Virus Research 323 (2023) 198974

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

A combined approach of rolling-circle amplification-single site restriction endonuclease digestion followed by next generation sequencing to characterize the whole genome and intra-host variants of human Torque teno virus

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ARTICLE INFO

Keywords:
Torque Teno Virus
Whole genome
Rolling circle-amplification
Restriction endonuclease digestion
Next generation sequencing
Intra-host variants

ABSTRACT

Torque Teno Virus (TTV) was initially associated with post-transfusion hepatitis, but growing evidence of its ubiquity in humans is compatible to no apparent clinical significance. TTV is a small non-enveloped virus with a circular single-negative-stranded DNA genome, belonging to the *Anelloviridae* family. Currently, TTVs are divided in seven phylogenetic groups and are further classified into 21 species. Studies about diversity of TTV in different conditions are receiving increasing interest and in this sense, sequencing of whole genomes for better genetic characterization becomes even more important. Since its discovery in 1997, few TTV complete genomes have been reported worldwide. This is probably due, among other reasons, to the great genetic heterogeneity among TTV strains that prevents its amplification and sequencing by conventional PCR and cloning methods. In addition, although metagenomics approach is useful in these cases, it remains a challenging tool for viromic analysis. With the aim of contributing to the expansion of the TTV whole genomes dataset and to study intra-host variants, we employed a methodology that combined a rolling-circle amplification approach followed by EcoRI digestion, generating a DNA fragment of ~4Kb consistent with TTV genome length which was sequenced by Illumina next generation sequencing. A genogroup 3 full-length consensus TTV genome was obtained and co-infection with other species (at least those with a single EcoRI cleavage site) was not identified. Additionally, bioinformatics analysis allowed to identify the spectrum of TTV intra-host variants which provides evidence of a complex evolution dynamics of these DNA circular viruses, similarly to what occurs with RNA viruses.

Torque Teno Virus (TTV) was first discovered in 1997 in a Japanese patient with acute post-transfusion hepatitis of unknown etiology (Nishizawa et al., 1997). Currently, TTV is globally spread with very high prevalence in human population and has not been associated to a clinical disease so far, being its role in pathogenesis largely unknown (Bendinelli et al., 2001; Okamoto, 2009). In fact, its widespread detection in the intestine, skin, oral and nasal cavities, pharynx, saliva, urine and blood, and the identification of viremic individuals independently of age group or health status, are hardly compatible with the concept of pathogenicity, unless associations of viral loads or genetic variants with particular conditions are specified (Lolomadze and Rebrikov, 2020). Interestingly, TTV loads have been recently proposed to be employed as an endogenous marker for the human immune status after solid organ transplantation (Rezahosseini et al., 2019; Schmidt et al., 2021). Additionally, TTV species in the follow-up of kidney transplantation have been analyzed by a metagenomic approach showing differences in diversity between recipients and donors (Kulifaj et al., 2020).

TTV is a small (30–50 nm), icosahedral non-enveloped virus with a circular single-stranded DNA (ssDNA) genome of negative polarity and is classified in the *Anelloviridae* family (Nishizawa et al., 1997). This viral family includes 30 genera but only *Alphatorquevirus* (TTV), *Betatorquevirus* (Torque teno Mini virus) and *Gammatorquevirus* (Torque teno

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https://doi.org/10.1016/j.virusres.2022.198974
Received 7 September 2022; Received in revised form 17 October 2022; Accepted 18 October 2022
Available online 19 October 2022

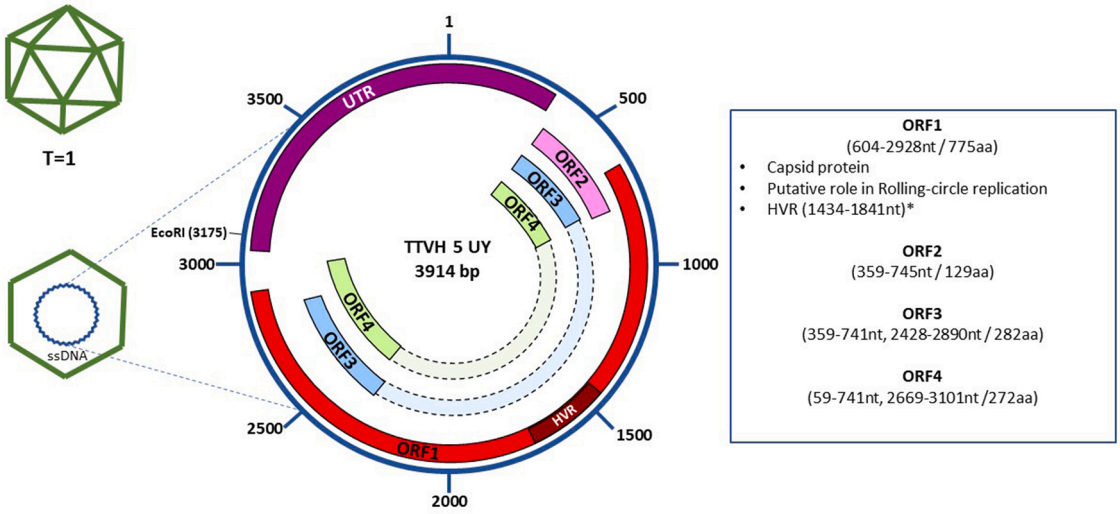

Fig. 1. TTV genome structure for the TTVH 5 UY consensus sequence. ORF1, ORF2, ORF3, ORF4, HVR and UTR nucleotide positions, amino acid length and putative function are shown. *HVR nucleotide position was indicated according to the reference TA278 isolate (Nishizawa et al., 1999).

Midi virus) have human hosts (ICTV, 2020). TTV genome is approximately 3.8 Kb in length, in which 1.2 Kb corresponds to the untranslated region (UTR) and 2.6 Kb belongs to the coding region (CDS) (Focosi et al., 2016; Wei et al., 2015). The CDS presents at least four partially overlapping open reading frames (ORF1–4) with small ORFs of unknown function, including a hypervariable region (HVR) and an N-terminal arginine fragment with a poly-A sequence (Kakkola et al., 2008; Mushahwar et al., 1999; Spandole et al., 2015). ORF1 (~700 aa) encodes the capsid protein and is presumably involved in TTV rolling-circle replication (Erker et al., 1999; Luo et al., 2002; Mushahwar et al., 1999; Tanaka et al., 2001). ORF2 (~200 aa), which is believed to be divided in several smaller ORFs, encodes a protein-tyrosine phosphatase (Peters et al., 2002).

TTV exhibits very high genetic variability, where the UTR is the most conserved region and the coding region presents a divergence of up to 70% at the amino acid level between strains (Biagini et al., 1999; Luo et al., 2002). TTV is currently divided in seven phylogenetic groups (G1-G7) and three subgroups were identified within G3 (3a, 3b and 3c) (Hsiao et al., 2021, 2016). For TTV grouping criteria, groups are separated by a nucleotide divergence of more than 30% or evolutionary distances >0.30 (Hsiao et al., 2016). Moreover, TTV is further classified into 21 species (Torque teno virus 1–29) based on the ORF1 nucleotide pairwise identity threshold of 69% (ICTV, 2020).

Due to the elevated genetic diversity of TTV, genetic characterization can be a great challenge. Indeed, few TTV complete genomes reported worldwide (137 according to the Genbank database april 2022), are available for genotyping studies. Former strategies for viral full-length sequencing involved mainly PCR and cloning methods followed by Sanger sequencing, which require a previous knowledge of the viral sequence (Daly et al., 2011; Leski et al., 2012; Valdazo-González et al., 2012). Therefore, this approach offers a limited potential to reveal the diversity present in the sample (Logan et al., 2014; Mullan et al., 2004, 2001). In this sense, next-generation sequencing (NGS) is receiving increasing interest, but virome approaches remain a challenging methodology to obtain complete genomes from individual samples. The combination of rolling-circle amplification (RCA) followed by single site restriction endonuclease digestion and NGS easily allows obtaining whole TTV genomes without previously knowing the sequence and at the same time, identifying intra-host single nucleotide variants (iSNVs) and insertions or deletions (INDELs) with a great coverage.

To achieve this, DNA obtained from a previously detected TTV positive serum sample from Uruguay (TTVH 5 UY) (Cancela et al., 2016) was subjected to RCA. This sample belonged to a 43-year-old male infected with hepatitis B virus. RCA is an isothermal process where ssDNA molecules can be synthesized from circular ssDNA templates with random hexamers primer and the bacteriophage phi29 DNA polymerase, a high-fidelity, elevated processivity and proofreading enzyme (Zhao et al., 2008). The amplification protocol was performed according to Niel et al. (2005) with modifications. In this study, 4 µl of DNA diluted in 6 µl of water was denatured at 95 °C for 3 min, cooled in ice and added to a mix containing 2 U phi 29 DNA polymerase (New England BioLabs, Inc), 1X exo-resistant random primer (Thermo Fisher Scientific, USA), 3.6 µl dNTPs 10 mM mix, 1X BSA and 1X phi29 DNA Polymerase Reaction Buffer in a final reaction volume of 25 µl. The reaction was incubated for 18 h at 30 °C and the enzyme was inactivated at 65 °C for 10 min. Afterwards, the amplification product was linearized with 10 U of *Eco*RI (Thermo Fisher Scientific, USA) for 3 h at 37 °C. The enzyme was inactivated at 65 °C for 10 min. This restriction endonuclease was previously reported for the digestion of group 3 TTV genome in a unique restriction site (Niel et al., 2005). However, an additional panel of enzymes could be selected in order to consider different genetic groups (Information provided in Suppl. Table 1). The obtained product of ~4Kb was purified from agarose gel (QIAquick Gel Extraction Kit, Qiagen) (Suppl. Fig. 1). The purified product was quantified with Qubit fluorometer (Qubit™ DNA-HS Assay kit). Nextera DNA Flex Library Preparation kit (Illumina, USA) with dual indexing was used from 100 ng of DNA. Control quality libraries were performed on a Fragment Analyzer 5200 system (Agilent Technologies, USA) using the Standard Sensitivity NGS Analysis Kit (Agilent Technologies, USA). Library was sequenced on an Illumina MiniSeq Genomic Platform at the Faculty of Sciences (UdelaR, Uruguay) using Mid Output Reagent Cartridge (300-cycles, 150 base-pair paired-end reads) by following standard Illumina protocols.

A total of 610,386 sequencing raw reads were demultiplexed automatically on the MiniSeq platform with the default settings. Adapter/quality trimming was performed with Trim Galore considering base quality ≥30. The cleaned reads were assembled with a *De novo* approach employing the software Spades, Abyss and IDBA-UD. The obtained contigs were BLAST-searched on NCBI database and the sequence that exhibited the highest nucleotide similarity was selected for reference alignment. The reference alignment was carried out with BWA-MEM program and the reads were mapped to Brazilian 3 h TTV genome (Genbank accession number AY823989). The consensus calling was obtained with Samtools and IVAR.

The consensus complete genome of TTVH 5 UY was successfully obtained with 3914 nucleotides long (Fig. 1), 55.7% GC content,

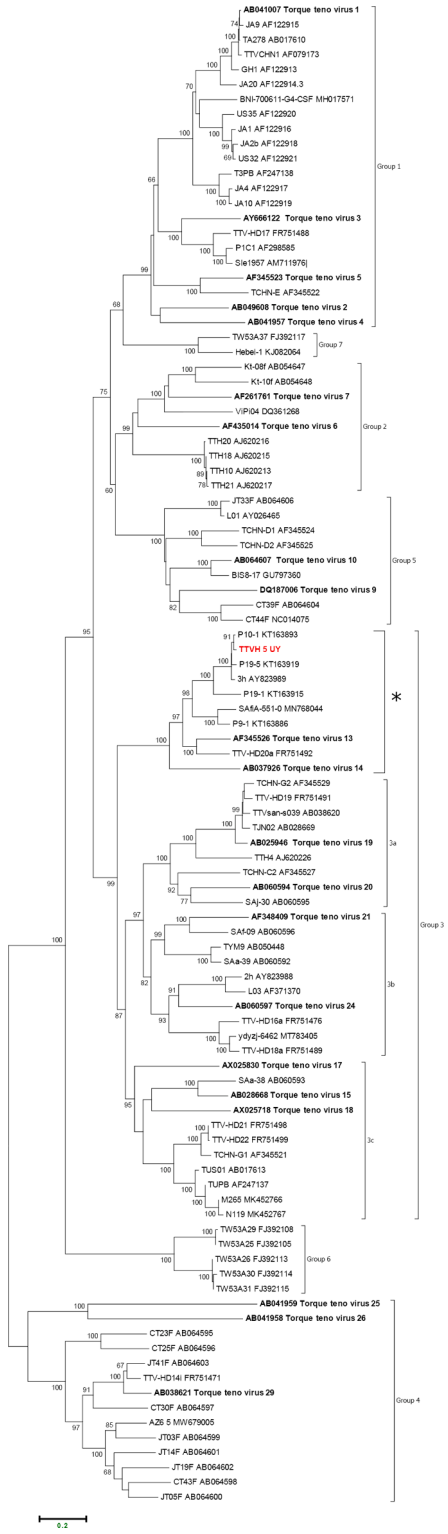

Fig. 2. Phylogenetic tree based on the full-length and nearly full-length TTV genomes from Group 1 to 7. Tree reconstruction was performed using the Neighbor-Joining method with Tamura-Nei model as the most suitable substitution model using Molecular Evolutionary Genetics Analysis (MEGA) v7.0 software. Robustness of the tree was determined by bootstrap analysis for 1000 replicates. Only values of ≥60% are shown. Isolates names and GenBank accession numbers are included. Uruguayan sequence (TTVH 5 UY) from this study is indicated in red. Closely related isolates and TTV species based on ICTV, 2020 were also incorporated. The unclassified cluster within group 3 is indicated with *.

Table 1
Summary of p-distances between TTVH 5 UY vs. TTV species. Phylogenetic groups (1–7) for each isolate are indicated. Closely related sequences to TTVH 5 UY from Genbank database are also included and are shown in bold.

Isolate name/ species	Accession Number	Country	Group	p-distance
Torque teno virus 1	AB041007	Japan	1	0.471
Torque teno virus 2	AB049608	Japan	1	0.444
Torque teno virus 3	AY666122	Finland	1	0.459
Torque teno virus 4	AB041957	Japan	1	0.438
Torque teno virus 5	AF345523	China	1	0.456
Torque teno virus 6	AF435014	Germany	2	0.477
Torque teno virus 7	AF261761	UK	2	0.471
Torque teno virus 9	DQ187006	USA	5	0.493
Torque teno virus 10	AB064607	Japan	5	0.476
Torque teno virus 13	AF345526	China	3	0.285
Torque teno virus 14	AB037926	Japan	3	0.313
Torque teno virus 15	AB028668	Japan	3 (Subtype 3c)	0.409
Torque teno virus 17	AX025830	NA	3 (Subtype 3c)	0.401
Torque teno virus 18	AX025718	NA	3 (Subtype 3c)	0.437
Torque teno virus 19	AB025946	Japan	3 (Subtype 3a)	0.412
Torque teno virus 20	AB060594	Japan	3 (Subtype 3a)	0.424
Torque teno virus 21	AF348409	China	3 (Subtype 3b)	0.452
Torque teno virus 24	AB060597	Japan	3 (Subtype 3b)	0.419
Torque teno virus 25	AB041959	Japan	4	0.545
Torque teno virus 26	AB041958	Japan	4	0.529
Torque teno virus 29	AB038621	Japan	4	0.490
TW53A26*	FJ392113	Taiwan	6	0.477
TW53A37*	FJ392117	Taiwan	7	0.461
P9–1	KT163886	USA	3	0.253
TTV-HD20a	FR751492	Germany	3	0.278
SAfiA-551–0	MN768044	Tanzania	3	0.249
P19–1	KT163915	USA	3	0.160
P19–5	KT163919	USA	3	0.037
P10–1	KT163893	USA	3	0.016
3h	AY823989	Brazil	3	0.033

NA, not available.
*non- assigned species.

491,176 mapped reads and 16,264 x coverage. The NGS data analysis revealed that consensus TTVH 5 UY sequence presented 146 nucleotide substitutions in comparison with the reference sequence AY823989. Twenty-eight mutations were located in the HVR and six of them were missense variants (A1468G, G1471A, C1525G, A1684G, A1773C and A1822T). In addition, TTVH 5 UY contains a deletion of 6 nucleotides corresponding to position 2790 in the reference genome, which does not affect the reading frame. This deletion is located in the overlapping region involving ORF1, ORF3 and ORF4, which results in a two-amino acid deletion for the three ORFs mentioned.

In our previous work, most of TTV Uruguayan UTR sequences formed monophyletic clusters closely related to group 3 reference isolates (Cancela et al., 2016). The phylogenetic reconstruction with the complete genome sequence confirmed that TTVH 5 UY clustered within group 3 with very high bootstrap values (Fig. 2). TTV group 3, the most

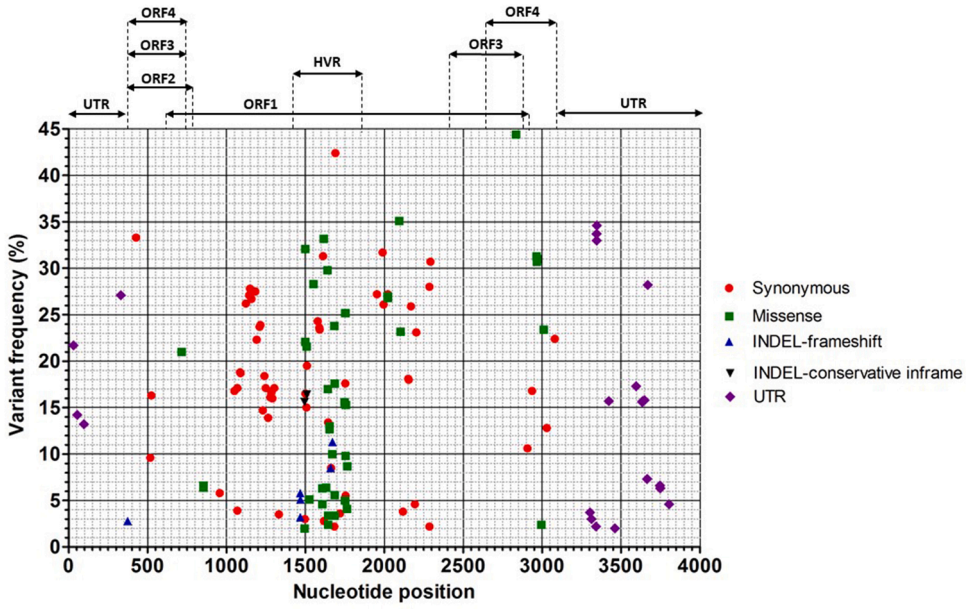

Fig. 3. Intra-host variants detected for TTVH_5_UY. Variant frequency for each position is shown. Synonymous, missense, INDEL-frameshift and INDEL-conservative inframe changes are also indicated. ORF1, ORF2, ORF3, ORF4, HVR and UTR regions are indicated.

diverse group of TTV, can be found in America, East Asia and Europe (Hsiao et al., 2021). The sequence obtained in this work together with the two Brazilian isolates (2 h and 3 h) are the only three whole genome sequences of TTV from South America. Brazilian 2 h isolate clustered with sequences from sub-group 3b. However, TTVH 5 UY and Brazilian 3 h sequence grouped into a separate and more divergent monophyletic cluster within group 3 (showed with an asterisk in Fig. 2). This cluster of sequences has not been classified within any subgroup (non 3a, 3b or 3c) (Hsiao et al., 2021). In order to further characterize the full-length TTV genome obtained, a p-distance matrix was constructed between TTV Uruguayan sequence and reference isolates (Table 1). The analysis between TTVH 5 UY and the closest related isolates (in bold in Table 1) revealed p-distance values ranging from 0.313 to 0.016. Even, a sub-cluster of the closest isolates with Uruguayan sequence, P10–1 (KT163893, USA), Brazilian 3 h (AY823989) and P19–5 (KT163919, USA) showed very low p-distances values (0.037–0.016) (Table 1, Fig. 2). Torque teno virus 13 (AF345526) is the most similar specie to these sequences and based on the analysis of the complete ORF1, TTVH 5 UY shared a nucleotide identity of 69% with this specie, which is the ICTV species demarcation threshold (ICTV, 2020).

On the other hand, taking into account the criteria reported by Laubscher et al., 2022 to identify TTV co-infection (≥75% of the ORF1 covered), other species (at least those with a single EcoRI cleavage site, Suppl. Table 1) were not identified by analyzing the reads obtained. Subsequently, an intra-host variants analysis was carried out in order to explore TTV intra-lineage diversity.

Although co-infection between several TTV species and genogroups in the same individual has been widely reported (Biagini et al., 1999; Chan et al., 2001; Jelcic et al., 2004; Kulifaj et al., 2020; Laubscher et al., 2022; Ninomiya et al., 2008), the TTV intra-host variants has not been well explored. We analyzed the spectrum of variants with the LoFreq tool to call the frequency variants present in the mapping reads using TTVH 5UY consensus sequence as reference. Since the estimated sequencing error rate with Illumina platform is approximated at 2% (Beerenwinkel et al., 2012; Stoler and Nekrutenko, 2021; Yang et al., 2018), to ensure high-quality and reliable variant calling the parameters were set as: frequency $\geq$ 2%, minimum coverage of 1000 reads and Bonferroni-corrected P-value $<$ 0.05. A total of 130 variants were identified in different genome positions (120 iSNVs and 10 INDELS). Most of them were found in the CDS region, while 22 were located in the UTR region (Suppl. Table 2). Variant frequency identified for each nucleotide genome position is shown in Fig. 3. The identification of iSNV and INDELs with frequencies between 2 and 44.4%, not present in the consensus sequence, could suggest a quasispecies dynamic of TTV as reported by other authors (Nishizawa et al., 1999). Even though many reports focus on the TTV co-infection detection, the analysis of intra-host variants is also worth exploring in order to start deepening the knowledge about its complex intra-host microevolution. The TTV circulation as quasispecies could allow the virus to evade the immune response and to establish persistent infection (Nishizawa et al., 1999). This hypothesis is consistent with postulations about possible existence of a healthy human virome being Anelloviruses one of the main components (Koonin et al., 2021).

The RCA-single site restriction endonuclease digestion followed by NGS represents a useful combined tool in order to expand the dataset of TTV complete genomes which could greatly contribute to better understand the viral epidemiology, particularly in South America, where available TTV genomes are very scant. Remarkably, this methodological strategy has the potential to obtain whole genomes from circular viruses like TTV with a very good coverage, instead of employing a complex virome approach which can result in poor mapping reads for the desired virus, especially for samples that may have low viral load. However, the main limitation of the procedure employed is that the TTV species diversity present in an individual (co-infections) could not be completely characterized, since it will depend on the single site restriction enzyme used. Therefore, this method is not suitable for a TTV species screening, unlike a viromic approach. Nevertheless, several enzymes could be used to obtain complete genomes of different species or groups (panel proposed in the Supp. Table 1). In addition, this methodology could also be used for DNA circular viruses with other genome length such as Torque teno Mini and Midi viruses (2.8Kb and 3.2Kb, respectively). The appropriate selection of the enzyme and the size of the purified DNA are crucial steps to enrich the sample with the virus of interest prior to Illumina sequencing. In fact, this becomes an advantage in order to identify intra-lineage variants. The high-throughput data provided by NGS can be employed to analyze the mutant spectrum of the viral population and to perform an in-depth characterization of the intra-host variants (Arroyo et al., 2013; Au et al., 2016; Barzon et al., 2011; Dube Mandishora et al., 2018; Fisher et al., 2015; Shen-Gunther et al., 2017; Wright et al., 2011; Yi et al., 2014). Two decades ago, TTV circulation as

quasispecies was reported by Nishizawa et al., 1999 using clone-based Sanger sequencing of 850 bp of ORF1, including HVR. However, TTV species had not been described at that time, not being able to correctly discriminate between intra- or inter-species diversity. The methodology used here in combination with bioinformatic tools allowed analyzing the intra-lineage variants throughout the entire genome as no co-infection between different species were identified in the sample sequenced.

In summary, in this work we employed a simple methodological pipeline (Suppl. Fig. 2) which enabled us: (i) to obtain a consensus genogroup 3 full-length TTV genome from Uruguay, confirming our previous findings; (ii) to describe in detail its genetic characteristics and phylogenetic relationship with a TTV Brazilian sequence (3 h) and (iii) to present novel insights into the intra-host viral dynamics of TTV.

Data availability

The complete genome sequence of TTVH 5 UY has been deposited in the GenBank database under the accession number OP168886. Illumina MiniSeq sequence raw reads were deposited in the NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA891042, BioSample accession SAMN31306976.

Ethics

Sample was processed in accordance with the requirements of the Ministry of Public Health of Uruguay and complied with the ethical standards of the Helsinki Declaration (1964, amended in 2008) of the World Medical Association.

Author contributions: NR and FC conceived the study. FC, NR, AM, YP, GB did the experiments. FC and NR analyzed the data. NR and FC wrote and edited the manuscript. YP, SM and JA reviewed the draft. All authors approved the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

Authors are grateful to Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Agencia Nacional de Innovación e Investigación (ANII) and Comisión Sectorial de Investigación Científica (CSIC).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198974.

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