genera (Supplementary Fig. S1). Thus, according to adopted recommendations for smacoviruses' taxonomy (Varsani and Krupovic, 2018), in which a 40% Rep amino acid sequence identity has been used as a genus delineator (VARSANI;KRUPOVIC, 2018), we propose the creation of a new genus within the *Smacoviridae*, named *Suismacovirus* (*Sui* = *suinus* [latin] = swine).

Subsequent genome-wide pairwise analysis showed that the sequences from this monophyletic clade (Fig. 2) formed 21 potential new species, of which 16 correspond to viral genomes recovered from pigs (Fig. 3b). It was observed that nine smacoviruses' Rep included in the new proposed genus "*Suismacovirus*", also share >40% pairwise identity with those of *Huchismacovirus* members. A similar finding was observed for a *Dragsmacovirus* representative, which showed >40% Rep identity with *Huchismacovirus*, though in a different genus. Regardless of such observations, in both cases, a strong phylogenetic support is indicating that those sequences belong to different genera. Thus, these findings highlight the need for different analyses for *Smacoviridae* taxonomic inferences, for which the phylogenetic analysis and Rep pairwise identity scores performed here are recommended.

Smacoviruses have been largely detected in fecal matter of several vertebrates (STEEL *et al.*, 2016; KRABERGER *et al.*, 2018; FONTENELE *et al.*, 2019; LIMA *et al.*, 2019), including pigs (CHEUNG *et al.*, 2013; SIKORSKI *et al.*, 2013; CHEUNG *et al.*, 2014; KIM *et al.*, 2014; CHEUNG *et al.*, 2015), and invertebrates (DAYARAM *et al.*, 2015; ROSARIO *et al.*, 2018). However, to date, this is the second study reporting smacovirus' genomes in pigs' serum (TOCHETTO *et al.*, 2020b). Notably, previous investigations on serum viromes of other animal species have not identified smacovirus genomes (ZHANG *et al.*, 2016; MOUSTAFA *et al.*, 2017; SADEGHI *et al.*, 2017; ZHANG *et al.*, 2017; WANG *et al.*, 2018; WEBER *et al.*, 2018). Interestingly, infection of Archaea with smacoviruses has been suggested (DIEZ-VILLASENOR;RODRIGUEZ-VALERA, 2019). The detection of smacovirus genomes in animal faecal matter and serum reinforces the hypothesis that these may likely infect animal cells, cause viremia and be shed in faeces. However, those may also have its origins from Archaea infecting the intestinal tract.

5.1.2 Redondoviridae – a new redondovirus genome

The *Redondoviridae* is a recently established viral family that comprises viral genomes recovered from oro-respiratory samples of humans; it was then proposed that these would primarily replicate in humans (ABBAS *et al.*, 2019). Redondoviruses genome are ~3 kb in length and contains three ORFs bidirectionally organized encoding a Rep, a CP, and a conserved hypothetical protein (ORF3) overlapping the CP. Currently, the family comprises a single genus, *Torbevirus,* with two species, *Brisavirus* and *Vientovirus* (ABBAS *et al.*, 2019). Phylogenetic analysis revealed that one genome obtained here clustered with members of *Redondoviridae* family, to which the name porcine redondovirus 1 (PoRV-1) is proposed (Table 1; Fig. 1; Fig. 4).

The PoRV-1 genome has ~3 kb and share the same genomic characteristics with previously reported redondoviruses (Table 1, Fig. 4a) (ABBAS *et al.*, 2019). At the 5' terminal of the *rep* gene, the canonical nonanucleotide TAGTATTAT is present at the apex of a predicted stem-loop structure marking a potential *ori.* Conserved RCR and SF3 helicase (including the arginine finger) motifs were found in all Rep protein sequences clustering with redondoviruses (Fig. 4d). Phylogenetic analysis of the Rep protein showed that PoRV-1 clusters with *Vientovirus* species, together with two unclassified CRESS DNA viruses recovered from pig faeces, named porcine stool-associated circular virus (PoSCV) 5 (KJ433989) (CHEUNG *et al.*, 2014) and PoSCV-BEL/15V010 (KY214434) (CONCEICAO-NETO *et al.*, 2017a) (Fig. 4b). PoRV-1 Rep amino acid sequence was ~51-57% identical to those of *Vientovirus* members and ~62-63% to those of PoSCV-5 (KJ433989) and PoSCV-BEL/15V010 (KY214434) (Fig. 4c, Supplementary Material S3).

Previous study proposing *Redondoviridae* family analyzed CP and ORF3 protein sequences in order to better characterize this taxon. Accordingly, in the present study, the CP identity of PoRV-1 varied between ~48-52% with others redondoviruses, ~88% with PoSCV-5 and ~84% with PoSCV-BEL/15V010 (Supplementary Material S4). A different phylogenetic

300 relatedness was observed among the CPs: PoRV-1, PoSCV-5 and PoSCV-BEL/15V010 formed
301 a distinct clade, apart from *Redondoviridae;* nevertheless, this clade was more closely related
302 to members of *Redondoviridae* than to other viral families (Supplementary Fig 2). Such
303 different relationships between Rep and CP phylogenies can be an indicative of recombination
304 (SCHIERUP;HEIN, 2000), which are a common event in CRESS DNA viruses (MARTIN *et*
305 *al.*, 2011; FONTENELE *et al.*, 2018; ANINDITA *et al.*, 2019).

306 Regarding ORF3, the pairwise identity ranged between ~34-40% with those of
307 redondoviruses and was higher with that of PoSCV-5 (~84%) and PoSCV-BEL/15V010
308 (~77%) (Supplementary Material S5). Although previous studies have not reported a putative
309 ORF3 in PoSCV-5 and PoSCV-BEL/15V010 genomes (CHEUNG *et al.*, 2014; ABBAS *et al.*,
310 2019), the current inspection detected a third ORF overlapping CP, homologous to ORF3 from
311 *Redondoviridae* family members (BLASTp search [PoSCV-5 ORF3: ~36% of amino acid
312 identity, e-value 4e-07, coverage 98%]; [PoSCV-BEL/15V010 ORF3 ~43% of amino acid
313 identity, e-value 0.011, coverage 53%]). PoSCV-5 and PoSCV-BEL/15V010 are currently
314 assigned to the *Circoviridae* family. However, based on the analyses performed here, such viral
315 genomes, together with PoRV-1, would be better placed taxonomically within the
316 *Redondoviridae* family. The genomic features of all these genomes were compared and are
317 available on Supplementary Table S2.

318

319 *5.1.3 Genomoviridae*

320 The *Genomoviridae* is a large viral family, currently divided in ten *genera*
321 *(Gemycircularvirus, Gemyduguivirus, Gemygorvirus Gemykibivirus, Gemykolovirus,*
322 *Gemykrogvirus, Gemykrozvirus, Gemytondvirus, Gemytripvirus and Gemyvongvirus)* and 237
323 species (VARSANI;KRUPOVIC, 2021). Genomoviruses genomes (~2 kb) are bidirectionally
324 organized (Type II) with two or three ORFs encoding a Rep (often spliced) and a RepA in the

complementary sense strand (unspliced smaller Rep-like protein with an endonuclease domain). In addition, a CP is encoded in the virion-sense (CHABI-JESUS *et al.*, 2020; VARSANI;KRUPOVIC, 2021).

The genomes of three novel genomoviruses here detected were named porcine genomovirus (PoGV) 1-3 (Table 1). All exhibited the typical organization of genomoviruses (Fig. 5a). A putative *ori* motif was identified at the apex of a potential stem-loop structure located at the large intergenic region (LIR). RCR and SF3 helicase motifs common to genomoviruses were found in the putative Rep of the three genomes (Supplementary Table S1). In addition, two additional conserved features among genomoviruses' Rep were identified: the fourth motif called "geminivirus Rep sequence" (GRS) between the RCR motifs II and II, and the lack of the arginine finger motif (Varsani & Krupovic 2017) (Supplementary Table S1).

By phylogenetic inference, PoGV-1 was classified in the *Gemykibivirus* genus, whereas PoGV-2 and PoGV-3 were classified in the *Gemykrogvirus* genus (Fig. 5b). Following the species demarcation criteria for *Genomoviridae* family members (78% genome-wide identity) (VARSANI;KRUPOVIC, 2017; VARSANI;KRUPOVIC, 2021), PoGV-1 was classified as *Gemykibivirus anima1* species. It shared >99% genome-wide identity with other genomoviruses previously detected in pig faeces (KY214433) (CONCEICAO-NETO *et al.*, 2017a) and arthropods (MN379617, MH545498) (ROSARIO *et al.*, 2018) (Supplementary Material S6). PoGV-1 Rep shared between 44-100% amino acid identity with gemykibiviruses, while the CP identity varied between ~29-100% with members of that genus (Supplementary Material S7-S8). PoGV-2 and 3 were assigned to *Gemykrogvirus carib1* species, sharing ~78% and ~96% genome-wide identity with gemykrogvirus recovered from faeces of caribou (KJ938717) (NG *et al.*, 2014) and from gila monster (MH378453), respectively (Supplementary Material S9). The Rep and CP of PoGV-2 and 3 shared between ~40-95% and

~33-99% amino acid identities, respectively, with those of all gemykrogviruses (Supplementary Material S10-S11).

Genomoviruses have been detected in eukaryotes (fungi, plants, insects, birds, mammals) (STEEL *et al.*, 2016; VARSANI;KRUPOVIC, 2017; KRABERGER *et al.*, 2019b) and in environment (KRABERGER *et al.*, 2015; PHAN *et al.*, 2015). Currently, only one species, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), was successfully cultivated *in vitro* in cells of a phytopathogenic fungus (*Sclerotinia sclerotiorum)* (YU *et al.*, 2010; YU *et al.*, 2013b) and a mycophagous insect (*Lycoriella ingenua*) (LIU *et al.*, 2016b). Previous studies have also reported genomoviruses in humans and other animal species.

5.1.4 Divergent CRESS DNA viral genomes

Nine CRESS DNA viral genomes, plus one circular DNA molecule identified in the present study, could not be assigned to any of the viral families currently described (Fig. 1; Fig. 6). The nine genomes vary in organization and size (1.8 to ~2.9 kb) (Table 1), although all of these encode putative Rep and CP. These were here named porcine associated circular DNA virus 2 to 10 (PoCV 2-10). The predominant genomic architecture observed was Type II (n = 7), although the Types IV (n = 1) e V (n = 1) have also been detected (Table 1; Fig. 6).

Most of the PoCV genomes recovered here exhibited a common putative *ori* marked by a nonanucleotide motif at the apex of a predicted stem-loop structure (n = 7). This structure was not found in the PoCV-4 and PoCV-8 genomes, though both exhibited an *ori* nonanucleotide motif (Table 1). All Rep encoding sequences harbor RCR and SF3 helicase motifs, with the exception of PoCV-8 which is apparently missing RCR motif I, and PoCV-7 which seems to lack RCR motif I and III (Supplementary Table S1). The BLASTp analysis of the putative CPs encoded by these CRESS DNA viruses is summarized in the Supplementary Table S3).

373 The phylogenetic tree, including representatives of *Cressdnaviricota* phylum, displayed
374 all of the previously established families as monophyletic with high statistical support (>0.9),
375 with exception of the *Bacilladnaviridae* clade (statistical support ~0.7) (Fig. 6). Such unstable
376 position of *Bacilladnaviridae* has been documented previously (KAZLAUSKAS *et al.*, 2019).
377 Of the divergent CRESS DNA viruses detected here, five genomes and the circular DNA
378 molecule were classified within four well-supported distinct clades (named 1 to 4 for
379 explanation purposes); three sequences clustered within the CRESSV1 clade and one clustered
380 within the CRESSV2 clade (Fig. 6).

381 PoCV-2, 3 and 4 grouped with CRESSV1 sequences, though in distinct clusters (Fig.
382 6). PoCV-2 and 3 exhibited a canonical putative *ori* marked by the nonanucleotide motif
383 TAGTATTAC at the apex of a predicted stem-loop structure. PoCV-2 and 3 shared ~70% Rep
384 pairwise identity between them and ~72% with bovine faeces associated circular DNA
385 molecule (KT862256) recovered in New Zealand (STEEL *et al.*, 2016). PoCV-4 lacked a stem-
386 loop structure but exhibited the *ori* nonanucleotide motif TATTTTTAC near to the 5' terminal
387 of the *cap* gene. It shared ~57% Rep and ~75% CP identity with an unidentified circular ssDNA
388 virus recovered from rhesus macaque feces (KU043446) in the USA. Rep pairwise identity
389 scores among CRESSV1 clade sequences evaluated here varied from ~20% to 93%, with a
390 ~35% average pairwise identity score (Supplementary Material S12).

391 PoCV-5 formed a cluster closer to CRESSV3 members (Clade 4 in Fig. 6) and was most
392 closely related to an unclassified circular ssDNA viral genome (KU043400) recovered from
393 feces of rhesus macaques in the USA (Rep and genome-wide pairwise identity: ~96% and
394 ~91%, respectively). The predicted CP amino acid sequence shared ~83% identity with
395 *Hudisavirus* sp. (MG522854) recovered from human feces in Ethiopia. Pairwise identity scores
396 of Rep indicate that sequences clustered in Clade 4 (Fig. 6) share more than 73% amino acid
397 identity, with an average score of ~87% (Supplementary Material S12). Most of the genomes

398 in Clade 4 exhibit Type IV genome architecture (except for four linear ssDNA genomes
399 obtained from GenBank: MG522854-MG522857; (ALTAN *et al.*, 2018)). A common putative
400 *ori* motif CAGT[G/A]TTAC near to the 3' terminal *rep* gene at the apex of a predicted stem-
401 loop structure (data not shown) was identified in all members (n = 12) of this clade. In addition,
402 Clade 4 members share a relatively high degree of genome-wide pairwise identity between them
403 (~62% to ~96%; average ~75%). Those findings are indicative that Clade 4 may represent a
404 new taxon within *Cressdnaviricota* phylum.

405 The additional PoCV genomes identified here, PoCV-6, PoCV-7 and PoCV-10
406 clustered within Clade 2. All of them have a putative *ori* marked by a nanonucleotide motif
407 TAGT[A/G]TTAC in the non-Rep strand, at the apex of a predicted stem-loop structure. PoCV-
408 6 was most closely related to odonata-associated circular virus (OdasCV) (KM598400; ~60%
409 Rep amino acid identity); PoCV-7, on its turn, was most closely related to tundra vole stool-
410 associated circular virus (MK738137) recovered from feces of *Tundra vole* in Russia (~47%
411 Rep amino acid identity); PoCV-10 was most closely related to marmot associated feces viruses
412 (Fig. 6) and shared the highest Rep amino acid identity (>57%) with two of them (MT181528
413 and MT181534). Regarding the putative CP of these three genomes (PoCV-6, 7 and 10), they
414 share the highest amino acid identity (>52%) with those of marmot associated feces virus.

415 PoCV-8 clustered within Clade 3 in the phylogenetic tree (Fig. 6); it exhibits a canonical
416 nonanucleotide motif TAGTATTAC marking the putative *ori* at the 5' terminal of *rep* gene,
417 but it lacked a stem-loop. PoCV-8 was most closely related to an unclassified circular ssDNA
418 virus (KU043409) detected in feces of rhesus macaque in USA (Rep pairwise identity: ~91%).
419 CP shared ~91% identity with a different unidentified circular ssDNA virus (KU043405).
420 Pairwise identity scores (Rep) indicate that all members of Clade 3 share more than 20%
421 identity, with an average of ~47%.

In PoCV-9, the nonamer AAGTATTAC is at the apex of a predicted stem-loop structure near to the 5' terminal of the *rep* gene. Phylogenetic inference shows close relationship between PoCV-9 and CRESS DNA sequences recently assigned to a newly proposed viral family, tentatively named *Nenyaviridae*, which includes the genera *Tetartoentvirus* and *Tritoentvirus* (Fig. 6) (KINSELLA *et al.*, 2020). PoCV-9 is most closely related to members of the *Tetartoentvirus* genus, sharing the highest genome-wide, Rep and CP amino acid identities (~95%, ~99% and ~81%, respectively) with *Entamoeba*-associated CRESS DNA virus 4 (MT293418) recovered from human stool in Netherlands (KINSELLA *et al.*, 2020) (Supplementary Material S13). In addition, PoCV-9 exhibits the same genomic features and shares similar Rep RCR and SF3 helicase motifs with members of *Tetartoentvirus* genus (Supplementary Table S4). This is the first report of an *Entamoeba*-associated CRESS DNA virus genome identified in serum of pigs. It has been suggested that members of this family infect *Entamoeba* parasites, which in turn can infect several vertebrates including humans and pigs and persist as commensal parasites in the intestinal tract (SOLAYMANI-MOHAMMADI;PETRI, 2006; URIBE-QUEROL;ROSALES, 2020). However, in some cases, these parasites can reach the blood circulation during phlebotomy, translocation from enteric environment, or systemic infections (URIBE-QUEROL;ROSALES, 2020). Thus, it is possible that the PoCV-9 genome detected in the present study reflects contamination of pigs with *Entamoeba*, eventually being released into the blood flow.

Finally, one circular DNA molecule with 1,049 nt and a single ORF encoding for a putative Rep was identified and is referred here to as porcine associated DNA molecule 1 (PoM-1) (Table 1). A potential stem-loop structure with the canonical nonanucleotide motif TAGTATTAC at its apex was identified near the 5' terminal of the *rep* gene. The Rep protein contains all RCR and SF3 helicase motifs characteristic of CRESS DNA viruses but lacks the arginine finger motif. In addition, the GRS motif was identified between RCR motifs II and III

(Supplementary Table 1). These findings are typical of Reps encoded by members of *Geminiviridae* and *Genomoviridae*, but not in *Bacilladnaviridae*, *Circoviridae*, *Nanoviridae*, and *Smacoviridae* (KRUPOVIC *et al.*, 2020). Phylogenetic analysis indicates that PoM-1 clusters within Clade 1, close to *Geminiviridae, Genomoviridae* and CRESSV6 members (Fig. 6). PoM-1 was most closely related to a CRESS DNA virus (MN621480) recovered from forest musk deer feces in China (~55% Rep amino acid identity). On contrary to all CRESS DNA genomic sequences forming Clade 1, PoM-1 exhibits a single ORF genome, a characteristic observed mainly in genomes of multipartite viruses from the *Nanoviridae* and *Geminiviridae* family, and satellite DNA molecules that require helper viruses for encapsidation (BRIDDON;STANLEY, 2006; KRABERGER *et al.*, 2019b).

5.1.5 Concluding remarks

Numerous studies have identified circular ssDNA viruses in feces of pigs. However, just a few have focused on the investigation of viromes in pigs' serum (QIN *et al.*, 2018; TOCHETTO *et al.*, 2020b; HE *et al.*, 2021; PAIM *et al.*, 2021). Nonetheless, CRESS DNA viral genomes have been reported in blood circulation of other mammals (ZHANG *et al.*, 2016; MOUSTAFA *et al.*, 2017; ZHANG *et al.*, 2017; WANG *et al.*, 2018; WEBER *et al.*, 2018). The presence of *Cressdnaviricota* viruses in serum and blood samples raises questions about their biology. For most of these viruses, there is no evidence that they can replicate in mammals host cells (KRUPOVIC *et al.*, 2020), but this is a possibility that should be evaluated in view of these results. On the other hand, their presence in serum could reflect systemic infection with still unknown cellular hosts which release their viruses into the bloodstream (WANG *et al.*, 2018). Another possibility is the translocation of viruses from the intestinal barrier into the blood (GORSKI *et al.*, 2006). For instance, the anelloviruses (also circular ssDNA viruses) are commonly detected in blood (WEBB *et al.*, 2020) and also in feces (BRASSARD *et al.*, 2008).

Such hypotheses should be further investigated, in order to allow a better understanding of the biology of CRESS DNA viruses in swine and other animal species.

Another relevant topic regarding viral genomes circulating in the blood is their potential role as causative of disease, or as part of its host's natural virome. The presence of viruses in the blood can interfere with the immune system and can be indirectly related to diseases' susceptibility or protection (CADWELL, 2015). Noticeably, the viral genomes recovered in this study were detected in pooled serum samples of domestic pigs with clinical signs of respiratory disease. However, since individual samples of healthy and diseased animals were not analyzed concurrently, it is not possible to infer associations between the occurrence of disease and the presence of such viral genomes in the hosts. These aspects should be addressed in further studies.

6. Conclusions

This study presented a snapshot of the CRESS DNA viruses circulating in serum of domestic pigs from Southern Brazil. A high diversity of viruses belonging to the families *Smacoviridae, Redondoviridae, Genomoviridae, Nenyaviridae* and unassigned viruses were identified. Remarkably, for the first time, viruses from the families *Redondoviridae* and *Nenyaviridae* are reported in pigs. In addition, this study proposes a new genus within the *Smacoviridae* family, named *Suismacovirus*, and several potential new species. Many CRESS DNA viruses identified here showed high identity with those identified in feces, raising up the question as to whether these could make their way to circulation via intestinal tract or whether these could be somehow mechanically transported by other hosts into the bloodstream. In addition, studies accessing their role as pathogens must also be performed. Nevertheless, this study expands the knowledge about CRESS DNA viruses confirming its

occurrence in commercially reared swine and providing additional evidence for the wide distribution of such genomes, with remarkable genetic and evolutionary diversity.

7. Author statements

7.1 Authors and contributors

Conceptualization: CT, FQM, SPC, DAL

Data curation: CT, SPC, APMV, FQM

Formal analysis: CT

Funding acquisition: PMR

Investigation: CT, SPC, CC, TFT, APMV

Resources: PMR

Supervision: FQM, PMR

Visualization: CT

Writing – original draft: CT, FQM

Writing – review & editing: SPC, FQM, PMR

7.2 Conflicts of interest

The authors declare that there are no conflicts of interest.

7.3 Funding information

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7.4 Ethical approval

This study was approved by the Ethics Committee on Animal Use from the Universidade Federal do Rio Grande do Sul (CEUA-UFRGS), protocol number 31670.

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Figures

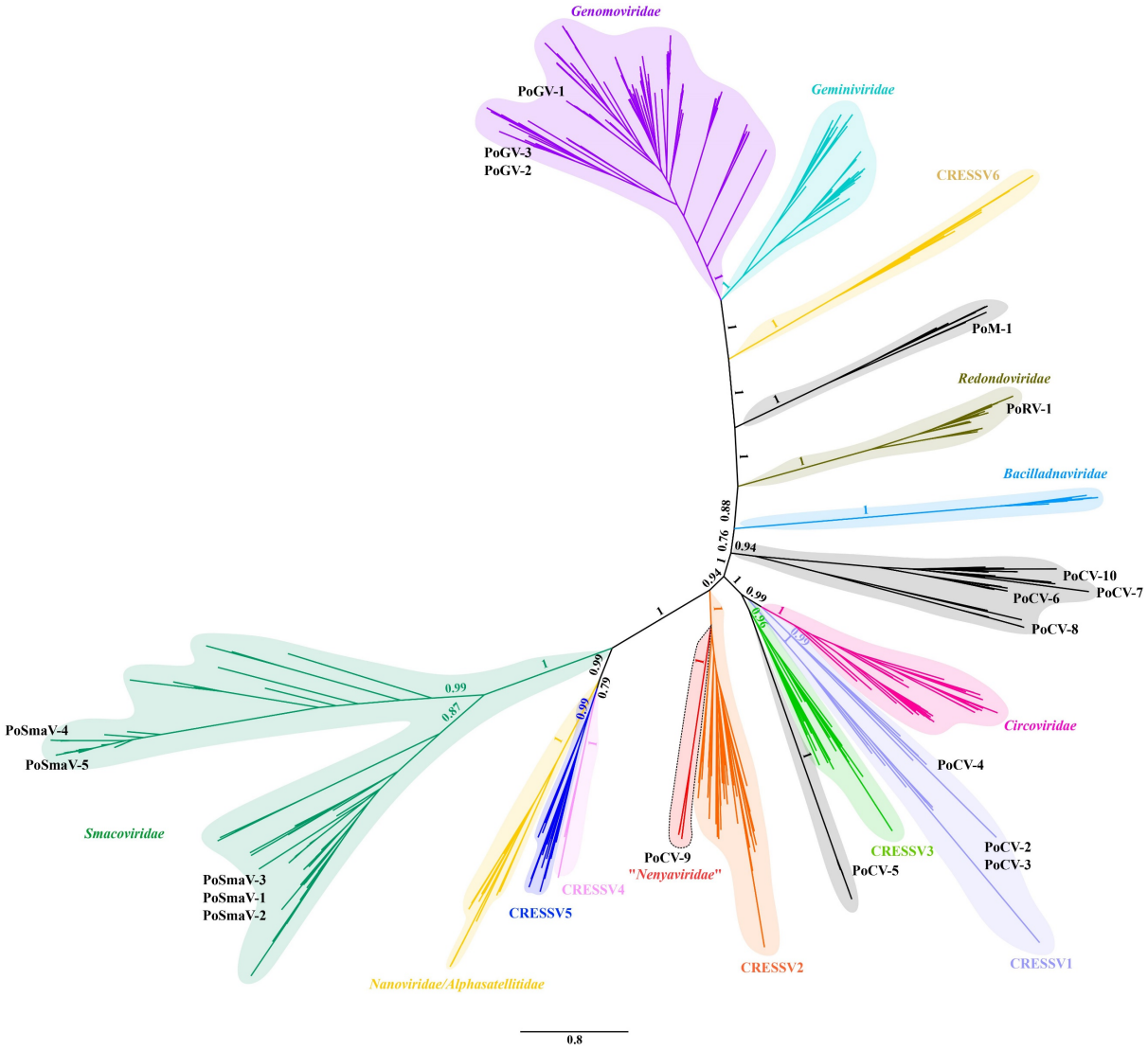


Fig. 1. Unrooted phylogenetic tree of the novel CRESS DNA viruses Rep proteins identified in serum of domestic pigs. Maximum likelihood phylogenetic tree based on the replication-associated protein (Rep) amino acid sequences of 556 CRESS DNA viruses, including those identified here (n = 19). The branch support was assessed using aBayes implemented in IQTREE. The viral genomes recovered here are shown near to the branch of its location on the tree.

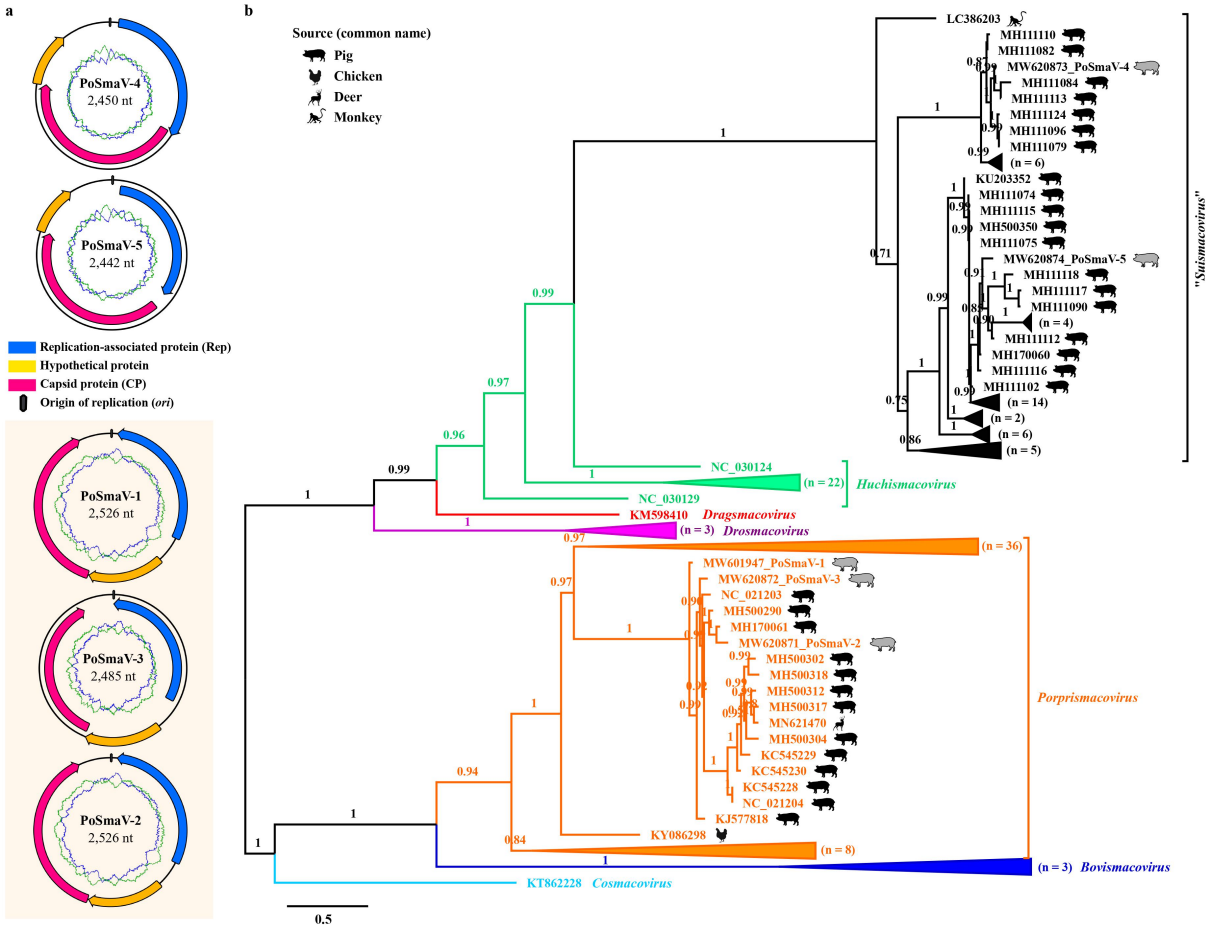


Fig. 2. Smacoviruses' genomes recovered from serum of domestic pigs. (a) Genomic organization of the smacovirus genomes detected here with their putative stem-loop structure containing the *ori* nonanucleotide motif. **(b)** Mid-point rooted phylogenetic tree based on Rep amino acid sequences of smacovirus and unclassified CRESS DNA viruses tentatively assigned to the novel genus "*Suismacovirus*" proposed here (n = 152). The branch support was assessed using the aBayes test (48) implemented in IQTREE. The grey pig pictures indicate the sequences recovered in this study.

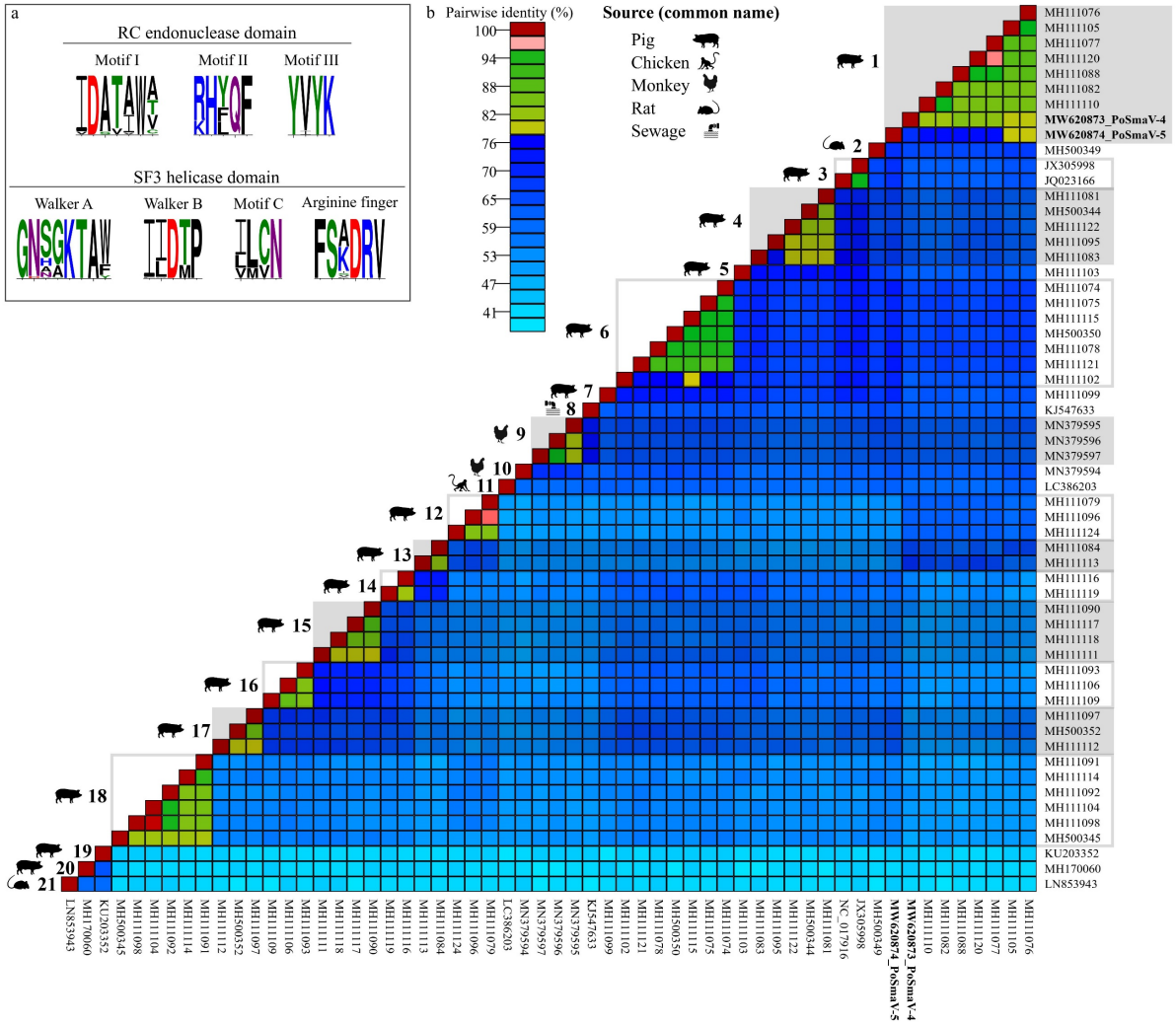


Fig. 3. Genome-wide pairwise identity plot of 58 genomes related to the new proposed *Smacoviridae* genus, "*Suismacovirus*". (a) Conserved RCR and SF3 helicase motifs of Rep protein from sequences grouped within the *Suismacovirus* genus. Residues are colored according to their chemical properties (polar, green; neutral, purple; basic, blue; acidic, red; hydrophobic, black). **(b)** Genome-wide identity (>77%) of smacovirus genomes, including the 21 potential new species identified in this study. The two genomes recovered here grouped with seven unclassified CRESS DNA virus genomes and are shown in bold. The identity scores are available on Suppl. Material S2.

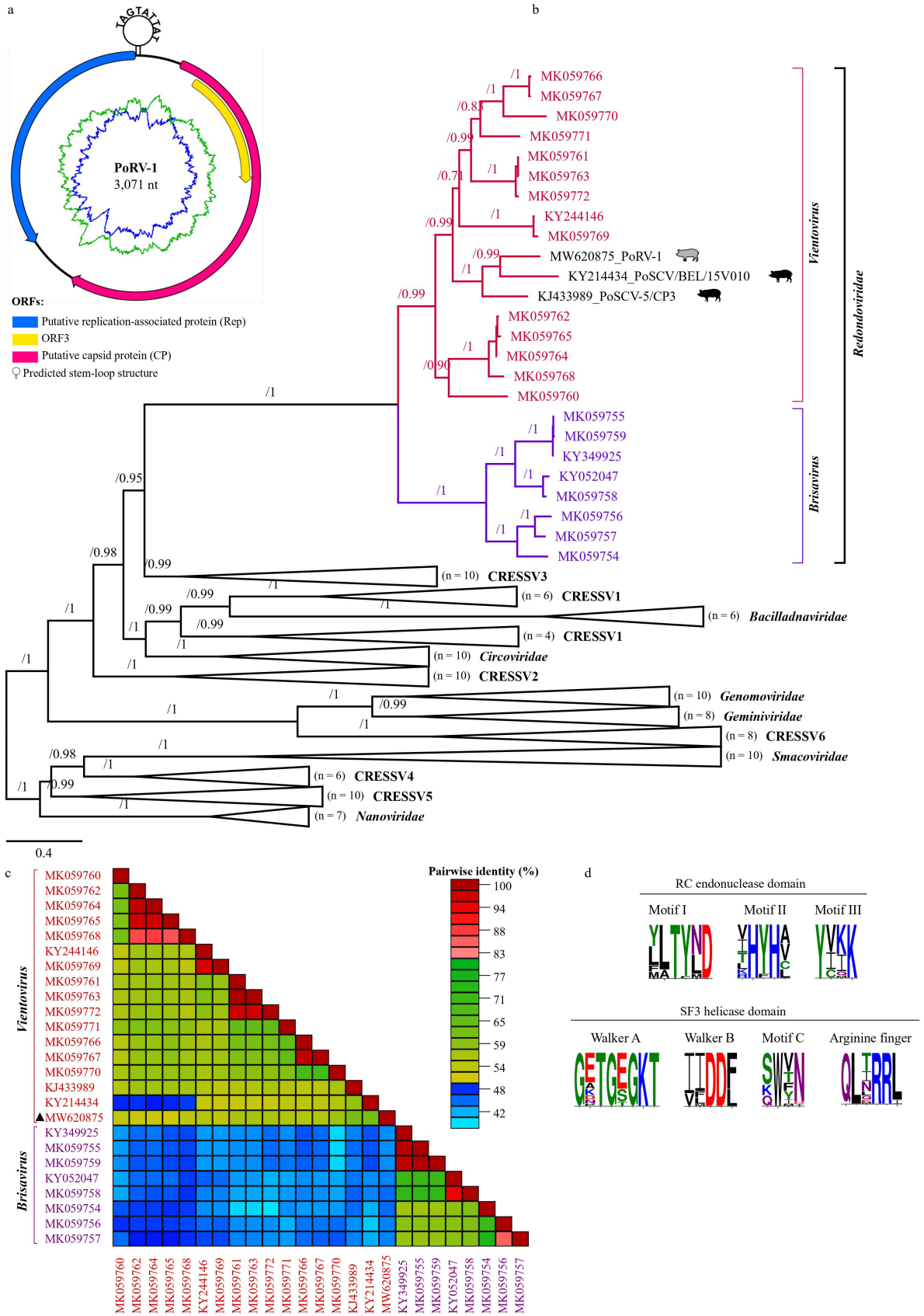


Fig. 4. Analysis of the redondovirus genome recovered in the present study (PoRV-1, porcine redondovirus). (a) PoRV-1 genome organization. A putative origin of replication (*ori*)

1135 is indicated by a predicted stem-loop structure with the canonical nonanucleotide motif located
1136 at its the apex. **(b)** Mid-point rooted maximum likelihood phylogenetic tree based on the Rep
1137 amino acid sequences from CRESS DNA viruses, including 22 Reps of members of
1138 *Redondoviridae* family. The number of Rep sequences included in each family/group varied
1139 between 6-10 (total = 129 sequences). The potential redondoviruses recovered from pigs are
1140 highlighted by a schematic pig; the one recovered in this study (PoRV-1) are in grey. **(c)**
1141 Pairwise identity matrix of Rep amino acid sequences from members of *Redondoviridae* family,
1142 PoRV-1 and two related unclassified CRESS DNA viruses (KJ433989, KY214434). **(d)**
1143 Conserved RCR and SF3 helicase motifs of Rep protein from redondoviruses including PoRV-
1144 1, PoSCV-5 and PoSCV- BEL/15V010. Residues are colored according to their chemical
1145 properties (polar, green; neutral, purple; basic, blue; acidic, red; hydrophobic, black).
1146

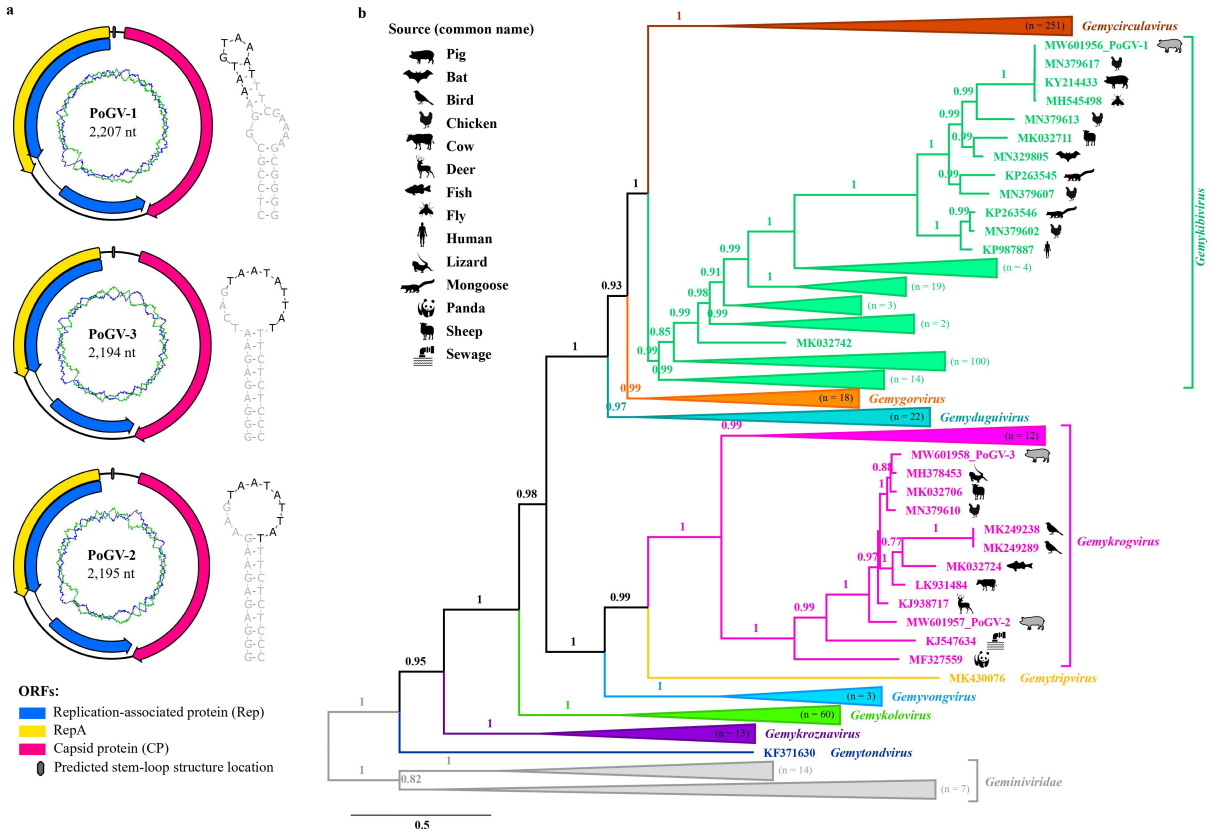


Fig. 5. Comparative phylogenetic analysis of the three genomoviruses detected in this study and other genomovirus genomes. (a) Genomic organization of the three genomovirus recovered in this study (PoGV, porcine genomovirus 1-3). The predicted stem-loop structures are shown next to each genome with the nonanucleotide motif at the apex marking the potential origin of replication (*ori*). **(b)** Maximum likelihood phylogenetic tree based on the Rep amino acid sequences of 569 CRESS DNA viruses and rooted with geminivirus sequences. The branch support was assessed using the aBayes test (48) implemented in IQTREE (values below 0.7 are not shown). Genomoviruses recovered here are marked with a schematic grey pig.

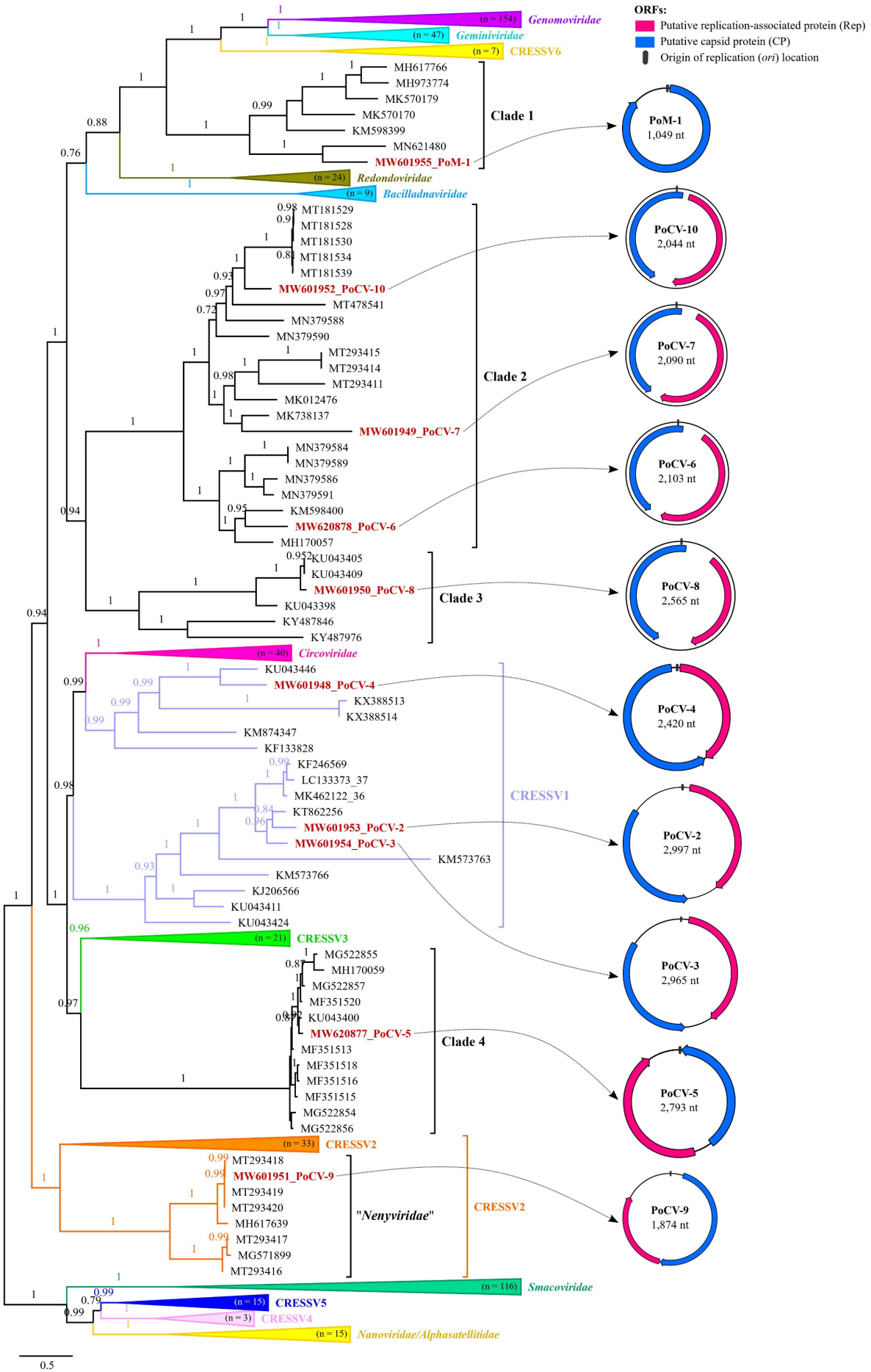
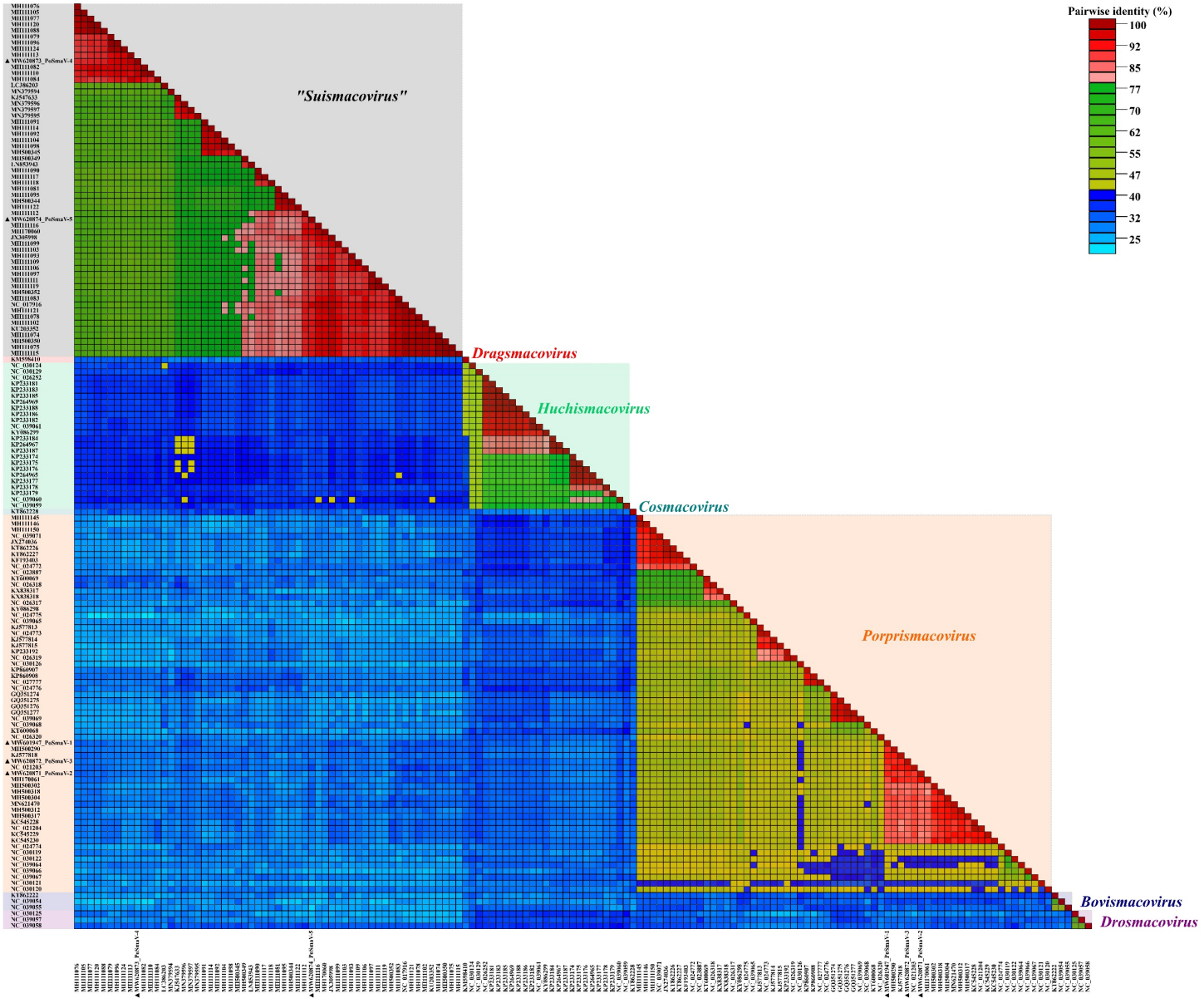
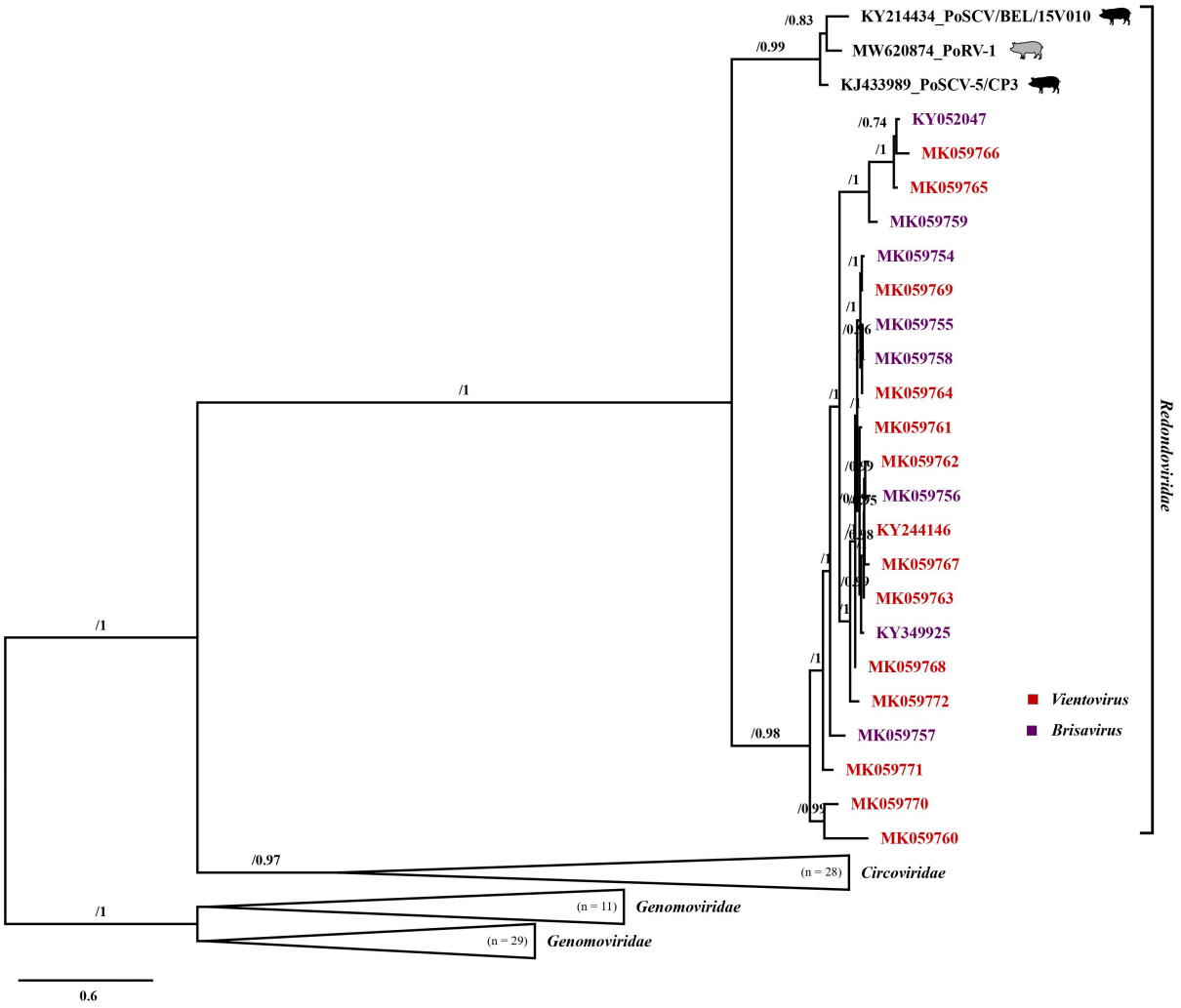

ORFs:
Putative replication-associated protein (Rep)
Putative capsid protein (CP)
Origin of replication (ori) location
(n = 154) Genomoviridae
(n = 47) Geminiviridae
(n = 7) CRESSV6
(n = 24) Redondoviridae
(n = 9) Bacilladnaviridae
(n = 40) Circoviridae
(n = 21) CRESSV3
(n = 33) CRESSV2
(n = 116) Smacoviridae
(n = 15) CRESSV5
(n = 3) CRESSV4
(n = 15) Nanoviridae/Alphasatellitidae
Clade 1
Clade 2
Clade 3
Clade 4
CRESSV1
CRESSV2
"Nenyviridae"
MH617766
MH973774
MK570179
MK570170
KM598399
MN621480
MW601955_PoM-1
MT181529
MT181528
MT181530
MT181534
MT181539
MW601952_PoCV-10
MT478541
MN379588
MN379590
MT293415
MT293414
MT293411
MK012476
MK738137
MW601949_PoCV-7
MN379584
MN379589
MN379586
MN379591
KM598400
MW620878_PoCV-6
MH170057
KU043405
KU043409
MW601950_PoCV-8
KU043398
KY487846
KY487976
KU043446
MW601948_PoCV-4
KX388513
KX388514
KM874347
KF133828
KF246569
LC133373_37
MK462122_36
KT862256
MW601953_PoCV-2
MW601954_PoCV-3
KM573763
KM573766
KJ206566
KU043411
KU043424
MG522855
MH170059
MG522857
MF351520
KU043400
MW620877_PoCV-5
MF351513
MF351518
MF351516
MF351515
MG522854
MG522856
MT293418
MW601951_PoCV-9
MT293419
MT293420
MH617639
MT293417
MG571899
MT293416
PoM-1
1,049 nt
PoCV-10
2,044 nt
PoCV-7
2,090 nt
PoCV-6
2,103 nt
PoCV-8
2,565 nt
PoCV-4
2,420 nt
PoCV-2
2,997 nt
PoCV-3
2,965 nt
PoCV-5
2,793 nt
PoCV-9
1,874 nt
0.5

Fig. 6. Phylogenetic tree and genomic organization of the nine divergent CRESS DNA viruses' genomes and one circular DNA molecule recovered from serum of domestic pigs in this study. Midpoint-rooted maximum likelihood phylogenetic tree of 556 Rep amino acid sequences of CRESS DNA viruses**.** The final alignment contained 416 amino acid sites. Clades belonging to the same viral family/group have the same color. The origin of replication (*ori*) location is highlighted in each genome illustration. All of them exhibited a predicted stem-loop structure, except PoCV 4-8. PoCV: porcine associated circular DNA virus; PoM: porcine circular DNA molecule.


Pairwise identity (%)
100
92
85
77
70
62
55
47
40
32
25
"Suismacovirus"
Dragsmacovirus
Huchismacovirus
Cosmacovirus
Porprismacovirus
Bovismacovirus
Drosmacovirus

Suppl. Fig. 1. Pairwise identity distribution of Rep amino acid sequences of smacoviruses. Sequences grouped according the genus demarcation criteria established for *Smacoviridae* family (>40% of Rep amino acid identity). Fifty-eight sequences had <40% pairwise identity to representatives of the six genera within *Smacoviridae* family and were tentatively assigned to a new *genus* of smacoviruses, "*Suismacovirus*". The black diamond indicates the sequences found here.



Suppl. Fig. 2. Phylogenetic tree based on the capsid protein (CP) amino acid sequences from members of *Redondoviridae* family (n = 22) and representatives of other CRESS DNA viruses families. The tree was constructed using IQTREE (NGUYEN *et al.*, 2015) with automatic selection of best-fit substitution model (*VT + F + G4*). Multiple sequence alignment was performed with MAFFT (options *"L-INS-i -leavegappyregion -ep 0.123"*) (45). The tree was rooted using *Genomoviridae* sequences as an outgroup. Potential redondoviruses recovered from pigs are highlighted by a schematic pig; the one recovered in this study (PoRV-1) is in grey.

Tables

Table 1. Genomic features of the 19 novel CRESS DNA viral genomes detected in this study.

Viral family/group	Genus	Acronym	Viral genome	Accession number	Length (nt)	Genome architecture	ORF orientation	Putative *ori* motif	Stem-loop (location)	Coverage (mean)[1]	CG content (%)
Smacoviridae	*Poprismacovirus*	PoSmaV-1	Porcine smacovirus-1 P20_50_BR	MW601947	2526	IV	ambisense	TAGTATTAC	2,518-22	77.5	42.8
		PoSmaV-2	Porcine smacovirus-2 P20_51_BR	MW620871	2526	IV	ambisense	TAGTATTAC	2,518-22	49.8	43
		PoSmaV-3	Porcine smacovirus-3 P20_52_BR	MW620872	2485	IV	ambisense	TAGTATTAC	2,468-35	63.2	43.9
Smacoviridae	*Suismacovirus*[2]	PoSmaV-4	Porcine smacovirus-4 P20_53_BR	MW620873	2450	V	unisense	TACTAGTAC	2,442-31	44.9	48.1
		PoSmaV-5	Porcine smacovirus-5 P20_54_BR	MW620874	2442	V	unisense	TACTAGTAC	2,434-31	41.6	47.9
Redondoviridae	*Torbevirus*	PoRV-1	Porcine redondovirus-1 P20_29_BR	MW620875	3071	II	ambisense	TAGTATTAT	3,062-20	40.6	36.8
Genomoviridae	*Gemykibivirus*	PoGV-1	Porcine genomovirus-1 P20_66_BR	MW601956	2207	II	ambisense	AATGTAAAT	2,198-24	60.2	50.2
Genomoviridae	*Gemykrogvirus*	PoGV-2	Porcine genomovirus-2 P20_60_BR	MW601957	2195	II	ambisense	TAATATTAT	2,183-18	85.9	50.1
		PoGV-3	Porcine genomovirus-3 P20_68_BR	MW601958	2194	II	ambisense	TAATATTAT	2,182-18	46.3	50.6
Unclassified CRESS DNA viruses	Unassigned	PoCV-2	Porcine associated circular DNA virus-2 P20_36_BR	MW601953	2997	II	ambisense	TAGTATTAC	2,989-18	36.9	14.0
		PoCV-3	Porcine associated circular DNA virus-3 P20_37_BR	MW601954	2965	II	ambisense	TAGTATTAC	2,948-20	28.7	40.5
		PoCV-4	Porcine associated circular DNA virus-4 P20_55_BR	MW601948	2420	II	ambisense	TATTTTTAC		43.9	36.5
		PoCV-5	Porcine associated circular DNA virus-5 P20_41_BR	MW620877	2793	IV	ambisense	CAGTGTTAC	2,783-17	138	50.3
		PoCV-6	Porcine associated circular DNA virus-6 P20_75_BR	MW620878	2103	II	ambisense	TAGTATTAC	2,094-16	29.6	33.7
		PoCV-7	Porcine associated circular DNA virus-7 P20_76_BR	MW601949	2090	II	ambisense	TAGTGTTAT	2,073-13	12.1	41.3
		PoCV-8	Porcine associated circular DNA virus-8 P20_47_BR	MW601950	2565	II	ambisense	TAGTATTAC		31.9	31.8
		PoCV-9	Porcine associated circular DNA virus-9 P20_85_BR	MW601951	1874	V	unisense	AAGTATTAC	1,860-21	43.7	41.0
		PoCV-10	Porcine associated circular DNA virus-10 P20_80_BR	MW601952	2044	II	ambisense	TAGTATTAC	2,037-15	33.5	30.5
Unclassified Circular DNA molecule		PoM-1	Porcine associated DNA molecule 1 P20_137_BR	MW601955	1049	VII	unisense	TAGTATTAC	1,026-24	60.9	44.8

[1]Mean coverage: the average number of reads that align to the assembled genome. Calculated with Geneious (R8.1.9) by mapping reads to assembled genome using low-sensitivity mode.

[2]Putative new genus.

Supplementary Table S1. CRESS DNA viral genomes recovered in this study, highlighting the HUH endonuclease (RCR motifs I, II, III) and superfamily 3 helicase (Walker A, Walker B, motif C, arginine finger) and GRS motifs identified in the putative replication-associated proteins (Reps).

Viral family/group	Genus	Genome	Accession number	HUH endonuclease domain				SF3 helicase domain			
				Motif I	Motif II	GRS motif	Motif III	Walker A	Walker B	Motif C	Arginine finger
Smacoviridae	*Poprismacovirus*	PoSmaV-1	MW601947	YMATIPH	KHIQC		YEKK	GNSGKTWL	IIDIP	CMTN	LSEDRW
		PoSmaV-2	MW620871	YMATIPH	KHIQC		YEKK	GNSGKTWL	IIDIP	CMTN	LSEDRW
		PoSmaV-3	MW620872	YMATIPH	KHIQC		YEKK	GNSGKTWL	IIDIP	CMTN	LSEDRW
Smacoviridae	*Suismacovirus*[1]	PoSmaV-4	MW620873	IDATIWV	RHLQF		YVYK	GNHAKTAF	ILDMP	LLVN	YFSKDRV
		PoSmaV-5	MW620874	VDATAWT	RHFQF		YVYK	GNSGKTAW	IIDTP	ILCN	YFSKDRV
Redondoviridae	*Torbevirus*	PoRV-1	MW620875	LYLTYLD	RHFHV		YCKK	GETGEGKT	IIDDF	SWVN	QLTRRL
Genomoviridae	*Gemykibivirus*	PoGV-1	MW601956	GLFTYSQ	THLHV	RKFDVEGFHPNIVPSLR	YATK	GRSRTGKT	VFDDI	WIMN	
Genomoviridae	*Gemykrogvirus*	PoGV-2	MW601957	FIITFPQ	VHYHV	TAFDYFGAHGNIKSIR	YVGK	GPTRTGKT	IFDDI	MCMN	
		PoGV-3	MW601958	LIITFPQ	VHYHV	TAFDYFGAHGNIKSVR	YVGK	GPTRTGKT	VFDDI	MCMN	
Unclassified CRESS DNA viruses	Unassigned	PoCV-2	MW601953	WTMTVKW	QHVHM		YLTK	GPSGSGKS	WFDEF	ISTI	QLFRRL
		PoCV-3	MW601954	WTITVKW	QHCHM		YLNK	GPAGSGKS	WFDEF	ISTI	QLFRRL
		PoCV-4	MW601948	WCITLRE	VHRHM		YAIK	GESGTGKS	LIEDF	ITTV	QLDRRI
		PoCV-5	MW620877	WCFTINN	PHYQG		YCRK	GPPGTGKS	VFEEF	ITSN	ALWDRL
		PoCV-6	MW620878	YQLTLND	EHVHI		YLTK	GGTGKGKT	IIEEF	ICSI	QFMRRI
		PoCV-7	MW601949		WHGHA			GPSGVGKS	VYDDF	ITSI	QWLRRM
		PoCV-8	MW601950		PHLHL		YCLK	GPTGAGKS	ILDEF	ISSC	QLIRRI
		PoCV-9	MW601951	WFLTENS	EHAHI		YITK	GPAGTGKS	MQDID	VTSN	AIYRRF
		PoCV-10	MW601952	FLLTLNE	EHIHI		YIRK	GPSGVGKT	IYDDF	ITSV	QWMRRV
Unclassified Circular DNA molecule		PoM-1	MW601955	WFCTWPQ	PHLHA	RFDLGDCHGDYRPAKS	YCMK	GPSNTGKT	YIDEY	VLSN	

PoSmaV: porcine smacovirus; PoRV: porcine redondovirus; PoGV: porcine genomovirus; PoCV: porcine associated circular DNA virus; PoM: porcine associated DNA molecule.
[1]Putative new genus.

Supplementary Table S2. Comparison of the genomic features of porcine redondovirus 1 (PoRV-1) recovered in this study and two currently unclassified CRESS DNA viral genomes (PoSCV-5 and PoSCV-BEL/15V010) downloaded from GenBank and previously reported members of the *Redondoviridae* family.

Feature	Redondoviruses	PoRV-1	PoSCV-5	PoSCV-BEL/15V010
Genome size (nt)	3,018-3,058	3,071	3,062	3,081
ORFs orientation	Ambisense	Ambisense	ambisense	ambisense
ORFs	CP, Rep, ORF3	CP, Rep, ORF3	CP, Rep, ORF3	CP, Rep, ORF3
Nonanucleotide motif	TA[T/G]TATTAT	TAGTATTAT	TAGTATTAT	TAGTATTAT
Ori location	non-Rep encoding strand	non-Rep encoding strand	non-Rep encoding strand	non-Rep encoding strand
Rep protein size (aa)	334-363	350	350	357
CP protein size (aa)	449-531	541	539	538
Translate ORF3 size (aa)	200	176	176	144

Supplementary Table S3. Closest matches for putative replication-associated protein (Rep) and capsid protein (CP) amino acid sequences of the divergent porcine associated circular DNA viruses (PoCV) and the porcine associated DNA molecule (PoM) identified in this study. Top hits based on the best *e-value* identified by BLASTp search.

Genome	ORF	BLASTp best hit	Query coverage	E-value	Identity (%)	Isolation source "host"	Country
PoCV-2	Rep	Circovirus sp.	88	0.0	84	*Sus scrofa*	China
	CP	Fur seal faeces associated circular DNA virus	95	0.0	88	*Sus scrofa*	China
PoCV-3	Rep	Bovine faeces associated circular DNA molecule	98	0.0	72	*Sus scrofa*	New Zealand
	CP	Fur seal faeces associated circular DNA virus	94	0.0	77	*Sus scrofa*	China
PoCV-4	Rep	Circular ssDNA virus sp.	50	2e-97	58	*Macaca mulatta*	USA
	CP	Circular ssDNA virus sp.	50	8e-81	76	*Macaca mulatta*	USA
PoCV-5	Rep	Circular ssDNA virus sp.	100	0.0	95	*Macaca mulatta*	USA
	CP	Hudisavirus sp.	97	0.0	83	*Homo sapiens*	Ethiopia
PoCV-6	Rep	Odonata-associated circular virus-17	99	3e-113	59	*Libellula quadrimaculata*	USA
	CP	Marmot associated feces virus 4	99	8e-119	59	*Marmota*	USA
PoCV-7	Rep	Tundra vole stool-associated circular virus	89	4e-64	47	*Tundra vole*	Russia
	CP	Marmot associated feces virus 4	100	2e-110	52	*Marmota*	USA
PoCV-8	Rep	Circular ssDNA virus sp.	100	0.0	90	*Macaca mulatta*	USA
	CP	Circular ssDNA virus sp.	100	0.0	91	*Macaca mulatta*	USA
PoCV-9	Rep	Entamoeba-associated CRESS DNA virus	100	0.0	99	*Homo sapiens*	Netherlands
	CP	Entamoeba-associated CRESS DNA virus	91	6e-94	80	*Homo sapiens*	Netherlands
PoCV-10	Rep	Marmot associated feces virus 3	93	5e-117	59	*Marmota*	USA
	CP	Marmot associated feces virus 4	98	1e-110	57	*Marmota*	USA
PoM-1	Rep	CRESS virus sp.	93	3e-102	55	*Forest musk deer*	China

Supplementary Table S4. Conserved genomic features of members of the previously proposed *Tetartoentvirus* genus (n = 4) (KINSELLA *et al.*, 2020), and the porcine associated circular DNA virus-9 (PoCV-9) genome detected here.

	Genome size	Genome Type	Nonanucleotide motif	Replication-associated protein						
				HUH endonuclease			Superfamily helicase			
				I	II	III	Walker A	Walker B	motif C	Arginine finger
Tetartoentvirus	1,874 – 1,876	V	AA[A/G]TATTAC	FLTEN	EHAHI	YITK	GPAGTGKS	MQDID	VTSN	YRRF
PoCV-9	1,874	V	AAGTATTAC	FLTEN	EHAHI	YITK	GPAGTGKS	MQDID	VTSN	YRRF

Supplementary Material

(Alguns arquivos suplementares não puderam ser inseridos na tese por questões de compatibilidade. Os mesmos podem ser acessados pelo link: https://www.dropbox.com/sh/nve2vjcoy2afm07/AAA2PSiyIcYDxRlS2EL6QDUla?dl=0)

Supplementary Material 1 – Pairwise identity scores of Rep amino acid sequences of smacoviruses. Obtained using SDT v1.2.

Supplementary Material 2 – Pairwise identity scores of the genomes assigned to *Suismacovirus* genus. Each box represents potential new species of smacoviruses.

Supplementary Material 3 – Pairwise identity scores of Rep amino acid sequences from members of *Redondoviridae* family. The sequences are colored according the two species of *Redondoviridae* (*Vientovirus* and *Brisavirus*).

Supplementary Material 4 – Pairwise identity scores of CP amino acid sequences from members of *Redondoviridae* family. The sequences are colored according the two species of *Redondoviridae* (*Vientovirus* and *Brisavirus*).

Supplementary Material 5 – Pairwise identity scores of ORF3 amino acid sequences from members of *Redondoviridae* family. The sequences are colored according the two species of *Redondoviridae* (*Vientovirus* and *Brisavirus*).

Supplementary Material 6 – Pairwise identity scores of genome sequences from members of the genus *Gemykibivirus* (Family: *Geminiviridae*). The identity score of genomes from the same species is delimited by light gray boxes.

Supplementary Material 7 – Pairwise identity scores of Rep amino acid sequences from members of the genus *Gemykibivirus* (Family: *Geminiviridae*).

Supplementary Material 8 – Pairwise identity scores of CP amino acid sequences from members of the genus *Gemykibivirus* (Family: *Geminiviridae*).

Supplementary Material 9 – Pairwise identity scores of genome sequences from members of the genus *Gemykrogvirus* (Family: *Geminiviridae*).

Supplementary Material 10 – Pairwise identity scores of Rep amino acid sequences from members of the genus *Gemykrogvirus* (Family: *Geminiviridae*).

Supplementary Material 11 – Pairwise identity scores of CP amino acid sequences from members of the genus *Gemykrogvirus* (Family: *Geminiviridae*).

Supplementary Material 12 – Pairwise identity scores of Rep amino acid sequences from the nine divergent CRESS DNA viral genomes identified in this study (PoCV 2-10, in red) plus the circular DNA molecule (PoM-1, in red). Representatives of the viral families *Bacilladnaviridae, Circoviridae, Geminiviridae, Genomoviridae, Nanoviridae/Alphasatellitidae, Nenyaviridae, Redondoviridae, Smacoviridae* and of the six groups of unclassified CRESS DNA viruses (CRESSV1-6) were also included.

Supplementary Material 13 – Pairwise identity scores of genome sequences from members of the family *Nenyaviridae*.

4 ARTIGO CIENTÍFICO 2

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Investigation on porcine circovirus type 3 in serum of farrowing sows with stillbirths

Caroline Tochetto[a], Diane Alves de Lima[a], Ana Paula Muterle Varela[a], Lucía Cano Ortiz[a], Márcia Regina Loiko[a,d], Camila Mengue Scheffer[a], Willian Pinto Paim[b], Samuel Paulo Cibulski[b], Cristine Cerva[c], Juliana Herpich[a], Candice Schmidt[a], Ana Claúdia Franco[a], Fabiana Quoos Mayer[c,*], Paulo Michel Roehe[a]

[a] Laboratório de Virologia, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básica da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[b] Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[c] Centro de Pesquisa em Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Departamento de Diagnóstico e Pesquisa Agropecuária, Secretaria de Agricultura, Pecuária e Desenvolvimento Rural, Eldorado do Sul, Rio Grande do Sul, Brazil
[d] Universidade Feevale, Novo Hamburgo, Rio Grande do Sul, Brazil

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ABSTRACT

Since its first identification in 2016, porcine circovirus 3 (PCV3) has been detected in healthy and/or diseased swine in many countries worldwide. In a previous study by our group, PCV3 was detected in sera of sows which had at least one stillborn piglet in the last parturition. As such, it became important to investigate if the presence of PCV3 in sows' sera could be associated to the occurrence of stillbirths. With that aim, the frequency of PCV3 infections and viral DNA loads in sows' sera was investigated through a real-time quantitative PCR in 89 serum samples of just farrowed sows with or without stillbirths. PCV3 genomes were identified in most samples, with genome loads ranging between less than 10 to 200,000 copies per mL of serum. No significant differences were observed either in the frequency of infection or PCV3 viral loads in sows with or without stillbirths. Thus, no association could be established between PCV3 infection of sows at farrowing and stillbirths' occurrence.

1. Introduction

The genus *Circovirus*, family *Circoviridae*, officially comprises three porcine circovirus (PCV) species: PCV1, PCV2 and PCV3. PCV1 was discovered in 1974 as a contaminant of a porcine kidney (PK-15) cell line and is nonpathogenic virus for swine [1]. In the nineties, PCV2 was discovered and subsequently associated with a number of syndromes, collectively named porcine circovirus diseases (PCVD) [2]. In 2016, PCV3 was discovered in USA in sows with clinical signs compatible with porcine dermatitis and nephropathy syndrome (PDNS) [3] and in pigs with cardiac and multisystemic inflammation [4]. Since then, it has been reported worldwide, including Asia, Europe and South America [5].

PCV3 has a circular, single-stranded DNA (ssDNA) genome, with approximately 2000 nt in length and two major ORFs in opposite directions, that encode the capsid (Cap) and the replicase (Rep) proteins [3,4]. Despite its recent discovery, retrospective studies showed that PCV3 has been circulating in pigs at least since 1993 in Europe [6], and since 2006 in South America [7]. Moreover, PCV3 was also detected in wild boars [8,9], cattle [10], dogs [11], chamois, roe deer and ticks (*Ixodes ricinus*) [12]. Regarding PCV3 origin, studies show that it shares a common ancestor with bat-derived circoviruses [13]. Furthermore, it is proposed a PCV3 classification into two genotypes "a" and "b", although this classification is still under debate [13,14].

Associations between PCV3 and different clinical/pathological conditions have been reported, including porcine dermatitis and nephropathy syndrome (PDNS) [3], reproductive failures [15–19], respiratory problems [20,21], diarrheal [22], PCVD [23] and multisystemic inflammation and myocarditis [4]. The pathogenic potential of the virus was experimentally demonstrated in a recent study in which infected animals exhibited a PDNS-like disease [24]. However, PCV3 has been also detected in apparently healthy animals [3,7,25,26]. On the course of investigations on viruses that may be associated to stillbirths in sows, our group identified PCV3 genomes in pooled serum

* Corresponding author.
E-mail address: fabiana-mayer@agricultura.rs.gov.br (F.Q. Mayer).

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samples of sows which had farrowed at least one stillborn piglet in the litter [27]. Therefore, it became important to examine more deeply the association between PCV3 and the occurrence of stillbirths. In order to do that, the frequency of PCV3 infections and PCV3 DNA loads were investigated in sera of sows either with or without stillbirths, collected immediately after farrowing.

2. Methods

2.1. Samples

This study was approved by the Ethics Committee on Animal Use from the Veterinary Research Institute Desidério Finamor (CEUA-IPVDF) under protocol number 16/2015. The procedures for collection of samples have been described in detail elsewhere [27]. Briefly, serum samples of just-farrowed sows from six commercial piglet-producing farms located in Southern Brazil were collected in 2015–2016 [27]. The samples were separated in two groups: one with sera from sows which had at least one stillborn piglet in that parturition (stillbirth – S; n = 46) and another with sera of sows with no stillbirths (healthy – H; n = 43). All sows were clinically healthy at the time of sampling.

2.2. DNA extraction and PCV3 detection by real-time qPCR

DNA was extracted from 200 μL of serum with a standard phenol-chloroform protocol [28]. The quality of DNA was verified by micro-volume spectrophotometry (Nanodrop, ThermoScientific, USA). Primers targeting PCV3 *Cap* gene were designed using Primer3Plus v.2.4.2 [29] for quantitative SYBR Green real-time PCR (qPCR). Primer sequences were Forward: 5′-AACGGTGGGGTCATATGTGTTG-3′, and Reverse: 5′-AGACGACCCTTATGCGGAAA-3' (genomic positions from 1441 to 1616), with an amplicon size of 175 bp. The primers' specificity was checked by an *in silico* evaluation with Primer Blast software. The qPCR limit of detection was evaluated through a standard curve obtained by cloning the amplicon in a TOPO plasmid (Invitrogen) followed by transformation in competent *Escherichia coli* [28]. The plasmids were extracted as previously described [28], quantified in a fluorimeter and used to construct a standard curve with DNA molecules ranging from 1×10^8 to 1×10^0. Samples and standard curves were run in duplicate; negative controls were prepared with the same reagents, except that the sample DNA was replaced by sterile water. The reactions were performed in a final volume of 12.5 μL containing 5 μL of DNA, 6.25 μL of 2x Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 25 pmol of each primer. Reactions were carried out in 96-well plates in a StepOne™ Real-Time PCR System (Life Technologies) under the following cycling conditions: 2 min at 50 °C, 2 min at 95 °C, and 40 cycles of amplification (95 °C for 15 s and 60 °C for 30 s). A melting curve was constructed by gradual increase in temperature (0.3 °C) from 60 °C to 95 °C. The number of copies of viral DNA was determined by comparison with the standard curve.

2.3. Statistical analysis

The frequencies of occurrence of PCV3 infections were compared between sows with and without stillbirths using a chi-square test. The viral DNA loads were compared using a Wilcoxon-Mann-Whitney *U* test. Significant differences were considered when $p < 0.05$. The statistical analyzes were performed in SPSS software v. 22.0.

3. Results

The qPCR proved to be specific through Blast analysis and the quantification limit was 10 copies of PCV3 DNA, with an efficiency of 99.4%. However, DNA amounts lower than 10 copies were also detected, although not quantifiable. Thus, the criteria to consider positive samples was any sample with cycle threshold lower than 39 and with specific melt temperature of 83.4 °C. Negative controls did not give rise to amplicons and all DNA samples were shown to have good quality, as evidenced by A260/A280 and A260/A230 ratios higher than 1.8 and 2.0, respectively. Out of the 89 samples tested, 57 (64%) were found to contain PCV3 genomes. The frequency of positive animals was 67.4% (31/46) in sows with stillbirths and 60.5% (26/43) in sows without stillbirths; no statistically significant differences were detected between the groups (chi-square; $p = 0.45$). Viral genomes were detected is samples from all farms tested (Fig. 1).

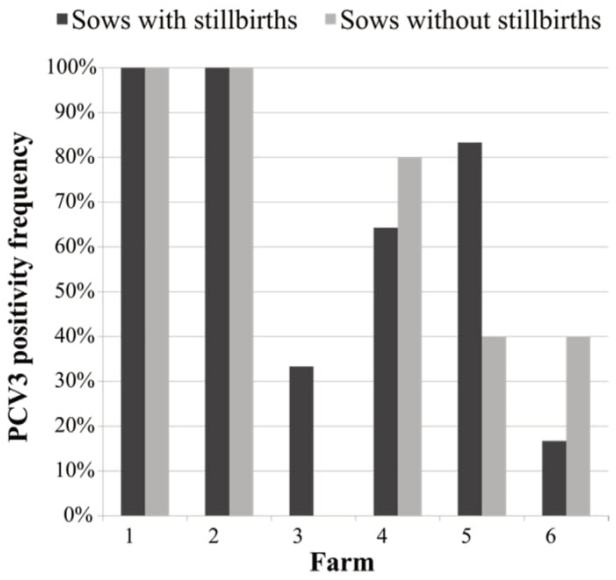

Fig. 1. Frequency of PCV3 in sampled farms. Dark grey bars represent sows with stillbirths and light grey bars sows without stillbirths. PCV3 DNA was detected in all tested farms and in both groups, with the exception of farm 3, where PCV3 was not detected in serum of sows with normal farrowing (without stillbirths).

In one of the farms (#3), all sows with normal farrowing (no stillbirths) were negative to PCV3. Of the 57 sera that contained PCV3 genomes, only 25 had quantifiable genome loads, that is, higher than 10 genome copies/mL, which was the quantification limit for qPCR. Out of those 25 samples, 14 corresponded to sows with stillbirths, whereas 11 were from sows without stillbirths. The median of detected DNA molecules per mL of serum was 448 and 426 in sows with and without stillbirths, respectively. No statistically significant differences were detected when comparing the PCV3 genome loads between these two groups (Wilcoxon-Mann-Whitney U; $p = 0.69$; Fig. 2).

4. Discussion

Previously, in a metagenomics study aiming to identify viruses that might be associated to the occurrence of stillbirths, PCV3 genomes were identified only in sows with stillbirths [27]. However, in that study, a clear association between PCV3 infection and stillbirths' occurrence could not be established. Here, this issue was re-addressed by individually examining each of the sows' samples. In order to do that, a qPCR was designed to determine the frequency of PCV3 infections and viral DNA loads in sera. The assay displayed 99.4% efficiency and was able to detect PCV3 DNA in samples previously negative at high throughput sequencing (HTS). Thus, pooling of samples for HTS, as previously performed [27], decreased the sensitivity of PCV3 genome detection, as here revealed by qPCR, and can introduce an important source of bias, particularly when the concentration of genomes in samples is low. This was indeed the case of the present study, in which most of the samples had PCV3 DNA loads of less than 10 copies per

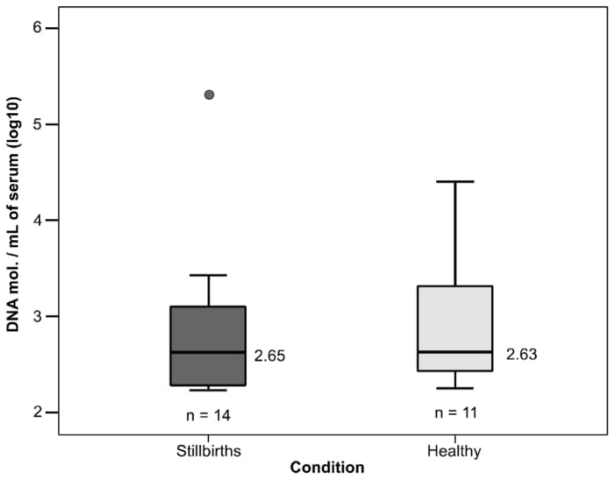

Fig. 2. PCV3 viral genome loads in sows with (dark grey) and without (light grey) stillbirths. Viral genome loads expressed as number of DNA molecules/mL of serum (log10). The median of detected DNA molecules per ml of serum was 448 in sows with stillbirths and 426 in sows without stillbirths. No statistically significant differences were observed.

reaction. Genomes in such scarcity were likely missed when HTS was performed. While HTS sensitivity in this case did not match that of qPCR, the technique still provides advantages, in that more detailed genomic information can be gathered from the full or partial genomes eventually detected [30,31].

PCV3 has been reported in pigs with reproductive failures as in abortions and stillborn piglets [15–19,23]. Indeed, in mummified fetuses, a high rate of co-infection was reported, including agents as porcine parvovirus (PPV), porcine circovirus 2 (PCV2) and *Leptospira* spp., not clarifying the role of PCV3 in such condition. In contrast, other studies reported PCV3 in healthy animals [6,23,25], in some cases, at higher frequency than in sick animals [7].

It is interesting that, here, PCV3 genomes were identified in sera of sows either with or without stillbirths. In one particular farm (#3) all ten sows with normal farrowing had no evidence of PCV3 infection in their sera; however, this was considered an exception, since all other farms had PCV3-infected sows in both groups (with or without stillbirths). The findings reported here reveal no significant differences in PCV3 DNA loads in sera of sows with or without stillbirths, suggesting that PCV3 is probably not associated to the occurrence of stillbirths in swine. However, it is important to note that examined sera were collected from sows just after farrowing; PCV3 infections may have occurred earlier in pregnancy – or previously. Infections with a number of other infectious agents in previous stages of gestation may lead to stillbirth occurrence [32]. Thus, it is still possible that PCV3 might play some role in the pathogenesis of stillbirths. Nevertheless, most striking is the detection of viral genomes in the sera of sows at farrowing in most sows, regardless of the occurrence of stillbirths. Further investigations on the role of PCV3 infections throughout pregnancy and in recently born and stillborn piglets could provide additional information about the potential involvement of the virus in this condition.

5. Conclusion

The present study provides evidence that just farrowed sows were infected with PCV3, as revealed by the identification of PCV3 genomes in the sows' sera. The occurrence of stillbirths seems not to be associated to the frequency of PCV3 infections or to the PCV3 genome loads in sows at farrowing. Further studies on PCV3 pathogenicity are needed to define its possible association – if any – with the occurrence of stillbirths in sows.

Author statement

Caroline Tochetto: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Original drafting. **Diane Alves de Lima, Ana Paula Muterle Varela, Lucía Cano Ortiz, Márcia Regina Loiko, Camila Mengue Scheffer, Willian Pinto Paim, Samuel Paulo Cibulski, Cristine Cerva, Juliana Herpich, Candice Schmidt, Ana Claúdia Franco:** Formal analysis; Investigation; Methodology; Writing – review and editing. **Fabiana Quoos Mayer:** Conceptualization; Data curation; Formal analysis; Investigation; Project administration; Supervision; Writing – review and editing. **Paulo Michel Roehe:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Project administration; Supervision; Resources; Writing - review and editing.

Declaration of competing interest

None.

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5 CONCLUSÕES

O presente estudo caracterizou o viroma de suínos com diferentes condições clínicas. Em suínos com sinais clínicos de doença respiratória, foi detectada uma grande diversidade de vírus DNA, especialmente os vírus CRESS DNA. Foram identificados vírus eucarióticos das famílias *Anelloviridae, Circoviridae, Genomoviridae, Nenyaviridae, Parvoviridae, Smacoviridae, Redondoviridae*, e também vírus procarióticos (*Microviridae* e *Inoviridae*). Além disso, um novo gênero, *Suismacovirus*, é proposto para a família *Smacoviridae* e, pela primeira vez, um vírus da família *Redondoviridae* foi descrito em suínos. No entanto, não foi possível realizar inferências sobre o potencial patogênico dos vírus descritos e futuros estudos devem acessar esta questão.

Análises iniciais em fezes de suínos com ou sem diarreia também revelaram uma extensa diversidade viral com destaque para os vírus eucarióticos das famílias *Circoviridae*, *Parvoviridae* e *Smacoviridae* e procarióticos *Microviridae* e *Inoviridae*, além de várias sequências de vírus não classificados. O estudo aprofundado dos genomas detectados e a análise comparativa do viroma são perspectivas deste estudo.

Além dos estudos de metagenômica, a presença do PCV3 no soro de matrizes com e sem natimortos foi avaliada. Os resultados mostraram que PCV3 está disseminado no soro dessas matrizes, não havendo associação com o desfecho de natimortalidade.

Finalmente, esse trabalho fornece uma compreensão mais abrangente da comunidade viral presente em suínos. Os resultados mostrados aqui podem ser utilizados como base para estudos futuros, na comparação do viroma detectado em diferentes condições clínicas e até mesmo em surtos de doenças infecciosas. Esse estudo contribui para a expansão dos bancos de dados virais, aumentando a informação genética disponível e, consequentemente, ajudando na compreensão da dinâmica e evolução viral, transmissão entre espécies e interação vírus-hospedeiro.

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APÊNDICES

APÊNDICE A – INVESTIGAÇÃO DE VÍRUS DE GENOMA DNA NO SORO DE SUÍNOS COM SINAIS CLÍNICOS DE DOENÇA RESPIRATÓRIA

Durante o estudo do viroma de suínos com problemas respiratórios, descrito no primeiro artigo dessa tese, outros achados foram identificados e serão comentados brevemente nesta seção. Além dos vírus *CRESS DNA* descritos no primeiro artigo, foram detectados genomas de vírus eucarióticos das famílias *Anelloviridae* e *Parvoviridae* e vírus procarióticos das famílias *Microviridae* e *Inoviridae*.

Tabela A1 – Dados obtidos no sequenciamento de alto desempenho realizado na plataforma *Miseq* (*Illumina*) do *pool* de soro de suínos com sinais clínicos de doença respiratória oriundo de granjas comerciais no Rio Grande do Sul. Foram pesquisados somente vírus de genoma DNA. As *reads* e *contigs* foram comparados ao banco de dados não redundante (*nr*) do NCBI utilizando o programa *diamond* com a opção *blastx*.

Total de *reads*	1.193.764	
Total de *reads* após trimagem	1.167.815	(97,83%)
***Reads* virais**	663.411	(56,81%)
Vírus eucarióticos	655.324	(98,78%)
Anelloviridae	281.640	(42,45%)
Parvoviridae	364.726	(54,97%)
Circoviridae	2.685	(0,40%)
Smacoviridae	2.155	(0,32%)
Genomoviridae	1.968	(0,30%)
Vírus CRESS DNA não classificados	2.094	(0,32%)
Alphaherpesvirinae	9	(0,00%)
Reoviridae	6	(0,00%)
Pneumoviridae	2	(0,00%)
Picornaviridae	1	(0,00%)
Marsellaviridae	38	(0,01%)
Vírus procarióticos	4.763	(0,72%)
Uncultured virus	9	(0,00%)
Unassigned viral reads	3.345	(0,50%)
***Reads* sem hit**	236.394	(20,24%)
Contigs* montados *de novo (SPAdes)	297	
Tamanho médio (nt)	1668.2	
***Contigs* sem hit**	67	(22,6%)
***Contigs* virais**	160	(53,9%)
Vírus eucarióticos	102	(63,8%)
Anelloviridae	86	(53,8%)
Parvoviridae	4	(2,5%)
Circoviridae	7	(4,4%)
Genomoviridae	4	(2,5%)
Smacoviridae	1	(0,6%)
Vírus CRESS DNA não classificados	13	(8,1%)
Vírus procarióticos	27	(16,9%)

Microviridae	26	(16,3%)
Inoviridae	1	(0,6%)
Unassigned viral sequences	18	(11,3%)

Tabela A2 – Genomas completos e parciais de Torque teno vírus suíno (TTSuV) detectados em soro de suínos oriundos de granjas comerciais no Rio Grande do Sul. A ORF1 das sequências obtidas nesse estudo foi comparada ao banco de dados não redundante (*nr*) do NCBI utilizando a ferramenta BLASTp (Mar/2021). O melhor hit representa a sequência com o menor *E-value*. (*) Genomas completos.

Contig	**Tamanho (nt)**	**ORFs detectadas**	**Gênero**	**Melhor *hit* no BLASTp (ORF1)**				
				Nome da sequência	**N. de acesso**	**Cobertura (%)**	**E-value**	**Identidade (%)**
TTSuV k2a P20_33_BR*	2.830	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus k2a	KY742736	100	0,0	98
TTSuV 1a P20_38_BR*	2.914	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus 1a	HM633251	100	0,0	99
TTSuV k2b P20_40_BR*	2.843	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus k2b	KY742732	100	0,0	96
TTSuV k2a P20_42_BR*	2.808	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus 1b	JX535335	100	0,0	98
TTSuV k2a P20_43_BR*	2.841	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus k2a	MG799364	92	0,0	95
TTSuV 1a P20_45_BR*	2.734	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus 1a	MH170065	92	0,0	96
TTSuV k2a P20_44_BR	2.807	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus k2a	MN272071	100	0,0	99
TTSuV k2a P20_46_BR	2.720	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus	MN272110	100	0,0	98
TTSuV 1a P20_49_BR	2.665	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus	MN272101	92	0,0	94
TTSuV 1a P20_56_BR	2.518	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus	MN272109	100	0,0	100
TTSuV 1a P20_57_BR	2.501	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus	MN272091	100	0,0	99
TTSuV k2a P20_58_BR	2.493	ORF1, ORF2, ORF1/1, ORF2/2	*Kappatorquevirus*	Torque teno sus virus k2a	KY742735	100	0,0	99
TTSuV 1a P20_59_BR	2.387	ORF1, ORF2, ORF1/1, ORF2/2	*Iotatorquevirus*	Torque teno sus virus	MN272103	100	0,0	99
TTSuV 1a P20_62_BR	2.362	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus 1a	MH170067	92	0,0	92
TTSuV 1a P20_63_BR	2.357	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus 1b	MG799359	100	0,0	100
TTSuV 1a P20_64_BR	2.348	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus 1b	MH170064	92	0,0	93
TTSuV 1a P20_65_BR	2.334	ORF1, ORF2	*Iotatorquevirus*	Anelloviridae sp.	MK012485	100	0,0	99
TTSuV 1a P20_67_BR	2.325	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272106	100	0,0	98
TTSuV 1a P20_69_BR	2.320	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272093	100	0,0	98
TTSuV 1a P20_70_BR	2.311	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272111	100	0,0	99
TTSuV 1a P20_71_BR	2.303	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus 1b	MH170070	100	0,0	100
TTSuV 1a P20_72_BR	2.275	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272100	100	0,0	100
TTSuV 1a P20_74_BR	2.258	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus 1a	HM633250	91	0,0	95
TTSuV 1a P20_77_BR	2.197	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272108	100	0,0	99
TTSuV 1a P20_79_BR	2.172	ORF1, ORF2	*Iotatorquevirus*	Torque teno sus virus	MN272101	100	0,0	99
TTSuV k2a P20_82_BR	2.036	ORF1	*Kappatorquevirus*	Torque teno sus virus 1b	HM633238	100	0,0	99
TTSuV 1a P20_83_BR	2.005	ORF1	*Iotatorquevirus*	Torque teno sus virus	MN272098	100	0,0	100

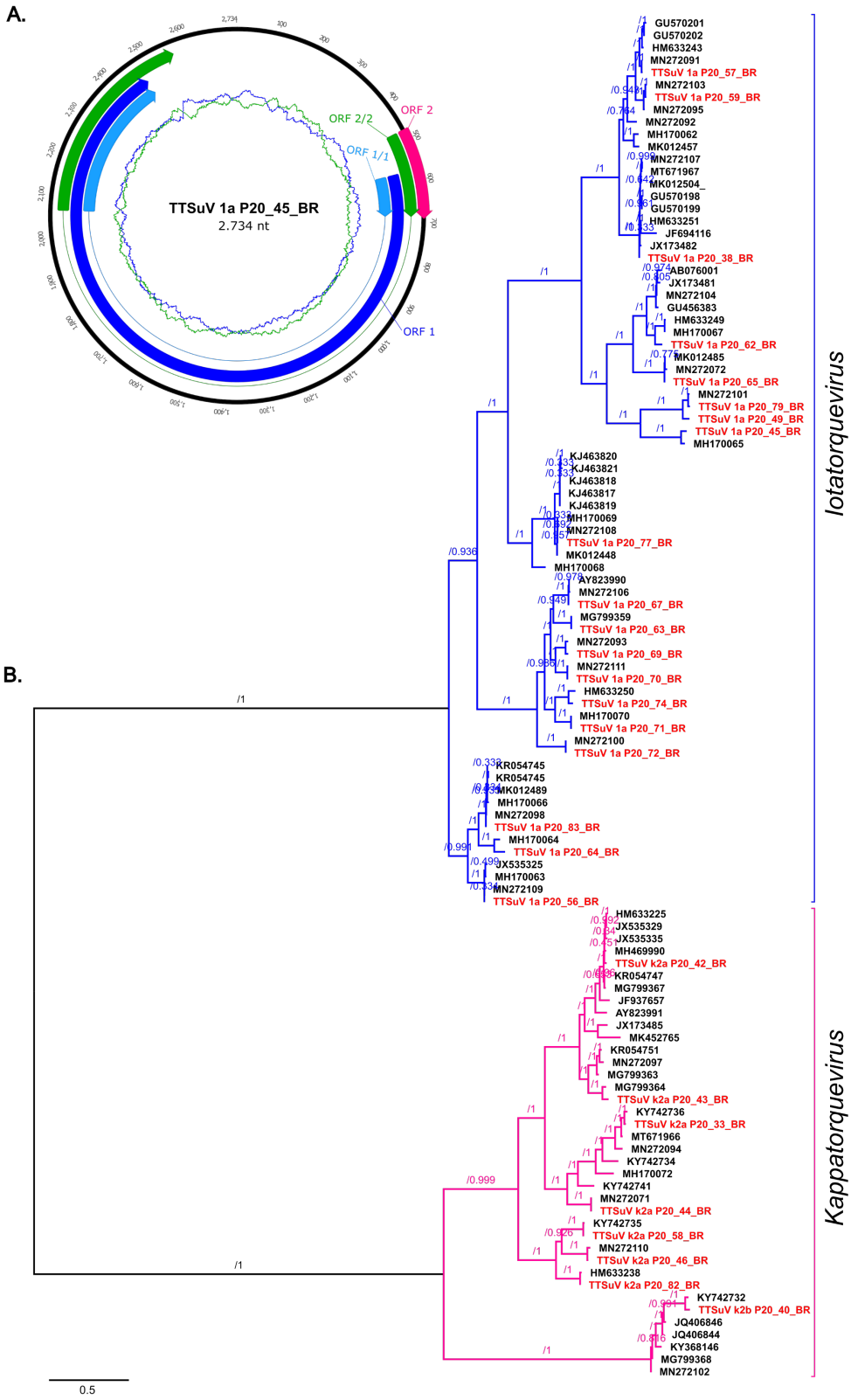

Figura A1 – Genomas de Torque teno vírus suíno (TTSuV) detectados no soro de suínos provenientes de granjas comerciais no Rio Grande do Sul. (A) Exemplificação de um dos genomas completos recuperados e as quatro ORFs típicas dos TTSuV. (B) Árvore filogenética com enraizamento por ponto médio (*midpoint rooted*) baseada na ORF1 traduzida de TTSuV. A árvore foi construída utilizando o método *Maximum Likelihood* (ML) com base no melhor modelo de substituição (LG + I + G4) inferido no programa IQTREE v.1.6.12 (NGUYEN et al., 2015). Os valores de suporte foram obtidos pelo teste de aBayes (ANISIMOVA et al., 2011). O alinhamento foi realizado utilizando o programa MAFFT v.7.471 (KATOH;STANDLEY, 2013) otimizado para alinhamento local acurado (parâmetros *"L-INS-I -leavegappyregion -ep 0.123")*. TTSuV 1a, k2a e k2b são as espécies de TTSuV detectadas.

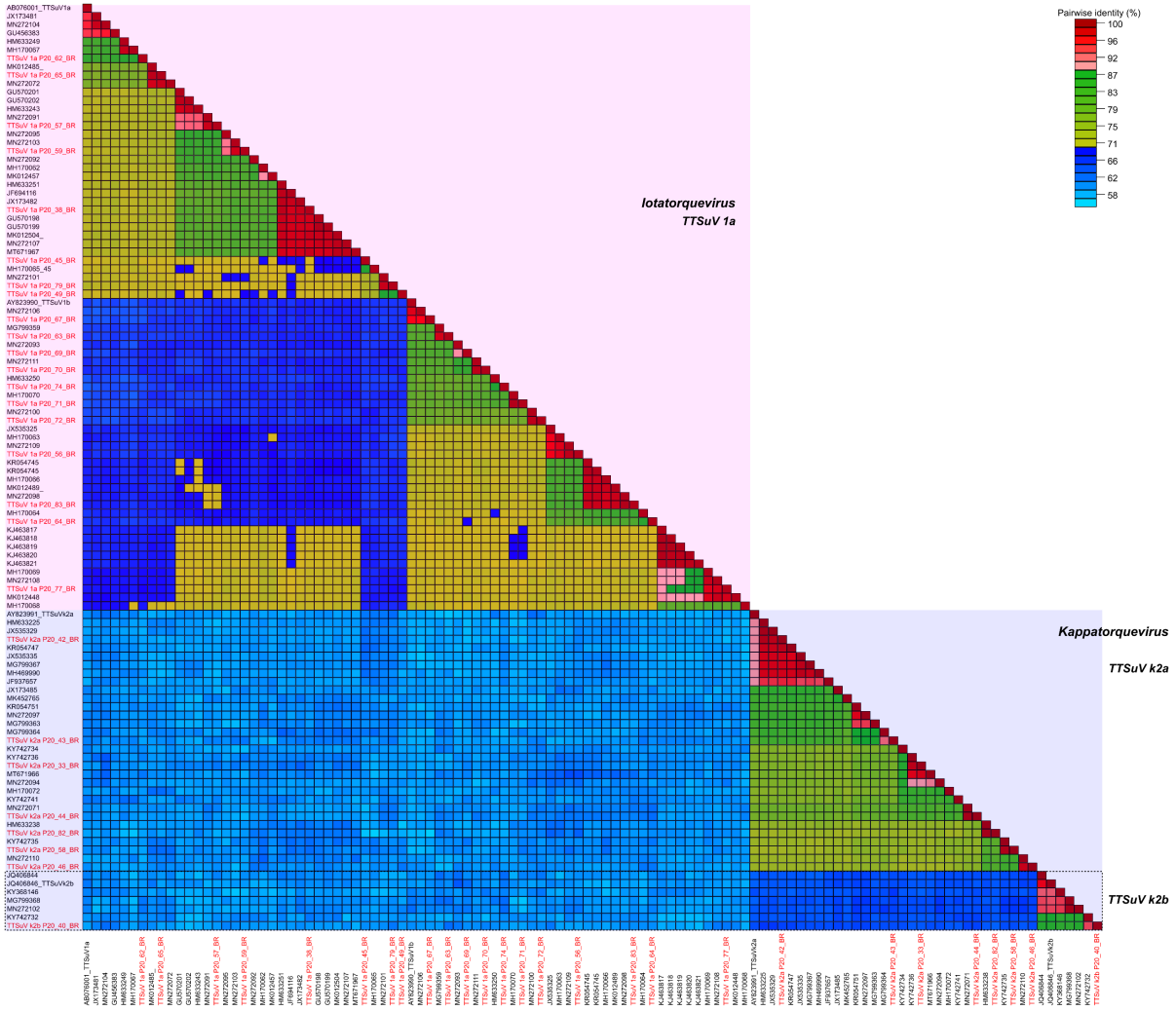

Figura A2 – Matriz de distribuição de identidade das sequências de nt da ORF1 de Torque teno vírus suíno (TTSuV). Foram comparadas 110 sequências, incluindo as 27 sequências de TTSuV identificadas nesse estudo (em vermelho). As duas caixas maiores demarcam os dois gêneros de anelovírus que infectam suínos: *Iotatorquevirus* (rosa) e *Kappatorquevirus* (roxo). TTSuV 1a, k2a e k2b são as três espécies reconhecidas atualmente. Matriz de identidade gerada pelo programa *Sequence Demarcation Tool (SDT)* v1.2 (MUHIRE *et al.*, 2014).

APÊNDICE B – INVESTIGAÇÃO DE VÍRUS DE GENOMA DNA NO SORO DE SUÍNOS CLINICAMENTE SAUDÁVEIS

Tabela A1 – Dados obtidos através do sequenciamento de alto desempenho realizado na plataforma *Miseq* (*Illumina*) do *pool* de soro de suínos clinicamente saudáveis, provenientes de granjas GRSC no Rio Grande do Sul. As amostras foram preparadas conforme descrito no Artigo 1. Foram pesquisados somente vírus de genoma DNA. As *reads* e *contigs* foram comparados ao banco de dados não redundante (*nr*) do NCBI (Maio/2020) utilizando o programa *diamond* (parâmetros *"blastx –f 102"*).

Total de reads	1,455,410	
Total de reads após trimagem	1,394,375	(95,81%)
Reads virais	889,111	(63,76%)
Vírus eucarióticos	888,801	(99,97%)
Anelloviridae	856,184	(96,30%)
Parvoviridae	31,009	(3,49%)
Circoviridae	4	(0,00%)
Vírus CRESS DNA não classificados	1551	(0,17%)
Alphaherpesvirinae	53	(0,01%)
Vírus procarióticos	3	(0,00%)
Unassigned viral reads	307	(0,03%)
Reads sem hit	445,795	(31,97%)
Contigs montados *de novo (SPAdes)*	344	
Tamanho médio (nt)	1356,3	
Contigs sem hit	149	(43,3%)
Contigs virais	26	(7,6%)
Vírus eucarióticos	22	(84,6%)
Anelloviridae	17	(65,4%)
Parvoviridae	3	(11,5%)
Vírus CRESS DNA não classificados	2	(7,7%)
Unassigned viral sequences	4	(15,4%)

APÊNDICE C – CARACTERIZAÇÃO DO VIROMA DE SUÍNOS COM OU SEM DIARREIA ATRAVÉS DE ABORDAGEM METAGENÔMICA E SEQUENCIAMENTO DE ALTO DESEMPENHO

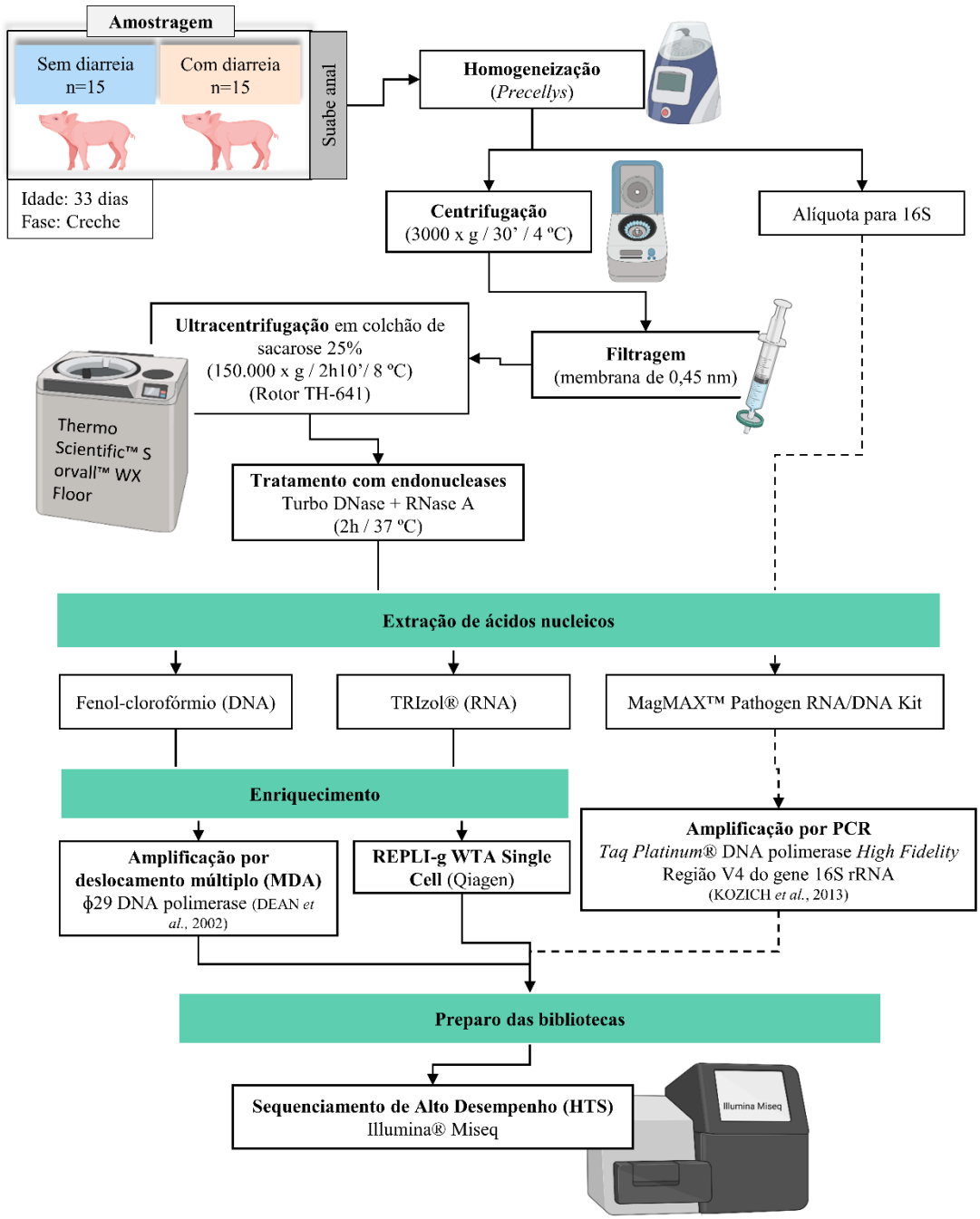

Figura C1 – Fluxograma do preparo e processamento das amostras de suabe anal coletadas de suínos com, ou sem diarreia, no Rio Grande do Sul. As amostras foram preparadas individualmente para pesquisa do viroma (sequenciamento *shotgun*) e da diversidade bacteriana (DNA *metabarcoding,* 16S).

Tabela C1 – Número de *reads* obtidas a partir do sequenciamento de alto desempenho (*Illumina*, *Miseq*) para o estudo do viroma de suínos com ou sem diarreia. Comparação entre o número de *contigs* obtidos por montagem *de novo* em dois programas diferentes: *MegaHIT* e *SPAdes*. D, suíno com diarreia; C, suíno sem diarreia (controle).

Amostra	***Reads***				***Contigs***	
	Total	**Após trimagem**	**Após remoção das *reads* do hospedeiro**	**Remanescente (%)**	**MegaHIT**	**SPAdes**
D1	179.032	161.622	161.400	90,15	2.056	1.632
D2	97.964	84.924	84.918	86,68	1.332	1.118
D3	104.222	79.008	78.986	75,79	3.511	2.610
D4	119.162	86.432	86.236	72,37	3.232	2.250
D5	151.880	129.120	128.986	84,93	1.752	1.475
D6	143.394	111.208	111.178	77,53	2.079	1.994
D7	112.016	94.256	94.238	84,13	1.344	1.031
D8	49.508	36.510	36.462	73,65	978	739
D9	90.236	58.792	54.190	60,05	2.855	2.591
D10	100.542	76.640	76.618	76,20	2.236	1.751
D11	107.350	84.996	83.884	78,14	1.329	1.085
D12	96.938	73.910	73.802	76,13	1.386	1.056
D13	130.340	107.906	107.812	82,72	2.743	2.058
D14	126.308	92.686	78.954	62,51	4.517	3.682
D15	86.416	67.484	67.058	77,60	1.882	1.514
C1	139.278	138.668	138.520	99,46	2.028	1.942
C2	88.572	72.494	72.484	81,84	971	853
C3	144.894	113.350	113.326	78,21	2.508	2.284
C4	146.010	128.656	128.648	88,11	1.821	2.361
C5	1.288.676	1.021.054	1.021.024	79,23	8.579	8.378
C6	90.212	66.548	66.538	73,76	1.488	1.238
C7	135.248	110.290	110.180	81,47	2.041	1.777
C8	118.122	98.702	92.082	77,95	2.405	1.731
C9	113.754	92.448	92.188	81,04	1.726	1.579
C10	32.420	27.724	27.654	85,30	560	436
C11	146.826	119.894	119.536	81,41	2.682	2.141
C12	119.842	96.690	96.524	80,54	2.017	1.478
C13	109.678	88.070	88.034	80,27	2.285	1.821
C14	124.044	95.118	85.598	69,01	2.924	2.252
C15	123.906	86.838	86.672	69,95	1.930	1.281

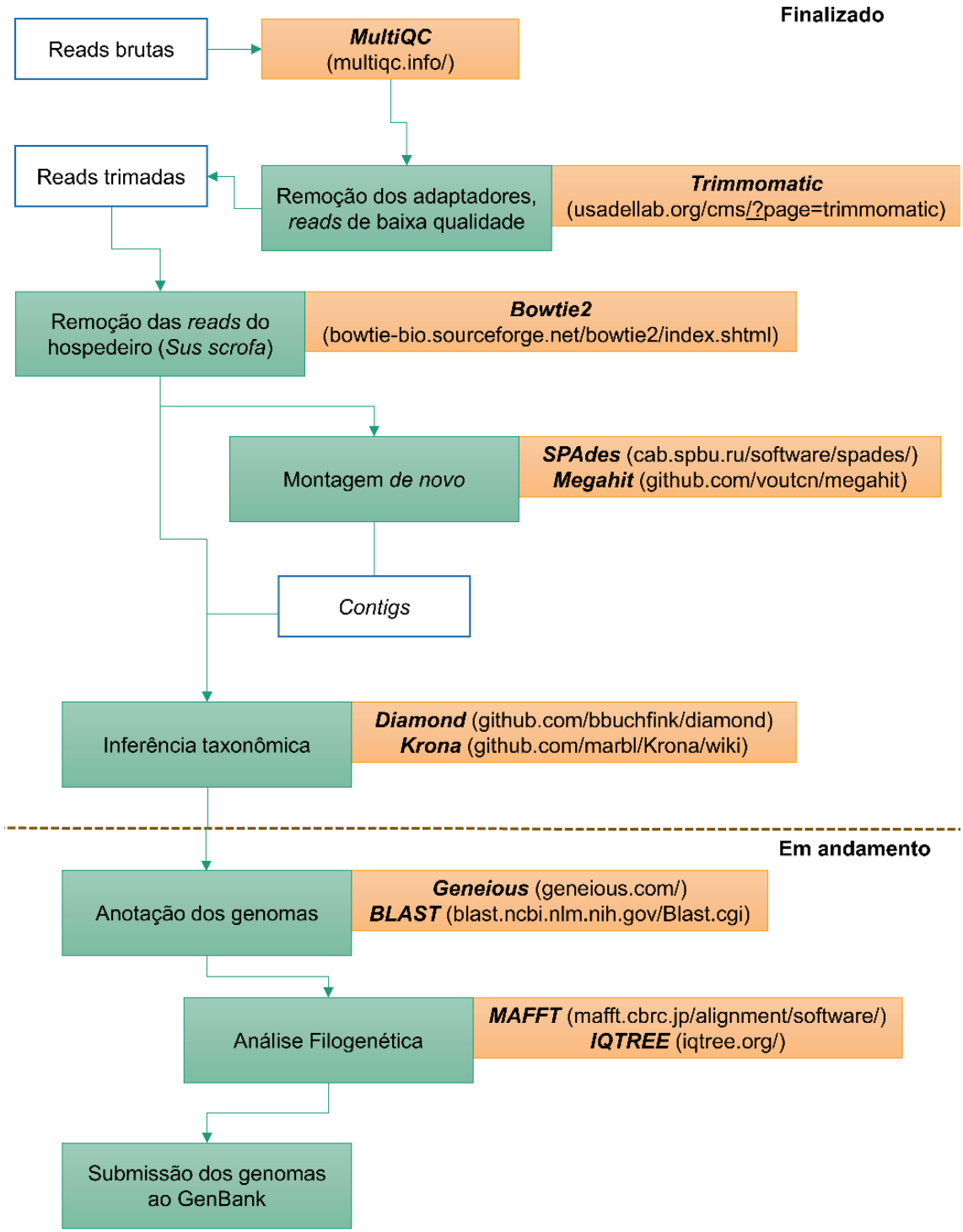

Figura C2 – Fluxograma da análise dos dados obtidos a partir do sequenciamento de alto desempenho do viroma de fezes de suínos com ou sem diarreia. Todas as ferramentas de bioinformática utilizadas estão disponíveis gratuitamente nos sites indicados, exceto o programa *Geneious*.

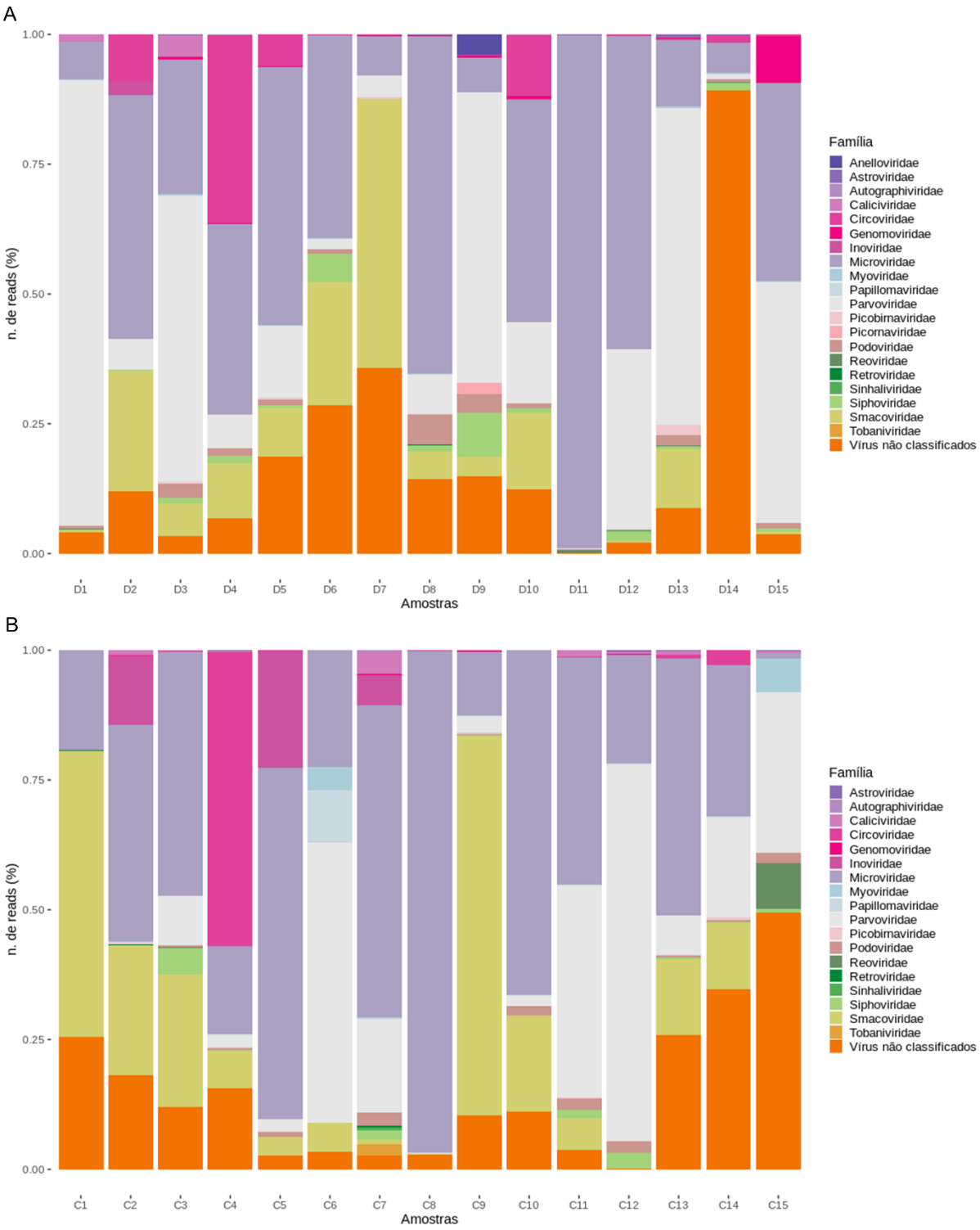

Figura C3 – Composição do viroma de fezes de suínos com diarreia (A, n = 15) e sem diarreia (B, n = 15) baseada no número de *reads* (%) (*paired* e *unpaired*) após trimagem e remoção das reads do hospedeiro. As *reads* foram comparadas ao banco de dados não-redundante (*nr*) do NCBI (Maio/2020) utilizando o programa *diamond* (parâmetros "*-blastx –f 102*"). As famílias virais que tiverem menos de 10 reads correspondentes não foram consideradas nessa análise.