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🌐 Amplicon Based Sequencing of a Human Monkeypox Virus Isolate V.2

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Abstract

The study details the genome sequencing of a human monkeypox virus isolate from Central Europe during the 2022 outbreak. The virus was isolated from skin lesions and procedures were conducted under BSL-4 conditions. The viral genome was sequenced using Oxford Nanopore Technologies and assembled by Raven and RagTag.

Attachments

XLSX	SVG	SVG
Table 1. Primers for...	xlsx.svg	xlsx.svg
9KB	2KB	2KB

Guidelines

All procedures with infectious materials were performed under BSL-4 conditions at the National Laboratory of Virology, University of Pécs. The virus was passaged once on CV-1 cells to reach a sufficient amount of infective particles.

Materials

- CV-1 (CCL-70, African green monkey, kidney) cell line,
- Minimum Essential Medium Eagle culture medium (MEM) with 10% fetal bovine serum (FBS),
- tissue culture flasks (CELLSTAR®; Greiner Bio-One GmbH, Frickenhausen, Germany),
- 1.5 mL Eppendorf Tubes® (Thermo Fisher Scientific, Inc.),
- 1 X PBS (Thermo Fisher Scientific),
- Direct-zol™ RNA Miniprep kit (Zymo Research),
- repliQa HiFi ToughMix® (Quantabio),
- SPRI beads (AMPure XP, Beckman-Coulter),
- Qubit 4 Fluorometer (Thermo Fisher Scientific),
- Ligation Sequencing Kit (SQK-LSK110, Oxford Nanopore Technologies),
- NEBNext Ultra II End Repair/dA-Tailing and Ligation Modules (New England Biolabs),
- R9.4.1 MinION Flow Cell (FLO-MIN106, ONT)

Troubleshooting

Safety warnings

> 🔴 All procedures with infectious materials have to be performed under BSL-4 conditions.

Ethics statement

The isolate was collected during routine diagnostics by the Czech Republic's national health authority (National Institute of Public Health, NIPH) without additional burden to the patient, anonymized, and archived at the NIPH. No personal data were used, in accordance with NIPH guidelines and the Declaration of Helsinki.

Cell line

1   Vero cell line (African green monkey kidney epithelial) was used which was obtained from American Type Culture Collection (ATCC CCL-81 Passage P3). For the experiment, 75 cm^2 tissue culture flasks (CELLSTAR®; Greiner Bio-One GmbH, Frickenhausen, Germany) were plated with 2×10^5 cells in Minimum Essential Medium Eagle culture medium (MEM) with 10% fetal bovine serum (FBS). The Vero cells were cultivated until ~80% (~1.2×10^6) confluency at 🌡 37 °C in a humidified 5% CO_2 atmosphere. Before the infection, the monolayer was washed with 1 X PBS (Thermo Fisher Scientific, Waltham, MA, USA).

Collection of the virus

2   The monkeypox virus (MPXV) was isolated from skin lesions of a patient in May, 2022 by Dr. Helena Jiřincová (Head of the Reference Laboratory at the National Institute of Public Health-NIPH, Prague, Czech Republic) and was deposited in the authorized collection of the NIPH's under the MPXV_NRL 4279/2022 identification number for diagnostic medical examination purposes in accordance with all applicable compliance of the locality. The isolate was kindly provided by Professor Daniel Růžek (Head of the Laboratory of Arbovirology, Biology Centre, České Budějovice, Czech Republic). The viral stock was used for further analysis in non-human cell-culture.

Propagation of the virus

3   All procedures with infectious materials were performed under BSL-4 conditions at the National Laboratory of Virology, University of Pécs. The virus was passaged once on Vero cells to reach a sufficient amount of infective particles. The same batch of working stock was used during the experiment. The viral titer of the working stock was determined with plaque assay on Vero cells. Non-infected control cultures were inoculated with MEM and treated the same way as the infected ones. For the infection, 2 ml MPXV with 5 plaque-forming units (pfu)/cell (MOI = 5) was used, which was diluted with MEM to reach the sufficient concentration. Cells were incubated with MPXV inoculum at 🌡 37 °C for 1 hour while were shaken gently every ten minutes.

The virus inoculum was removed, then the cell monolayer was washed once with 1 x PBS. For the flasks, 10 mL MEM medium was added which was supplemented with 2% FBS, 2 mM of L-glutamine, and 1% penicillin and streptomycin solution. The cells were incubated at 37 °C for 1, 2, 4, 6, 12, and 24 hours in a humidified 5% CO_2 atmosphere. After the incubation, the supernatant was removed, and the cells were washed with PBS. The dry flasks were stored at −80 °C until further processes. The

cells were washed and scraped down into lysis buffer and transferred to 1.5 mL Eppendorf Tubes® (Thermo Fisher Scientific, Inc.).

Nucleic Acid Isolation and Amplification

4   Viral DNA was extracted from infected cell cultures using Direct-zol™ RNA Miniprep kit (Zymo Research) according to the manufacturer's protocol except the DNase I treatment. Multiplex PCR primers were designed using PrimalScheme (Quick J et al. 2017) based on a MAFFT-aligned reference genome. Twenty-two primer pairs were designed (Table 1.) to generate ~10 kb long amplicons with 50-100bp overlappings, organized into two pools for PCR. Primer pools (10 µM) were used in reactions with repliQa HiFi ToughMix® (Quantabio). PCR was run for 45 cycles (98°C for 10 sec, 68°C for 100 sec).

4.1   Multiplex PCR reaction. Set up two reactions for the two primer pools

Component	Volume
repliQa HiFi ToughMix (2X)	12.5 µL
Primer pool 1/2 (10 µM)	2.5 µL
Nuclease free water	7.5 µL
Template	2.5 µL
Total reaction	25 µL

4.2   Set up the following conditions in a thermal cycler

Step	Temperature	Time	Cycles
Denaturation	🌡 98 °C	🕑 00:00:10	25-45
Annealing and elongation	🌡 68 °C	🕑 00:01:40	25-45
Final hold	🌡 4 °C	∞	

5   After PCR amplification, amplicons were cleaned using SPRI beads (AMPure XP, Beckman-Coulter) at a 1:0.4 sample-to-beads ratio, washed twice with 80% ethanol, and eluted in 50 µL nuclease-free water. DNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific), yielding 326 ng/µL and 276 ng/µL for the two pools.

5.1   SPRI bead clean-up

Add SPRI beads to the PCR products in a 0.4:1 sample-to beads ratio in a LoBind Eppendorf tube.

Incubate the reaction for 5 minutes at room temperature.

Place the tube onto a magnetic rack, and let the beads collect for 2 minutes (or until they are completely cleared).

Discrad the fluid (without disturbung the beads) and add 250 µL 80% Ethanol to the tube.

Mix the beads with the Ethanol, then let the beads clear.

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Mix the beads with the Ethanol, then let the beads clear.

Discard the fluid then let the beads air-dry for few minutes until the bead pellet loses it's shine.

Elute the beads into 50 µL nuclease free water, incubate at room temperature for 5 minutes without the magnetic rack.

Place the tube back to the magnetic rack and let the beads collect for 2 minute (or until they are completely cleared).

Pipette the elute carefully out of the tube without disturbung the beads, into a clean LoBind Eppendorf tube.

Measure the concentration of the elute and take forward 200 fmol into the End-Prep reaction

Library preparation and Sequencing

6   Libraries were prepared using the Ligation Sequencing Kit (SQK-LSK110, Oxford Nanopore Technologies, ONT) with barcoding from the EXP-NBD196 kit (ONT). DNA (~200 fmol) was processed with NEBNext Ultra II End Repair/dA-Tailing and Ligation Modules (New England Biolabs, NEB). SPRI bead cleanups were performed at each step, replacing ethanol with Small Fragment Buffer (SFB, ONT) after motor protein ligation. The final library (4.6 ng/µL) was eluted in 15 µL Elution Buffer (EB, ONT) and loaded onto an R9.4.1 MinION Flow Cell (FLO-MIN106, ONT). Sequencing ran for 34 hours on a MinION Mk1b device.

6.1   In a PCR tube set up the following reaction for the End-Prep step

Component	Volume
200 fmol PCR product	12.5 µL
Ultra II End Prep Reaction Buffer	1.75 µL
Ultra II End Prep Enzyme Mix	0.75 µL
Total reaction	15 µL

Incubate at room temperature for 10 minutes
Incubate at 65 °C f for 10 minutes
Incubate on ice for 1 minute

6.2 In a PCR tube set up the following reaction for the Barcoding step

Component	Volume
Whole End-Prep reaction	15 µL
EXP-NBD196 barcode	2.5 µL
Ultra II Ligation Master Mix	10 µL
Ligation Enhancer	0.3 µL
Total reaction	27.8 µL

Incubate at room temperature for 20 minutes
Incubate at 65 °C for 10 minutes in a thermal cycler
Incubate on ice for 1 minute

6.3 SPRI Bead clean-up

Pool the amplicon pools togehter and repeat 5.1 step with reducing the eluation volume to 30 µL.

6.4 In a clean tube set up the following reaction for the Adapter Ligation

Component	Volume
Barcoded amplicon pools	30 µL
Ligation buffer (LNB)	10 µL
Adapter Mix (AMX-F)	5 µL

Quick T4 DNA Ligase	5 µL
Total reaction	50 µL

Incubate at room temperature for 20 minutes.

6.5   SPRI Bead clean-up

Add SPRI beads to the PCR products in a 1:1 sample-to beads ratio in a LoBind Eppendorf tube.

Incubate the reaction for 5 minutes at room temperature.

Place the tube onto a magnetic rack, and let the beads collect for 2 minutes (or until they are completely cleared).

Discrad the fluid (without disturbung the beads) and add 250 µL Long Fragment Buffer (LFB) to the tube.

Mix the beads with the LFB, then let the beads clear.

Discrad the fluid (without disturbung the beads) and add 250 µL LFB to the tube.

Mix the beads with the LFB, then let the beads clear.

Discard the fluid then let the beads air-dry for few minutes..

Elute the beads into 15 µL Elution Buffer (EB), incubate at room temperature for 5 minutes without the magnetic rack.

Place the tube back to the magnetic rack and let the beads collect for 2 minute (or until they are completely cleared).

Pipette the elute carefully out of the tube without disturbung the beads, into a clean LoBind Eppendorf tube.

Measure the concentration of the elute and take forward 50 fmol to the final library.

Make up the final library in 12 µL.

6.6   Final library preparation and Flow Cell loading

Library preparaation

Component	Volume
Sequencing Buffer II (SBII)	37.5 µL
Library Beads II (LBII)	25.5 µL
Final library	12 µL
Total reaction	75 µL

Buffer mix for the flow Cell loading

Component	Volume
Flush Buffer (FB)	whole tube
Flush Tether (FLT)	30 µL

Vortex the buffer mix thoroughly.

Load 800 µL from the buffer mix onto the Flow Cell through the Priming Port carefully, without introducing any air bubbles into the Flow Cell.

Let the Flow Cell equilibrate at room temperature for 5 minutes.

Load 200 µL from the buffer mix through the Priming Port but with the Spot-On port open. Load in a manner that some fuild comes up in the Spot-On port, but carefully without introducing any air into the Flow Cell.

Load the 75 µL final library onto the Flow Cell through the Spot-On port by dripping the liquid into the port (carefully, without touching the port).

Start the sequencing with the appropriate kit selected.

Assembly

7   Raw FAST5 files were basecalled using Guppy (v6.5.7) [https://nanoporetech.com/document/Guppy-protocol#guppy-basecall-server] with the super-accurate model (dna_r9.4.1_450bps_sup.cfg). Barcodes were trimmed using Guppy barcoder (v6.5.7). Read quality was assessed with NanoPlot v1.41.0 [https://github.com/wdecoster/NanoPlot], and filtering was done with NanoFilt v2.8.0 [https://github.com/wdecoster/nanofilt] , removing reads <5000 bp or with Q-score <11. The average amplicon length was 9034 bp with a standard deviation of 1460. D*e*

novo assembly was performed using Raven 1.8.3 [https://github.com/lbcb-sci/raven] (Vaser, R., Šikić, 2021), without a reference genome, achieving approximately 99.2% coverage. The draft assembly consisted of two contigs, including an internal gap and incomplete terminal regions. These contigs were subsequently merged into a single sequence using RagTag v2.1.0 [https://github.com/malonge/RagTag] (Alonge et al., 2022).

8   Complete termini could not be generated using amplicon sequencing alone. Therefore, additional direct RNA sequencing reads (Kakuk et al., 2023) were used to reconstruct the genome ends. Reads aligning to the terminal regions were extracted and used to build consensus sequences using Samtools [https://github.com/samtools] with the following parameters:

```
samtools consensus -f fasta -o  -m simple -Z 10000 --min-MQ 0 --
show-del yes --show-ins yes --mark-ins -@ 12 -c 0.5
```

The resulting consensus matched the terminal sequences of the closely related, high-quality OXO44336.2 genome (Monzon, Varona, Negredo et al., 2024).

9   The final consensus genome was aligned to NC_063383 and ON563414.3 reference genomes for comparative verification. Gene annotation was performed with Liftoff v1.6.3 [https://github.com/agshumate/Liftoff] (Shumate & Salzberg, 2021), generating a GFF annotation file. The GFF file was curated manually and the Genome Annotation Generator [https://genomeannotation.github.io/GAG/] (Scott et al. 2018) was used to transform it for GenBank submission.
Nextclade v3.10.2 [https://github.com/nextstrain/nextclade] (Aksamentov et al. 2021) analysis using the **nextstrain/mpox/all-clades** database (https://clades.nextstrain.org) representing 1000 full genome MPOX sequences from the GenBank and the default settings were used for phylogenomics analysis to determine the clade and lineage. BLASTn algorithm was used to analyze the closest relatives of the MPXV_NRL_4279/2022 isolate.

10 All analyses were performed on Ubuntu 22.04 LTS. Default software parameters were used unless otherwise specified. Comparative genomic analysis was done using Nextclade [https://github.com/nextstrain/nextclade] (Aksamentov et al. 2021) and Geneious Prime [**https://www.geneious.com/**]. Repeat search was conducted by Tandem Repeat Finder v4.09.1 [https://github.com/Benson-Genomics-Lab/TRF] (Benson, 1999).

Data availability

11 The sequence has been deposited in the NCBI Viral Genomes database under the GenBank accession number PV424067 and is publicly available at [https://www.ncbi.nlm.nih.gov/nuccore/PV424067.1]. Raw sequencing reads are available under BioProject ID PRJNA1241293 and have been deposited in the

Sequence Read Archive (SRA) under accession number SRP572719, both accessible via [**https://www.ncbi.nlm.nih.gov/bioproject/1241293**].

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