Infectious Subgenomic Amplicons method to expedite reverse genetics of SARS-CoV-2 and other coronaviruses

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Abstract

There is a need for simple reverse genetics methods to decipher the biological properties of animal and human coronaviruses. Here, we attempted to rescue the recently emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the Feline enteric coronavirus (FeCoV) using the rapid "Infectious-Subgenomic Amplicons" (ISA) method. For each virus, transfection into permissive cells of eight overlapping subgenomic cDNA fragments covering the entire genome allowed reconstruction of the complete virus genome and generated infectious viral particles. Rescued viruses replicated the phenotypic and genotypic characteristics of the original isolates. In conclusion, the ISA method, which had been previously used for RNA viruses with shorter genomes (e.g., flaviviruses, alphaviruses and enteroviruses) can be used to rescue viruses with substantially longer genomes and usefully complements pre-existing methods for reverse genetics of coronaviruses. Its extreme simplicity and versatility makes it a strong option to decipher the biological properties of coronaviruses circulating in human, domestic or wild fauna populations.

Introduction

The order *Nidovirales* represents a large group of single-stranded positive-sense RNA viruses ((+) ssRNA) characterized by the size of their genomes, which represented the largest of the RNA virus in the world. This order is subdivided into nine suborders including the *Cornidovirineae* to which belongs the *Coronaviridae* family [1]. Coronaviruses (CoV) are widespread and infect humans, domestic animals and wild fauna populations. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; genus: *Betacoronavirus*) has emerged in 2019 in Wuhan, China and has spread worldwide with more than 16 million people infected in the world at the end of July 2020 [2]. SARS-CoV-2 is responsible of COVID-19, a disease that has been associated in a proportion of patients with a severe pneumonia leading to respiratory distress and possibly death [3,4]. Feline enteric coronavirus (FeCoV; genus: *Alphacoronavirus;* species: *Alphacoronavirus-1*) is a ubiquitous veterinary pathogen circulating in felids population and responsible for common enteritis [5]. In case of chronic or intermittent infections, genomic mutations can occasionally occur in FeCoV and lead to a severe systemic disease called feline infectious peritonitis (FIP) [6,7].

Reverse genetics methods enable to engineer wild-type or genetically modified viruses. In the case of coronaviruses, rescue of infectious virus can be obtained by the transfection of full- length cDNAs using vaccinia virus vectors or bacterial artificial chromosomes [8–11].

Alternatively, *in vitro* or in-yeast viral genome assembly followed by *in vitro* RNA production can lead to virus rescue by transfection of full-length cDNAs in cells [12,13]. Due to the complexity and large size of CoV genomes, the assembly and modification of full-length genomic cDNAs or RNAs remains laborious and time-consuming (*eg.* toxicity of clones, undesirable mutations, difficulty in driving a full-length 30 kb RNA transcripts *in vitro*).

The Infectious Subgenomic Amplicons (ISA) method is a simple and rapid bacterium-free method that has been developed in recent years and allowed rescuing viruses with relatively small (+) ssRNA genomes, from the *Flaviviridae*, *Togaviridae* and *Picornaviridae* families [14–18]. Unlike conventional reverse genetics systems that require cloning steps, the ISA method is based on the simple transfection of overlapping subgenomic DNA fragments, covering the entire virus genome into permissive cells. DNA recombination and production of full-length viral genomic RNA, under transcription signals, are produced by the cellular machinery.

Prior to the current study, the ISA method had been used for the reverse genetics of (+) ssRNA viruses with genomes 7,500 to 14,000 nucleotides long. Reverse genetics methods are powerful tools to study viral functions or develop attenuated strains for vaccine development [19,20], it was crucial to investigate whether it could be used for viruses with longer (+) ssRNA genomes such as coronaviruses. In the current study, we report the successful rescue of SARS-CoV-2 and FeCoV infectious particles using the ISA method. Genotypic and phenotypic characterization of recovered viruses were performed using the original isolates for comparison.

Results

Characterisation of SARS-CoV-2 and FeCoV clinical isolates

Attempts to rescue coronaviruses using the ISA method were based on genetic fragments derived from the genome of SARS-CoV-2 and FeCoV isolates. SARS-CoV-2 was passaged two times on VeroE6 and FeCoV was passaged five times on FeA cells. Sequencing of the complete genome was performed at the first and last passage from cell culture supernatant. No evolution in the consensus sequence was identified, suggesting previous adaptation to cell culture for both strains.

Design of reverse genetic systems

From each full-length sequence, 8 overlapping subgenomic fragments with an average size of 3,900 nucleotides were designed (Figure 1) and cDNA were obtained using PCR amplification of synthetic subgenomic viral fragments. During *de novo* synthesis, the human cytomegalovirus promoter (pCMV) was inserted upstream the first fragment to initiate transcription. Downstream the last fragment, the hepatitis delta ribozyme followed by the simian virus 40 polyadenylation signal (HDR/SV40pA) was added to allow transcription termination and RNA maturation (see previous studies [14,16]). Successful virus rescue was obtained after two passages using the following criteria: (i) observation of cytopathic effect (CPE), (ii) measurement of the molecular viral load in cell supernatant medium using a real time qRT-PCR assay, (iii) measurement of the infectious viral load in cell supernatant using TCID50 assays, and (iv) assessment of genome integrity using complete genomic sequencing. Except for sequencing which was done only once for each virus, each virus recovery was tested in triplicate.

Phenotypic and genotypic characterization of rescue SARS-CoV-2

For SARS-CoV-2, we transfected 3 µg of an equimolar mix of the 8 subgenomic amplicons on sub-confluent BHK-21 cells and added a suspension of VeroE6 cells after 24 hours; 5 days after transfection, the cell culture supernatant was passaged two times on VeroE6 cells monolayer. At the second passage, a clear CPE occurred 2 days post-infection (dpi), as observed with the original strain. The molecular and infectious viral loads at 2 dpi in the cell culture supernatant were 6.8±/-x0.5 log10 RNA copies per mL and 6.0±/-0.2 log10 TCID50 per mL, respectively, which is similar to viral loads obtained after culture of the original strain using a MOI (multiplicity of infection) of 0.001 (7.1±/-0.2 log10 RNA copies per mL and 7.6±/-0.1 log10 TCID50 per mL, respectively) (Supplementary Table 1a). Comparative replication kinetics were performed to further characterize the replicative fitness of the original and rescued strains. At a MOI of 0.001, virus growth was assessed every 12 hours by measurement of the molecular viral load in the supernatant of Vero E6 cells. Despite significant differences observed at early collection points, replication kinetics displayed similar shape, leading to no significant difference at the endpoint (48 h pi) between molecular viral loads (4.6±/-0.2and 4.9±0.01 log10 RNA copies per mL for the rescued and original SARS-CoV-2 strains, respectively) (*N*=3; student's test; P> 0.01) (Figure 2A). The sequencing of the complete genome showed no consensus mutation and only one minor synonymous mutation was detected (Supplementary Table 2). This indicates that the integrity of the genome structure of the rescued SARS-CoV-2 strain was preserved.

Phenotypic and genotypic characterization of rescued FeCoV

For FeCoV, 5 days after transfection of 3 µg of an equimolar mix of the 8 subgenomic amplicons on a confluent co-culture of BHK-21 and FeA cells, supernatant was passaged two times on FeA cells. At the second passage, a clear CPE was observed 2 dpi (similar to the original strain). The molecular and infectious viral loads at 2 dpi in the cell culture supernatant were 6.7±/-0.5 log10 RNA copies per mL and 5.8±/-0.6 log10 TCID50 per mL, respectively, which is similar to viral loads obtained after culture of the original strain using a MOI of 0.01 (7.0±/-0.1 log10 RNA copies per mL and 6.3±/-0.3 log10 TCID50 per mL, respectively) (Supplementary Table 1b). Comparative replication kinetics at a MOI of 0.01 showed no significant difference at the endpoint (2 dpi) between molecular viral loads (7.5±/-0.7 and 7.1±/-0.2 log10 RNA copies per mL for the rescued and original FeCoV strains, respectively) (*N*= 3; student's test; P> 0.01) (Figure 2B). The sequencing of the complete genome indicated that the genome structure integrity of the rescued FeCoV strain was conserved (no consensus mutations observed) (Supplementary Table 2).

Discussion

The ISA reverse genetics method had been used in the past for a variety of viruses with a positive-stranded RNA genome such as flaviviruses, alphaviruses or enteroviruses [14–18]. The method was proven to be extremely simple, rapid and easy to implement, but all previous results were obtained with "short" genomes (*i.e.* <15,000 nucleotides) (Figure 3). It was not certain that the method could be applied to viruses with a substantially longer and complex genome, such as coronaviruses (>25,000 nucleotides). Indeed the methods relies on the use of a larger set of subgenomic DNA fragments *in cellula* - 3 for alphaviruses *vs* 8 for coronaviruses – thus possibly reaching technical limits of the method as 7 simultaneous recombination events are necessary for coronaviruses instead of 2 for alphaviruses. If we consider that transcription initiation and termination or RNA maturation signals are efficient, this implies at least two constraints: cells generating infectious RNA should receive all fragments covering the entire virus genome, and that they all recombine together to reconstitute of a full-length DNA. An obvious consequence of this mechanism of action is that the probability for an individual cell to receive simultaneously upon transfection all subgenomic fragments decreases when the number of fragments increases. We previously showed that for some small (+) ssRNA viruses such as enteroviruses, the number of fragments used can be balanced by increasing in parallel the total amount of DNA to be transfected [15]. However, in the case of coronaviruses, given the length of the genome, this requirement had to be met with amounts of DNA that do not jeopardize cell biological functions, growth, and subsequent replication of the virus.

Unexpectedly, the implementation of the technique turned out to be quite simple when applied to the two tested coronaviruses, namely SARS-CoV-2 and FeCoV. Here, the initial design using 8 fragments allowed to obtain swiftly the *de novo* synthesis of subgenomic fragments (all of them <5800 nucleotides) and we successfully used classical amounts of DNA (3 µg) for transfection. This corresponds approximately to 1.1E+5 molecules of each subgenomic fragment per cell upon transfection and proved to be sufficient to obtain the recombination of all segments in individual cells. However, when we tried to rescue the SARS-CoV-2 and FeCoV using the ISA-derived Haiku [17] method by providing the pCMV and theHDR/SV40pA as two additional amplicons that overlapped with the 5’ extremity and 3’ termini of the genome respectively, this increased the number of segments to 10 and experiments remained unsuccessful. This strongly suggests that the number of 8 segments was close to the limit allowing successful full genomic recombination in individual cells under the experimental conditions considered.

In conclusion, the ISA method represents an extremely simple method for the reverse genetics of coronaviruses when using 8 subgenomic fragments and cells that can be efficiently transfected. The actual advantages of the method are that there is no need for cloning steps or *in vitro* assembly of a full-length genome and that the genome sequence can be completely identical to that of the targeted virus (no restriction sites or other genome modifications needed). In the case of SARS-CoV-2 and FeCoV, we conveniently obtained the rescued viruses under standard operating conditions and showed that their genomic sequence and characteristics in terms of growth and production of cytopathic effect were those of the original "natural" strains. In previous studies, we demonstrated the capacity of the ISA method to generate modified DNA fragments to analyse the phenotypic effects of engineered sequences [21,22].

Mutagenesis of the original sequence (*eg.* insert of fluorescent reporter gene [13] for drug screening and antiviral activities) can be very simply operated using modified subgenomic sequences without jeopardizing subsequent genomic assembly. This may offer a simplified way to produce recombinant viruses to further decipher, in the case of coronaviruses, the mechanisms involved in transmission, pathogenesis, immune interventions[23,24] or to design therapeutic strategies, as previously performed using traditional techniques[25–31].

Methods

Cells and antibodies.

Baby Hamster Kidney 21 cells (BHK-21; ATCC CCL-10) were grown in Minimal Essential Medium (MEM; Life Technologies) with 5% heat-inactivated foetal bovine serum (FBS), 1% L-glutamine (200 Mm; Life Technologies), 5% Tryptose Phosphate Broth (TPB; Life Technologies) and 1% P/S (5000 U/mL; 5 mg/mL). VeroE6 cells (ATCC CRL-1586) were grown in the same medium as BHK-21 cells supplemented with 1% non-essential amino acids (MEM NEAA; Life Technologies). Feline embryonic fibroblast cells (FeA) were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) with 10% FBS and 1% P/S. FeA cells were kindly provided by VIRBAC laboratory. Feline pulmonary epithelial cells (AK-D; ATCC CCL-150) were grown in a mix of Ham's F12 (F12) and L-15 medium (v/v) with 7% FBS and 1% PS. Additional cell lines and conditions are described in Supplemental Text.

Viral strains.

SARS-CoV-2 was provided courtesy of Pr. Christian Drosten (Charité, Berlin) from the European Archive Collection (human isolate BetaCoV/Germany/BavPat1/2020 p.1; reference: 026V-03883). FeCoV, isolated from a cat died after FIP, was obtained from the American Type Culture Collection (ATCC reference: VR-2126). All experiments were conducted in a BSL3 laboratory.

Virus full genome sequencing.

SARS-CoV-2 and FeCoV clinical samples were first passaged on VeroE6 and FeA respectively. Clarified cell supernatants were used to determine full viral genome sequences. Extraction of nucleic acid was performed using the EZ1 advanced XL machine (Qiagen) with the EZ1 Virus Mini Kit v2.0 (Qiagen). A first random amplification of the viral genome nucleic acids was performed as previously described [14]. Amplification was also performed using specific primers and the RT-PCR kit. SuperScript™ IV One-Step RT-PCR System kit (ThermoFisher Scientific) was used. Sequences of primers designed from the full-length sequences are available in Supplemental Table 3. PCR products were purified (Monarch® PCR & DNA Cleanup Kit; New England Biolabs) and pooled in equimolar proportions.

After Qubit quantification using Qubit® dsDNA HS Assay Kit and Qubit 2.0 fluorometer (ThermoFisher Scientific) amplicons were fragmented (sonication) into fragments of 200bp long. Libraries were built adding barcod, for sample identification, and primers to fragmented DNA using AB Library Builder System (ThermoFisher Scientific). To pool equimolarly the barcoded samples a quantification step by real time PCR using Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) was realised. An emulsion PCR of the pools and loading on 530 chip was realised using the automated Ion Chef instrument (ThermoFisher).

Sequencing was performed using the S5 Ion torrent technology v5.12 (Thermo Fisher Scientific) following manufacturer's instructions [32]. Consensus sequence was obtained after trimming of reads (reads with quality score <0.99, and length <100pb were removed and the 30 first and 30 last nucleotides were removed from the reads) mapping of the reads on a reference (determine following blast of De Novo contigs) using CLC genomics workbench software v.20 (Qiagen). *De novo* contigs were produced to ensure that the consensus sequence was not affected by the reference sequence and no coronavirus mutated sequences were present in the samples. Quasi species with frequency over 5% were studied.

Preparation of subgenomic cDNA fragments.

For each viruses, eight overlapping fragments were design as showed in. The first and last fragments were directly flanked at their 5' and 3'extremities by the pCMV and the HDR/SV40pA respectively during *de novo* synthesis (Genscript manufacturer's for FeCoV; Thermofisher manufacturer's for SARS-CoV-2). cDNA were amplified from these *de novo* synthetic viral fragments as templates. Super Fidelity PCR polymerase kit (Thermofisher Scientific) was used. Primers sequences are described on Supplemental Table 4**.** The final mixture contained 25 µL of Reaction Mix, 3 µL of DNA (1ng/µL), 100 nM of each primer and 20 µl nuclease-free water. RT-PCR and PCR reactions were performed on a Biometra TProfessional Standard Gradient thermocycler. Amplicons were purified (Monarch® PCR & DNA Cleanup Kit; New England Biolabs) and size of PCR products were verified by gel electrophoresis.

Cell Transfection.

The optimal cell lines for SARS-CoV-2 and FeCoV transfection were determined using different conditions described in Supplemental Table 5. An equimolar mix of subgenomic cDNA fragments of SARS-CoV-2 and FeCoV were transfected into sub-confluent BHK-21 cells and a co-culture of BHK-21 + FeA respectively using Lipofectamine 3000 (ThermoFischer Scientific) for 24 hours. For SARS-CoV-2, a suspension of VeroE6 cells was added 24 hours after transfection and then incubated for 5 days at 37°C, 5% CO2. For FeCoV, fresh medium was added 24 hours after transfection and then incubated for 5 days at 37°C, 5% CO2. Cell supernatant were harvested and serially passaged 2 times to ensure the complete disappearance of the DNA used during the transfection. Passages were performed by inoculating clarified supernatant media onto subconfluent VeroE6 and AK-D cells for SARS- CoV-2 and FeCoV respectively:

after 1 h of incubation, cells were washed twice using Hanks' Balanced Salt solution (HBSS; Gibco), fresh medium was added, and plates were incubated 3 days. After the last passage, cell supernatant media were harvested, clarified by centrifugation, aliquoted and stored at -80 ˚C. These virus stocks were used to perform quantification of viral RNA, TCID50 assay, whole-genome sequencing and determined kinetic growth.

Real time RT-PCR assay for virus detection.

Viral RNA was isolated from 100 µL of cell supernatant using a QIAamp Viral RNA kit and RNase-Free DNase Set on the automated QIAcube (Qiagen) automate, following manufacturer's instructions. Relative quantification of viral RNA was performed using the express One-Step SuperScript® qRT-PCR (Invitrogen). The mixture contained 5 µL of express qPCR SuperMix Universal, 0.25 µL of each primer (500 nM), 0.1 µL of probe (200 nM), 1 µL of express SuperScript® RT mix and 3,5 µL of extracted nucleic acids. Assays were performed using the QuantStudio 12K Flex Real-Time PCR machine (Life technologies) with the following conditions: 50 °C for 15 min, 95°C for 20 sec, followed by 45 cycles of 95°C for 3 s, 60°C for 30 s. Data collection occurred during the 60°C step. The amount of viral RNA was calculated from standard curves using synthetic RNA. Primers used are described in the Supplemental Table 6.

Tissue Culture Infectious Dose 50 (TCID50) assay.

Subconfluent cultures of VeroE6 and AK-D cells in 96-well culture microplate were used for SARS-CoV-2 and FeCoV respectively. Cells were inoculated with 10-fold serial dilutions of clarified cell culture supernatants and incubated 3 days for each viruses. Each row included 6 wells of the dilution and two negative control. The presence of CPE in each well was used to determinate TCID50/mL. The determination of the TCID50/mL for both viruses was performed using the Reed and Muench method [33].

Virus replication kinetics.

Infections at MOI 0.001 and 0.01 were performed using subconfluent VeroE6 or AK-D -cells for SARS-CoV-2 and FeCoV, respectively. Cells were washed twice (HBSS) 4 and 1 hours after the infection with SARS-CoV-2 and FeCoV respectively and fresh medium was added. Cell supernatants were sampled every 12 hours up to 48 hours , respectively, clarified by centrifugation, aliquoted and stored at − 80 °C. They were then analysed using the real-time RT-PCR assay as described above. Each experiment was performed in triplicate.

Statistical analyses.

Exploratory analysis were performed using a two way ANOVA multiple comparisons with Sidak's multiple comparisons test. Statistical analysis and graphical representation were performed using Graph Pad prism 7.00.

Declarations

Acknowledgments

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Author Contributions

J.M., B.C., A.N. and X.D.L. conceived and designed the study. J.M., G.P. and T.F. performed the experiments.

J.M., F.T., G.P and A.N. analysed the data.

J.M., G.P. and F.T. contributed to reagents/materials/analysis tools. J.M., A.N., and X.D.L. wrote the paper.

Competing Interests: The authors declare no competing interests.

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Figures

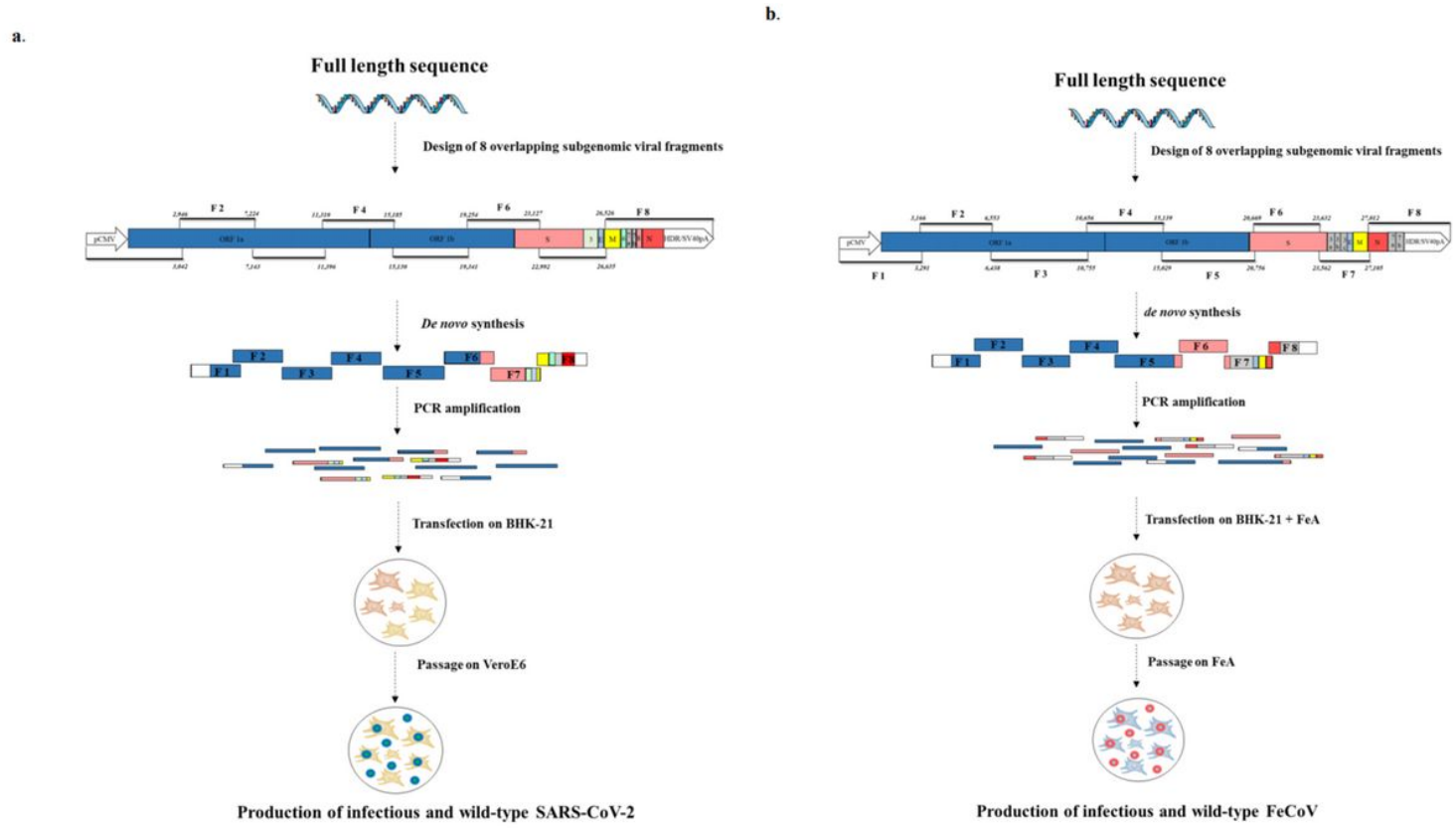

Figure 1

The ISA method to rescue SARS-CoV-2 and FeCoV. For each coronaviruses, full-length sequences were used to design eight overlapping subgenomic viral fragments covering the full genome of SARS-CoV-2 (a) and FeCoV (b). Nucleotide position on genome are indicated in italic black.

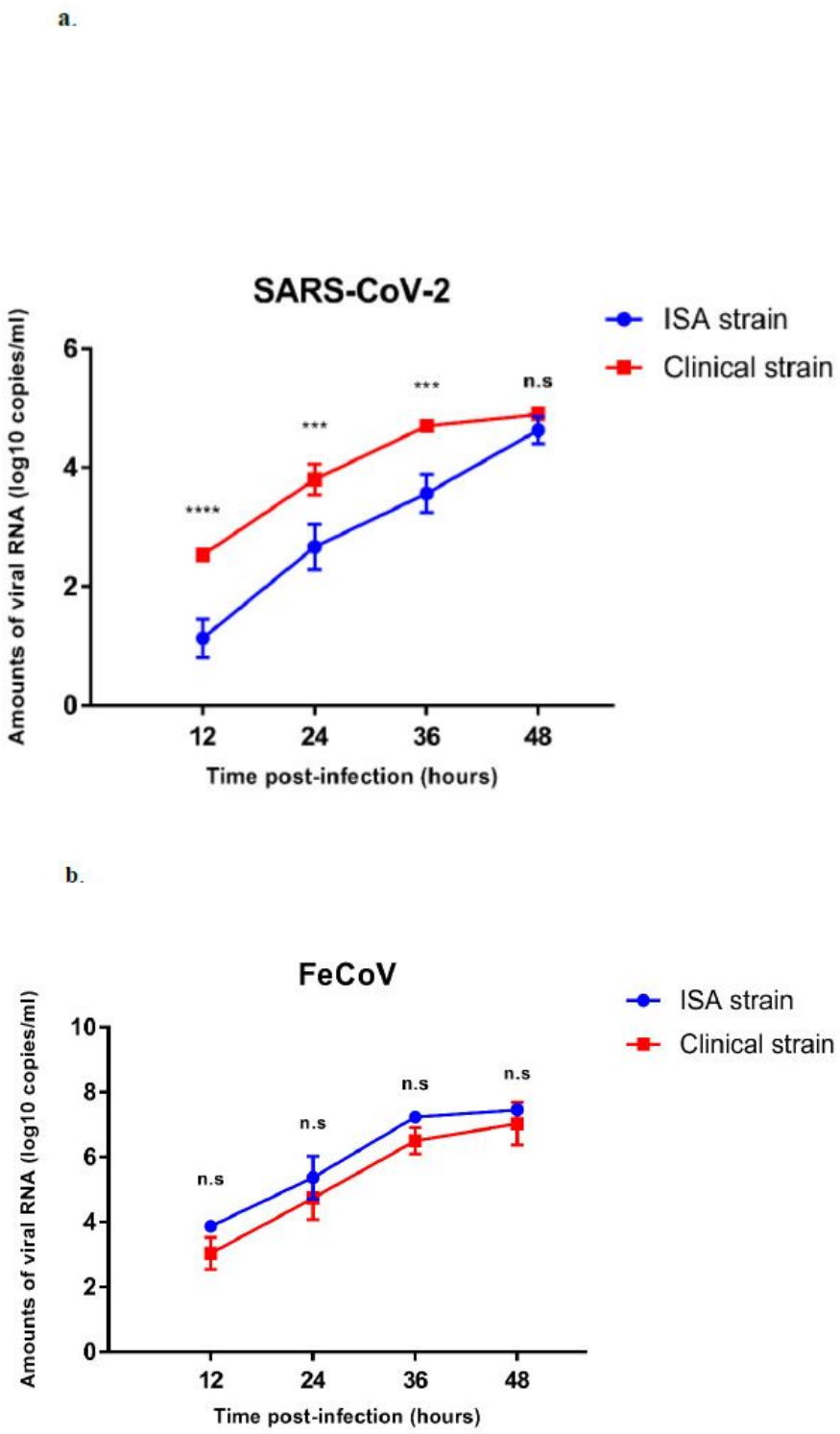

Figure 2

Virus replication kinetics of rescued and isolated strains. An MOI of 0.001 and 0.01 were used to infect VeroE6 and FeA -Sn cells with rescued or isolated SARS-CoV-2 (a) and FeCoV (b) respectively. Data are represented as mean ± SD. Each experiment was performed in triplicate (N=3). Exploratory analyses were performed using Student's T-test exact test.

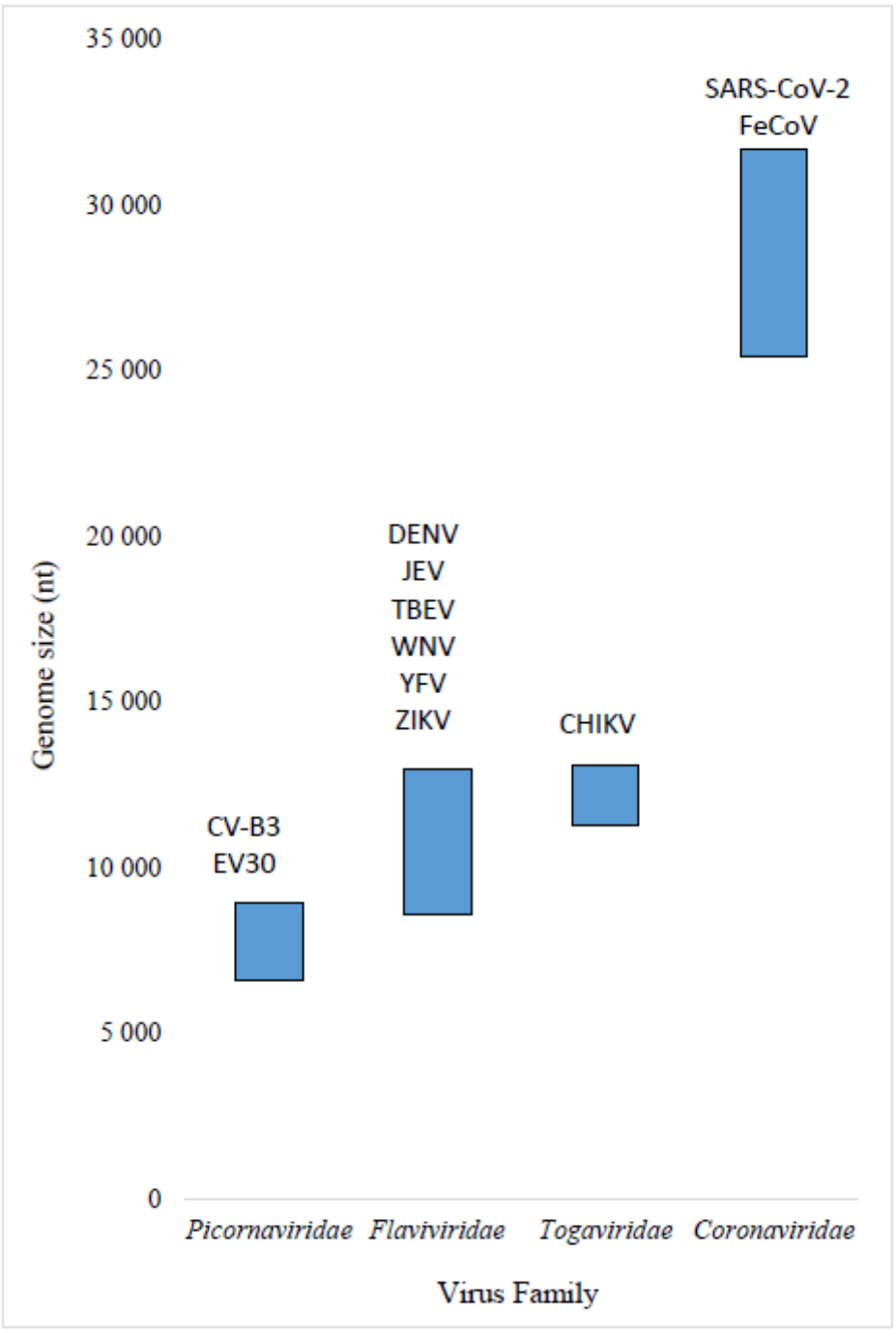

Figure 3

Viruses obtained using the ISA method. Single-stranded (+) RNA viruses from Picornarviridae, Flaviviridae, Togaviridae and Coronaviridae families rescue using the ISA method with their corresponding genome sizes were represented. SARS-CoV-2 and FeCoV were rescued in this study. Blue bars indicates the genome size in nucleotides (nt) (determined using the National Center for Biotechnology Information (NCBI)). CV-B3: Coxsackievirus B3; EV30: Echovirus 30; DENV: Dengue virus; JEV: Japanese encephalitis virus; TBEV: Tick-borne encephalitis virus; WNV: West Nile virus; YFV: Yellow fever virus; ZIKV: Zika virus; CHIKV: Chikungunya virus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; FeCoV: Feline enteric coronavirus

Supplementary Files

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