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# Review of virological methods for laboratory diagnosis and characterization of monkeypox virus (MPXV): lessons learned from the 2022 Mpox outbreak

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**Abstract**

Monkeypox virus (MPXV), originally endemic in West Africa (Clade II) and Central Africa (Clade I), has recently emerged worldwide and has reinforced the need for rapid and accurate MPXV diagnostics. This review presents and critically discusses the range of virological methods for laboratory diagnosis and characterization of MPXV as well as related lessons learned and practical experience gained from the 2022 Mpox global outbreak. Real-time PCR is currently considered the diagnostic gold standard and ensures accurate and timely confirmation of suspected Mpox cases based on suspicious skin lesions, and digital PCR improves the precision of MPXV DNA quantification. Whole genome sequencing reveals the diversity within the Clade IIb outbreak and highlights the role of microevolution in the adaptation of the virus to the human host. Continuous genomic surveillance is important for better understanding of human-to-human transmission and prevention of the emergence of variola virus–like strains. Traditional virological methods such as electron microscopy and virus isolation remain essential for comprehensive virus characterization, particularly in the context of vaccine and antiviral drug development. Despite the current challenges, serological tests detecting a range of anti-MPXV antibodies are important adjunct diagnostic and research tools for confirmation of late-presenting or asymptomatic MPXV cases, contact tracing, epidemiological studies, seroepidemiological surveys, and better understanding of the role of IgG and neutralizing antibodies in the immune response to infection and vaccination. A multidisciplinary approach combining advanced molecular techniques with traditional virological methods is important for rapid and reliable diagnosis, surveillance, and control of the outbreak.

**Keywords:** monkeypox virus, MPXV diagnostics, whole genome sequencing, isolation of MPXV, electron microscopy, MPXV serology

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## Introduction

Monkeypox virus (MPXV) belongs to the genus of Orthopoxviruses (OPV) within the family *Poxviridae* and is the etiological agent of the human disease known as Mpox. In the past, MPXV was endemic in certain regions in West and Central Africa (1). However, sporadic outbreaks in non-endemic countries such as the United States in 2003, the United Kingdom in 2018, Israel in 2018, and Singapore in 2019 have been linked to recent travel to endemic areas or contact with infected animals (2–5). The current Mpox outbreak, characterized by high human-to-human transmission, has spread in several non-endemic countries in Europe and North America since May 2022 (6). The first cases were detected in the United Kingdom, prompting the World Health Organization (WHO) to declare it a Public Health Emergency of International Concern (PHEIC) in July 2023 due to the rapid increase of cases worldwide (7). As of December 14th, 2023, Mpox cases have been reported in 116 countries, with 91,788 confirmed cases and 167 deaths (8).

The MPXV genome is a double-stranded DNA molecule of about 197 kb. Sequences are divided into two main clades in endemic regions: Clade I (formerly the Central African clade), which includes strains from the Democratic Republic of the Congo, and Clade II (formerly the West African clade) (9). Clade II is further divided into two subclades: Subclade IIa, which includes West African strains, and Subclade IIb, which contains only genomes associated with the 2022 outbreak. Genomic surveillance conducted throughout the Mpox outbreak indicates that the MPXV sequences from 2022 form a cohesive group but are phylogenetically distinct from the Nigerian strain, leading to their placement in the separate Subclade B.1 (10, 11). To date, several thousand MPXV genome sequences have been deposited in public sequence repositories, and they have formed several B.1 lineages with unique mutational and evolutionary characteristics (11, 12).

The significant increase in Mpox cases in non-endemic countries recorded during the 2022 outbreak, the newly identified transmission route, and constant concerns related to potential resurgence of smallpox (the only officially eradicated human disease) have reinforced the need for rapid and accurate MPXV diagnostics. This review presents and critically discusses the range of virological methods for laboratory diagnosis and characterization of MPXV as well as related lessons learned from the 2022 Mpox outbreak. In addition to the standard-of-care molecular methods and some innovative molecular approaches (such as digital PCR), the practical experience gained in 2022/2023 with virus isolation in cell culture, detection of MPXV by electron microscopy, and monitoring of the serologic response after MPXV infection and smallpox vaccination are summarized.

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## Molecular detection of MPXV and MPOX case confirmation

### Sample collection for MPXV detection

The selection of appropriate samples is crucial for reliable diagnosis of Mpox. Swabs from lesion exudates, roofs, and crusts or tissue biopsies are most recommended for MPXV detection because testing from such samples has the highest clinical sensitivity (from 91% to 100%) (13, 14). It is advisable to collect several skin/mucosal lesions with the same/similar morphology from different body areas in a single tube. Blood samples are not recommended for diagnostic purposes because they may lead to false negative results due to the brief viremia that usually occurs before the appearance of characteristic skin lesions. For research purposes, additional sample types such as saliva, rectal swabs, nasopharyngeal swabs, semen, urine, fecal samples, and vitreous or cerebrospinal fluid samples may provide insight into viral shedding and the role of body fluids in transmission, especially in close contact; for example, sexual activity (13, 15–22).

As previously reported, all suspected clinical cases of Mpox in Slovenia were tested in the laboratory for the diagnosis of zoonoses at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. Suspected cases were those with clinical symptoms and an epidemiological link to exposure to MPXV. Swabs of skin lesions or biopsy samples were collected in commercially available universal transport media (UTM) for MPXV DNA testing. A total of 144 different clinical samples from 129 suspected cases were molecularly tested by real-time polymerase chain reaction (PCR), and Mpox was confirmed in 49 patients (23).

### MPXV molecular detection using real-time PCR

Confirmation of an Mpox case involves detection of MPXV DNA by either real-time PCR and/or sequencing. Laboratory protocols usually involve two steps: in the first step, OPV is detected, although the species cannot be identified. This is followed by the second step, in which MPXV is specifically detected by real-time PCR or sequencing. Alternatively, some laboratory protocols are based on the initial generic detection of MPXV by real-time PCR followed by specific real-time PCR to differentiate between MPXV clades. Although final Mpox case confirmation usually relies on real-time PCR due to its high accuracy and rapid turnaround time, whole genome sequencing provides important additional comprehensive information on the molecular characteristics and origin of the virus (13, 24–26).

Real-time PCR is considered the "gold standard" laboratory method for virological confirmation of MPXV infection (13, 27). Several validated real-time PCR protocols have been developed for the detection of OPV and specifically MPXV, some of which differentiate between Clade I and Clade II. Various primer and probe sequences for OPV and MPXV detection have been published, allowing suitably equipped and experienced laboratories to develop their own in-house assays (18, 19, 28–32). Since the last emergence of MPXV, several commercial real-time PCR test kits with high sensitivity and specificity are available on the market for the detection of OPV or specifically MPXV. The tests have been validated using well-characterized clinical samples to ensure the quality and robustness of the tests (16, 33–39).

Frequently used targets for MPXV molecular diagnostics are the genes *D14L* (28), *G2R* (28), *B7R* (40), *F3L* (18, 34, 41, 42), *B6R* (31), and *N3R* (41), and the tumor necrosis factor (TNF) receptor gene (43, 44). Most assays have a detection limit of 10 to 250 copies per reaction (27). However, a single nucleotide polymorphism (SNP) observed in 2022 MPXV sequences has significantly reduced the sensitivity of some PCR assays. The SNP was located at the binding site of the reverse primer and caused an approximate increase in cycle threshold (Ct) value by 6.8 or 100-fold loss in sensitivity (45).

The first Mpox case in Slovenia was confirmed on May 23rd, 2022, following the algorithm shown in Figure 1. First, OPV was detected using a previously described real-time PCR protocol (29). In the next step, confirmation of MPXV, particularly the genetic

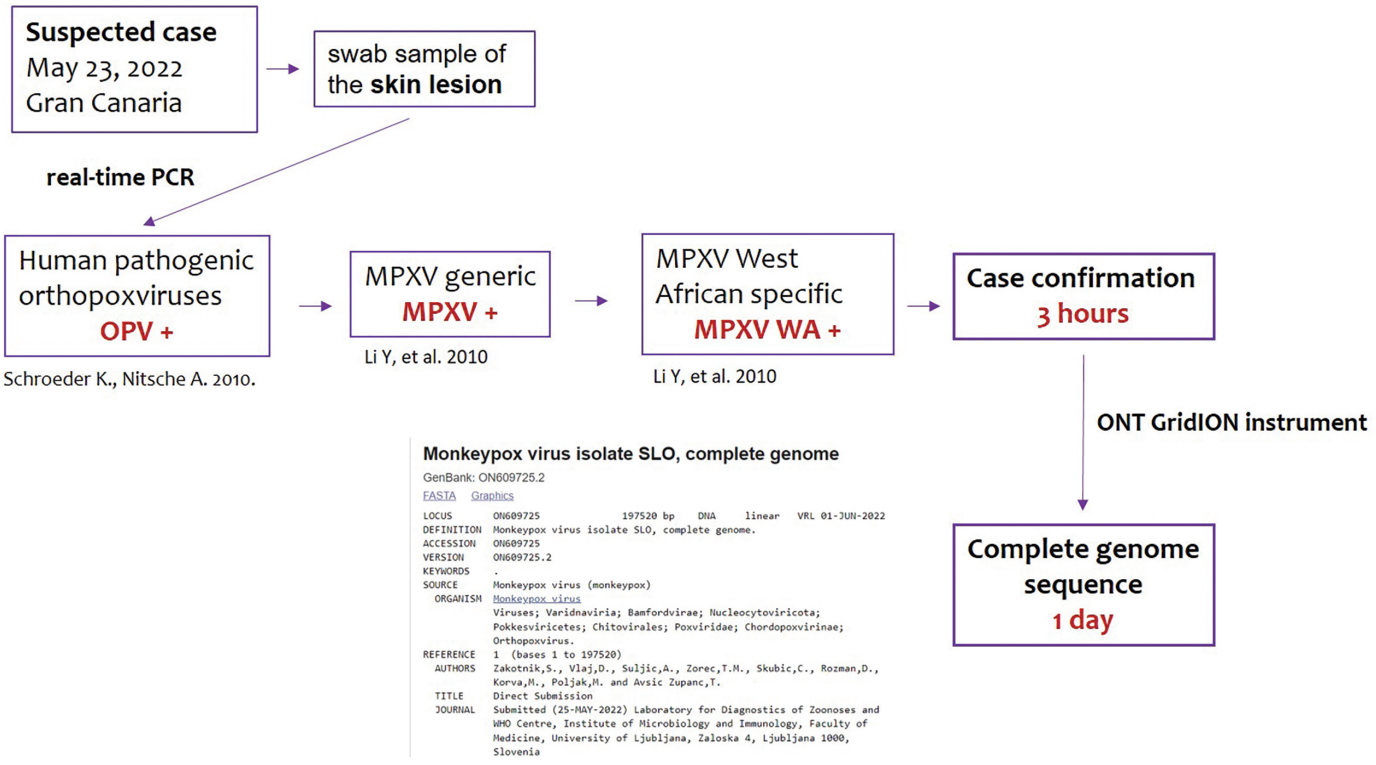


Figure 1 | Laboratory algorithm used for confirmation of the first Mpox case in Slovenia.

lineage of Clade II (former West Africa), was performed using established generic and West Africa–specific RT-PCR assays (28). The suspected Mpox case was finally confirmed within 3 hours of receipt of the sample at the laboratory. Subsequently, within 24 hours of confirmation, the complete genome sequence was generated using the ONT GridIon instrument and submitted to the National Center for Biotechnology Information (NCBI). Our diagnostic approach is in line with routine practice in the great majority of other European countries (17).

In order to establish a specific, rapid, and cost-effective molecular diagnostic approach for the detection of MPXV in clinical samples, we tested seven PCR assays using lesion swabs obtained from nine Slovenian Mpox patients. In addition to the three in-house RT-PCR protocols already mentioned (28, 29), four commercial kits were evaluated for confirmation of OPV and/or MPXV: the LightMix Modular Assay targeting OPV and MPXV-specific LightMix Modular Assay (Roche, TIB MolBiol, Berlin, Germany), the RealStar OPV PCR kit (Altona Diagnostics, Hamburg, Germany), the and MPXV Real Time PCR Kit (Bioperfectus, Shanghai, China).

The LightMix Modular Assay targets a 113 bp fragment of the 14 kDa gene specific for OPVs, and the MPXV-specific LightMix Modular Assay amplifies a 106 bp fragment of the J2L/J2R gene. Both assays have a limit of detection (LOD) of < 10 copies per reaction. RT-PCR reactions were performed according to the manufacturer's instructions using the TaqMan Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Waltham, MA, USA).

The RealStar OPV PCR kit uses technology for the simultaneous detection and differentiation of non-variola OPV species (including MPXV) and variola virus (VARV)–specific DNA. Details of LOD and gene detection region(s) are not available. The testing was performed following the manufacturer's instructions.

In the MPXV Real Time PCR Kit, the specific primers and probes target the *F3L* gene regions of MPXV. The kit also uses amplification of the human housekeeping gene (RNase P) to control for sample adequacy and efficiency of nucleic acid extraction. The assay's LOD is five MPXV copies per reaction. The assay was performed according to the manufacturer's instructions.

All seven assays listed above were performed using the QuantStudio 7 Pro Real-Time PCR System (ThermoFisher Scientific). The assays were compared on the basis of the mean Ct value, which showed clear differences when comparatively evaluated on the same set of samples. The in-house OPV/parapoxvirus (PPV) multiplex real-time PCR assay (29) yielded the highest mean Ct value (24.3), significantly higher than the commercial RealStar OPV PCR kit (Ct 15.7; $p = 0.005$; one-way ANOVA with Tukey's multiple comparison test) and the commercial OPV LightMix Modular assay (Ct 17.0; $p = 0.019$; one-way ANOVA with Tukey's multiple comparison test; Fig. 2). For the assays specifically targeting MPXV, no statistically significant performance differences were observed, with similar mean Ct values obtained for the different methods: Ct 19.5 with the in-house MPXV generic test (28), Ct 20.0 with the in-house MPXV WA–specific test (28), Ct 20.9 with the MPXV-specific LightMix Modular Assay, and Ct 19.8 with the MPXV Real Time PCR Kit. During the 2022 outbreak, a combination of the in-house MPXV generic and the MPXV WA—specific assays (28) was used in Slovenia for detection and final confirmation of Mpox cases. In addition, commercially available OPV and MPXV LightMix Modular assays were used when available. All 49 Mpox cases in Slovenia were confirmed based on positivity of at least two different real-time PCR tests listed above.

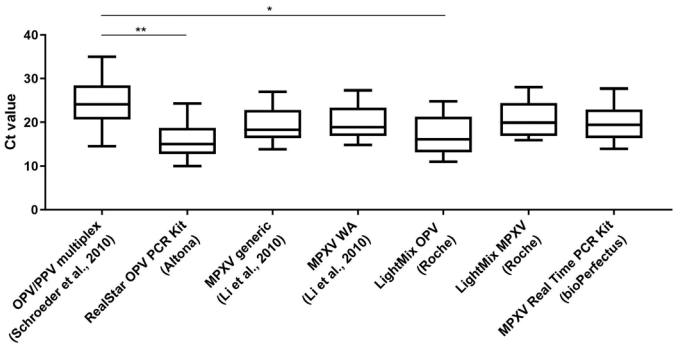


Figure 2 | Comparison of seven different PCR assays/protocols (three in-house and four commercial) for the detection of OPV or specific MPXV. The mean Ct value of each assay was compared with the mean Ct value of each other assay. One-way ANOVA with Tukey's multiple comparison test was used to test for significant differences between the assays. Statistically significant differences are indicated by an asterisk; $p < 0.019$ (*) and $< 0.005$ (**).

## Digital PCR quantification

Digital PCR technology (dPCR) offers advantages for precise quantification and better comparability between different laboratories and platforms. In contrast to conventional quantitative PCR (qPCR), dPCR allows absolute quantification of DNA copies in samples without relying on standard curves. The microfluidic design generates numerous reaction partitions that function like individual reactions. Using the Poisson distribution of positive or negative fractions, dPCR determines the absolute target concentration in copies per microliter (46–48).

During the Mpox 2022 outbreak, dPCR was used for the absolute quantification of MPXV in clinical samples (46), to analyze the kinetics of viral DNA in body fluids during the acute phase of Mpox (49), and to quantify virus growth *in vitro* (50). A recent study demonstrated a strong correlation between MPXV DNA levels measured by dPCR and Ct levels determined by qPCR (51). However, further studies are needed to determine the utility of dPCR for absolute MPXV quantification in clinical samples (46).

We successfully quantified MPXV in clinical samples by preparing a MPXV standard by dPCR according to the following protocol. Nanoplate-based microfluidic dPCR was performed using the QIAcuity Probe PCR Kit (Qiagen, Hilden, Germany) in a 40 µl reaction mixture containing a 5 µl sample of DNA, 10 µl of 4× Probe PCR Master Mix, 0.9 µM of primers G2R-G_F and G2R-G_R, 0.25 µM of probe G2R-G_P (28), 0.025 U/µl of restriction enzyme Anza 52 PvuII (Thermo Scientific), and nuclease-free water. Five microliters of nuclease-free water were added in place of a DNA template as a non-template control. The reaction mixture was prepared in a pre-plate, transferred to the QIAcuity Nanoplate 26k 24-well plate (Qiagen), sealed with the QIAcuity Nanoplate Seal (Qiagen), and then loaded into the QIAcuity Four automated digital PCR instrument (Qiagen) as specified by the manufacturer. The workflow included 1) a priming and rolling step with partitioning of the wells, 2) a thermocycling step under the following conditions: initial DNA denaturation and enzyme activation for 2 minutes at 95 °C, followed by 45 amplification cycles of 15 s at 95 °C and 30 s at 58 °C, and 3) an imaging step with image acquisition of the wells on the green channel (exposure time: 500 ms, gain: 6 dB). The data were analyzed using QIAcuity Software Suite v2.2.0.26 (Qiagen). To improve the accuracy of the concentration calculation in each well, Volume Precision Factor v4.0 (Qiagen) was applied according to the manufacturer's instructions. The MPXV-positive sam-

ple was tested in three consecutive 10-fold dilutions, the measurements of which were then used to determine the MPXV DNA concentration in the original sample. Based on such analysis and calculation of the average value of the measurements, the MPXV DNA concentration in the MPXV2.8 sample was determined to be $2.14 \times 10^5$ copies/µl (Fig. 3 and Table 1).

Using the established MPXV standard, we generated standard curves for both the generic MPXV and the MPXV WA–specific in-house assays (28) (Figs. 4A and 4B). The standard curve for the generic MPXV assay was calculated by five 10-fold standard dilutions ranging from $8.39 \times 10^5$ to 8.39 copies/µl ($R^2 = 0.998$; efficiency 85.366%), represented by the equation $y = -1.62\ln(x) + 38.932$. In addition, the standard curve for the MPXV WA–specific assay was derived from five 10-fold standard dilutions ranging from $7.34 \times 10^5$ to 7.34 copies/µl ($R^2 = 0.995$; efficiency 88.614%), with the equation $y = -1.549\ln(x) + 39.359$.

Therefore, we successfully quantified MPXV in clinical samples using both MPXV generic and MPXV WA–specific in-house assays. The results showed no statistically significant difference between the assays ($p = 0.1338$). The median amount of MPXV detected by the generic MPXV assay was $1.12 \times 10^5$ copies/µl (min $1.42 \times 10^2$ and max $5.46 \times 106$ copies/µl), and the median amount detected by the specific WA assay was $2.21 \times 10^5$ copies/µl (min $2.21 \times 10^2$ and max $7.68 \times 10^6$ copies/µl) (Fig. 5).

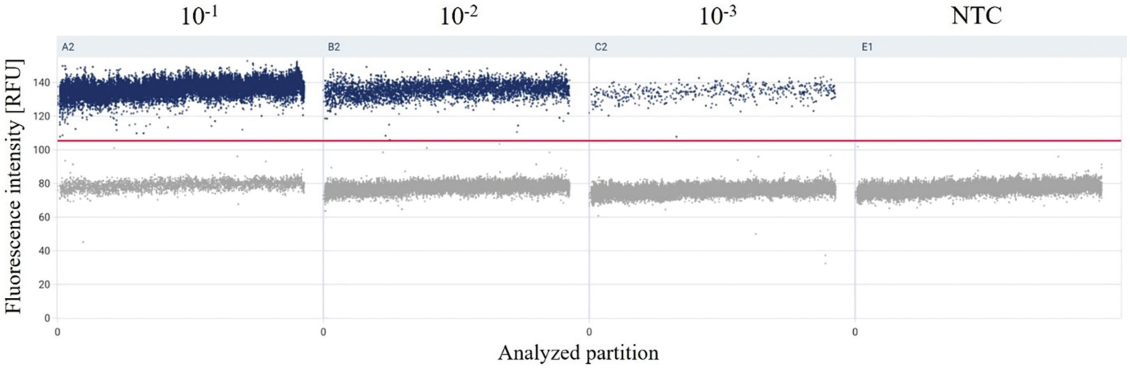


Figure 3 | One-dimensional scatter plots of fluorescence partition intensity when testing three consecutive 10-fold dilutions of MPXV-positive sample MPXV2.8 and the non-template control (NTC) with the digital PCR assay for quantification of MPXV DNA. Blue dots represent positive partitions, gray dots negative partitions, and the red horizontal line the threshold for partition positivity.

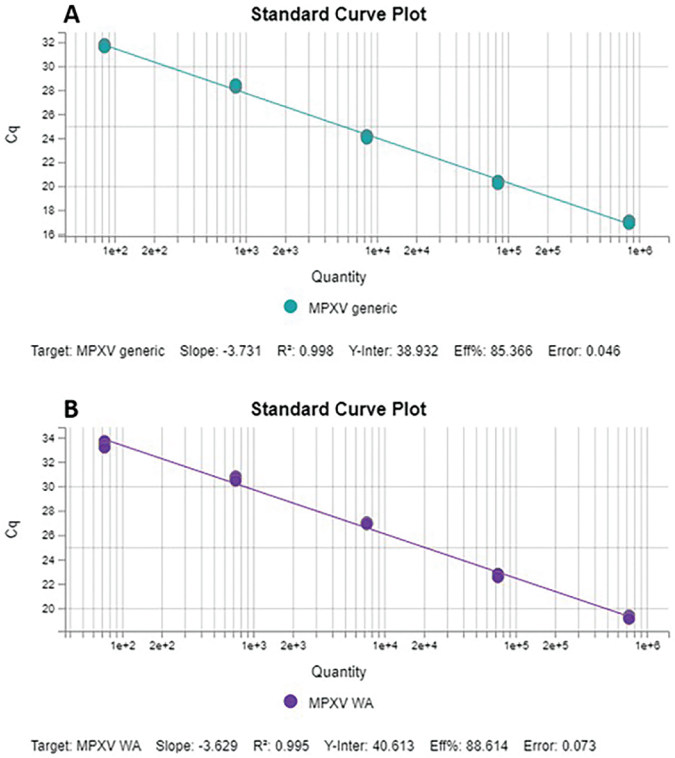


Figure 4 | Plotting of the MPXV standard curve using the MPXV generic PCR in-house real-time assay (A) and the MPXV WA–specific PCR in-house real-time assay (Clade II) (B). Five standard dilutions from $10^5$ to $10^1$ were used to calculate the curve.

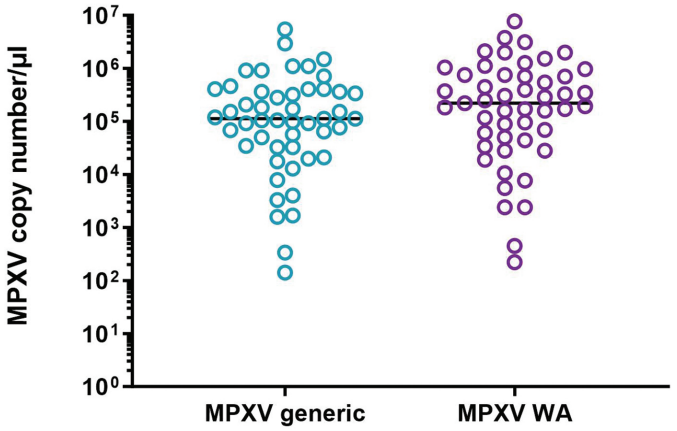


Figure 5 | MPXV copy number / µl in 49 samples from 49 Slovenian Mpox patients measured with the MPXV generic PCR and the MPXV WA (Clade II)–specific in-house real-time PCR assays. The median value is marked in the graph. For statistically significant differences between the groups, the non-parametric Mann–Whitney test was used. No statistically significant difference between the assays ($p = 0.1338$) was obtained.

## Whole genome sequencing

Whole genome sequencing is not commonly used in clinical diagnostics due to the high cost of reagents and infrastructure, the time required, and the need for specialized personnel. However, it is an indispensable tool for tracking the evolution of pathogens and transmission patterns during an outbreak, as the SARS-CoV-2 pandemic has shown (52). Sequencing protocols using metagen-

**Table 1 | Quantification of MPXV DNA in three consecutive 10-fold dilutions of MPXV-positive sample MPXV2.8 using digital PCR.**

| Sample dilution | Dilution factor | CI (95%) | Valid partitions | Positive partitions | λ | MPXV DNA concentration in (copies/µl) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Sample dilution | Original sample |
| MPXV2.8 $10^{-1}$ | 10 | 1.6% | 25,465 | 22,679 | 2.213 | 21,740.80 | $2.17 \times 10^5$ |
| MPXV2.8 $10^{-2}$ | 100 | 2.9% | 25,385 | 4,706 | 0.205 | 2,124.00 | $2.12 \times 10^5$ |
| MPXV2.8 $10^{-3}$ | 1,000 | 8.8% | 25,447 | 500 | 0.020 | 212.16 | $2.12 \times 10^5$ |

omics or amplicon approaches and next-generation sequencing tools such as Illumina or MinION were used to generate MPXV whole genome sequences from clinical samples (12, 53–58). Ongoing genomic surveillance of circulating lineages has proven helpful in supporting public health and government decisions to contain MPXV transmission (27). As of December 14th, 2023, a total of 6,501 MPXV IIb lineage whole genome sequences from 52 countries have been collected in the Gisaid database (https://gisaid.org/hmpxv-variants/; accessed December 14th, 2023). The greatest number of sequences, 3,187 of them, were contributed by the United States, followed by 786 sequences from Germany and 503 from Portugal (https://gisaid.org/hmpxv-variants/; accessed December 14th, 2023). Other platforms such as Nexstrain, mpoxSpectrum, and MPoxVR also provide a global number of uploaded MPXV sequences and genomic data (59).

As outlined previously, all but one Slovenian MPXV detected in clinical samples have been fully sequenced, and the comprehensive protocols for this process and subsequent phylogenetic analyses have been described in detail previously (23). The Slovenian sequences are well positioned in Subclade IIb, where all sequences from the 2022 global outbreak are located. Subclade IIb includes lineages A.1, A.1.1, A.2 (further subdivided into three sublineages), A.3, C.1 (https://nextstrain.org/mpox/clade-IIb; accessed December 14th, 2023), and B.1, which is responsible for the Mpox outbreak in Europe in 2022 and subsequent spread to the Americas, Oceania, and Asia (11). The B.1 lineage has further adapted and diversified during the outbreak into 14 sublineages (https://nextstrain.org/mpox/clade-IIb; accessed December 14, 2023). Although it has more mutations than lineage A relative to the presumed ancestor, the data show greater variability within lineage A than within B.1, with all B.1 isolates being closely related (12, 60).

The mutation rate detected in MPXV strains belonging to the current outbreak exceeds previous estimates for poxviruses at one to two nucleotides/genome/year, likely due to the activity of the human APOBEC cytosine deaminase enzyme and adaptation of the virus to the human host (12, 61). Microevolutionary events within a short period of time, such as SNPs, gene loss or duplication, and recombination, particularly at the MPXV genomic ends during the outbreak, may contribute to virus evolution, adaptation to the human host, and human-to-human transmission (12, 62–67).

According to the Nextstrain classification, MPXV Clade-IIb comprises 24 different lineages and sublineages. In various studies on the MPXV strains derived from the 2022 outbreak in Europe, several unrelated introductions into the continent have been identified, suggesting a chain of transmission within a country (23, 68–71). In the European region, lineages B.1, B.1.7, and B.1.10 were found with significant frequency (69). Five different genetic lineages were identified in Slovenia: B.1, B.1.14, B.1.2, B.1.3, and A.2.1. The divergence of MPXV evolution in the European region was calculated to be about $5.70 \times 10^{-5}$ substitutions per site per year (69). Monitoring changes in the MPXV genome is crucial to prevent the emergence of an epigone of variola virus. However, such monitoring requires high-quality genomic data and meticulous annotation (63).

Although whole genome sequencing and subsequent phylogenetic analysis significantly contributed to viral characterization at the molecular level, electron microscopy and isolation of viable viruses in cell cultures remain essential for complete viral characterization, especially in the context of vaccine and antiviral drug development.

## Electron microscopy

After the invention of transmission electron microscopy (TEM) in the 1930s, vaccinia virus (VACV) was one of the first viruses to be visualized by electron microscopy (EM). Continuous advances in EM have played a critical role in elucidating the morphology, size, ultrastructure, and characterization of the viral replication cycle,

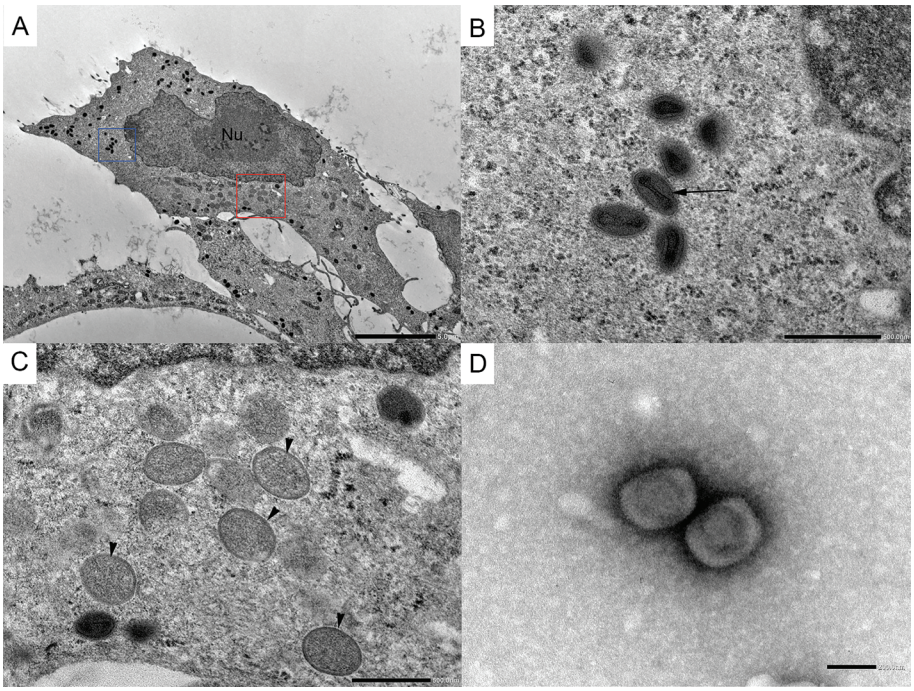


Figure 6 | Panel A shows VERO E6 cells infected with MPXV under a thin-section electron microscope. The cell nucleus is labelled "Nu+" and the scale corresponds to 5 µm. Panel B shows a closer view of the mature virions within the highlighted area in the blue box of panel A. A lateral body within a maturing virus particle is indicated by the arrow, and the scale is set at 500 nm. Panel C enlarges the area within the red box in panel A and provides higher magnification. Here the viral inclusion bodies, also known as "virus factories", consist of spherical, immature virus particles (arrowhead). The scale for panel C is 500 nm. Finally, panel D shows brick-shaped virions that have been negatively stained with 2% phosphotungstic acid at a scale of 200 nm.

including cellular changes induced by MPXV (72–74). In addition to research, EM has also proven to be a valuable diagnostic tool, with the simple negative contrast method being widely used in smallpox diagnostics due to its speed and ability to reliably distinguish between OPV, PPV, and the more common, less severe herpesviruses (72, 75). However, the high cost of purchasing and maintaining EM equipment, combined with the need for very specialized skills of laboratory personnel and the development of highly sensitive, high-throughput molecular assays, have resulted in reduced use of EM for diagnostic purposes (75–78).

Various clinical samples, such as vesicle fluids from skin lesions or solid tissues such as scabs or biopsies, can be examined with EM for the detection of poxviruses. The practical resolution limit of EM for biological material is 2 nm, so that the fine structures of the viral particles are clearly visible. The ultrastructural analysis contributes to a better understanding of the evolution and spread of MPXV (79). OPVs are assembled in the host cytoplasm in "viral factories" and exist in intermediate compartments such as the immature, mature, and extracellular envelope virions. Mature OPVs generally have an ovoid or brick-shaped morphology, ranging from 220 to 450 nm in length and 140 to 260 nm in width, making them among the largest known animal viruses. However, OPVs cannot be distinguished morphologically (75, 80).

After confirmation of the first Mpox case in Slovenia, the morphological characteristics of MPXV were determined and studied by TEM directly on clinical specimens with negative staining and after isolation of the virus in a cell culture by ultra-thin EM (Fig. 6).

#### Negative staining electron microscopy

In negative staining EM, also known as negative contrast, an aqueous solution of heavy metal salts is applied to a sample. When the solution dries in the air on the grid, it forms an electron-tight "glass" around the objects on the grid. Biological materials, which have a lower electron density, appear as lighter structures against the darker background of the stain. The process of negative staining involves three steps: adsorption, washing, and staining (75).

The clinical samples were centrifuged in UTM viral transport medium for 10 minutes at low speed (2,000 *g*) to remove larger particles and cell debris. Formvar-coated grids were exposed to a drop of UTM medium for 5 minutes and then negatively stained with 2% phosphotungstic acid. In addition, the UTM medium was ultracentrifuged (Airfuge; Beckman Coulter, Indianapolis, IN, USA) at high speed (100,000 *g*) to concentrate the viruses directly on the grid, and negative staining was performed using the same procedure. Examination of the grids was performed using a TEM (JEM-1400 Plus; JEOL, Tokyo, Japan) at 120 kV. The OPVs were identified based on their characteristic brick-shaped morphology and a size of approximately 350 × 270 nm (Fig. 6D).

#### Ultrathin section electron microscopy

After successful MPXV isolation in cell culture (see details below), ultrathin sections of MPXV-infected VERO E6 cells were prepared. Two days after inoculation, the cells were fixed with a modified Karnovsky fixative. Fixation was followed by post-fixation in 1% (w/v) osmium tetroxide for 1 h. Samples were afterwards dehydrated in a graded series of ethanol and embedded in Epon (Serva Electrophoresis, Heidelberg, Germany). Ultrathin sections (60–80 nm) were cut with an ultramicrotome from the resin block embedded with samples and contrasted with uranyl acetate and lead citrate using an automatic contrasting system (EM ACE 20; Leica, Wetzlar, Germany). Finally, the grids and stained sections were examined with JEM-1400 Plus TEM at 120 kV.

The EM showed both mature and immature virus particles, indicating virus replication in the cytoplasm of the infected cell. Viral inclusion bodies ("viral factories"), composed of round, spherical, and less dense immature virus particles, were visible inside the cytoplasm next to mature poxvirus particles with a typical oval profile and dumbbell core (Fig. 6A, 6B, 6C). Our EM results are in accordance with other published Mpox cases, which have demonstrated the importance of TEM for study of replication and pathogenesis as well as characterization of this emerging pathogen (4, 42, 74, 81–84).

### Virus isolation

MPXV was originally isolated in cell culture in 1958 from pustules of cynomolgus monkeys showing non-lethal smallpox-like symptoms (85), and it was later detected in infected humans in the Democratic Republic of the Congo in 1970 (86). Initially, viral culture was considered the MPXV diagnostic gold standard and confirmed the Mpox diagnosis in 67% of suspected cases after the eradication of smallpox in the Democratic Republic of the Congo in the 1980s (87). Lately, molecular assays targeting viral DNA have become the most reliable MPXV diagnostic method due to their rapid turnaround time and higher sensitivity.

#### MPXV isolation: practical considerations

Only highly trained personnel should perform *in vitro* testing with viable MPXV using full personal protection in biosafety level 3 (BSL-3) laboratory facilities. Several mammalian cell lines (VERO, VERO E6, VERO 76, LLC-MK2, BSC-40, BSC-1, HeLa, PEK, HEP-2, A549, MRC-5) have been shown to be susceptible to MPXV infection and support its replication (14, 88–90). The ability of poxviruses to infect cells without specific receptors contributes to this broad susceptibility of cell lines (91–93). However, VERO cells are particularly useful because MPXV replicates in these cell lines with a higher titer compared to HEP-2 and PEK cells (86).

Clinical samples with low Ct values when tested with PCR and thus with anticipated high virus concentration (plaque-forming units [PFU]/ml)—for example, from skin lesions—are most appropriate for virus isolation (14). Viable MPXV has been efficiently isolated from a range of clinical specimens, including semen samples collected 6 days after symptom onset and from anal and urethral swabs (89, 94). Successful virus growth in cell culture is most frequently associated with samples with PCR Ct values up to 30 (14, 88, 95, 96). However, the Ct values can vary significantly between different laboratories and different PCR assays (Fig. 2), thus no single Ct value can be used as an eligibility cut-off for viral culture. Extensive cytopathic effects (CPE) usually occur 1 to 4 days after inoculation, with unique CPE patterns observed in specific cells, often with widespread cellular detachment and degeneration (88). Upon passage of the virus on VERO cultures, CPE is observed as infectious foci (96).

During the 2022 Mpox outbreak, samples from six PCR-confirmed Slovenian Mpox patients (swabs of vesicle fluid from four patients and biopsy samples from two patients) were inoculated into the VERO E6 cell line in the BSL-3 laboratory at our facility. The LightMix modular MPXV PCR Ct values ranged from 16.9

to 30.4. For each patient, 500 µl of the samples was inoculated directly into the VERO E6 cell growth medium (ATCC-LGC; CRL-1586) consisting of Dulbecco's modified eagle medium (DMEM; Gibco, Thermo Fisher Scientific) with 5% fetal bovine serum (FBS; Euroclone, Pero, Italy) and antibiotics (penicillin, streptomycin, amphotericin B). The inoculated cells were incubated in a T25 flask (TPP, Trasadingen, Switzerland) at 37 °C and 5% $CO_2$. The inoculated cells were monitored daily for CPE, and an intense poxvirus-specific effect was observed on the 2nd day (Fig. 7). Isolation of viable MPXV was successful in five out of six samples tested, with the exception of a sample with a PCR Ct value of 30.4. In addition, when we intended to sequence the whole MPXV genome from this sample, we were only able to cover 40% of the genome, suggesting that the MPXV DNA was substantially degraded in this sample and the virus was most probably not infectious (23).

Although virus isolation is not recommended or routinely performed in MPXV diagnostics, it is essential for the study of viral pathogenesis and for research on future diagnostic methods, antiviral drugs, and vaccines. Determination of a patient's infectivity is an important adjunct tool in infection control, patient isolation protocols, public health guidelines, and cases of prolonged viral shedding (88). Viral cultures remain essential for the detection of rare and/or emerging viral pathogens, especially in samples with presumptive high viral concentrations (95).

### MPXV inactivation

Ensuring safe laboratory conditions when handling highly pathogenic viruses, such as OPVs, requires appropriate and reliable virus inactivation procedures. Heat treatment is a widely used method due to its ability to denature the secondary structures of viruses. Although literature data on inactivation methods were previously only available for VACV and VARV viruses among the OPVs, recent research by Batejat et al. in 2022 on MPXV recommends a 30-minute treatment at 60 °C for the Clade II and Clade I genetic lineages using viral transport media (VTM) and FBS (97). Further evaluation of heat treatment under different conditions (30 minutes at 65 °C, 15 minutes at 65 °C, or 15 minutes at 95 °C) by Fischer in 2022 revealed no plaque formation after such a procedure, providing viable protocols for MPXV inactivation (98). Of note, heat treatment at 60, 70, or 95 °C only minimally affected the detection of MPXV DNA by qPCR, indicating that inactivated samples are suitable for molecular diagnostics without a significant decrease in Ct values (97).

In addition, evaluations of commonly used reagents in clinical laboratories such as Trizol and commercially available lysis buffer AVL with ethanol were performed, showing inactivation of MPXV after a 10-minute incubation at room temperature (98). A study testing seven commercial diagnostic buffers confirmed inactivation of MPXV at different incubation times, and heat (56 °C and 60 °C) and sodium dodecyl sulfate detergent were also found to be effective, highlighting the importance of matching the protocol to downstream biological processes (99).

As a result of the initial absence of literature information on MPXV inactivation, an in-house viral inactivation protocol was developed at our institution. A 500 µl MPXV sample suspended in cell culture medium was heat inactivated at 60 °C for 1 hour. Subsequently, three passages of the inactivated virus were performed on VERO E6 cells, where no CPE was observed and the Ct values of an MPXV generic assay (28) performed on all passages increased from 15.8 to 32.6 after three passages, supporting the effectiveness of the heat inactivation protocol developed for the subsequent MPXV PCR (Fig. 8).

Prolonged stability of MPXV DNA is crucial for shipping samples between laboratories to be used as positive controls (especially at outbreak onset) and for referral purposes. Room-temperature shipment substantially simplifies transport logistics and reduces associated costs, facilitating the establishment of a global

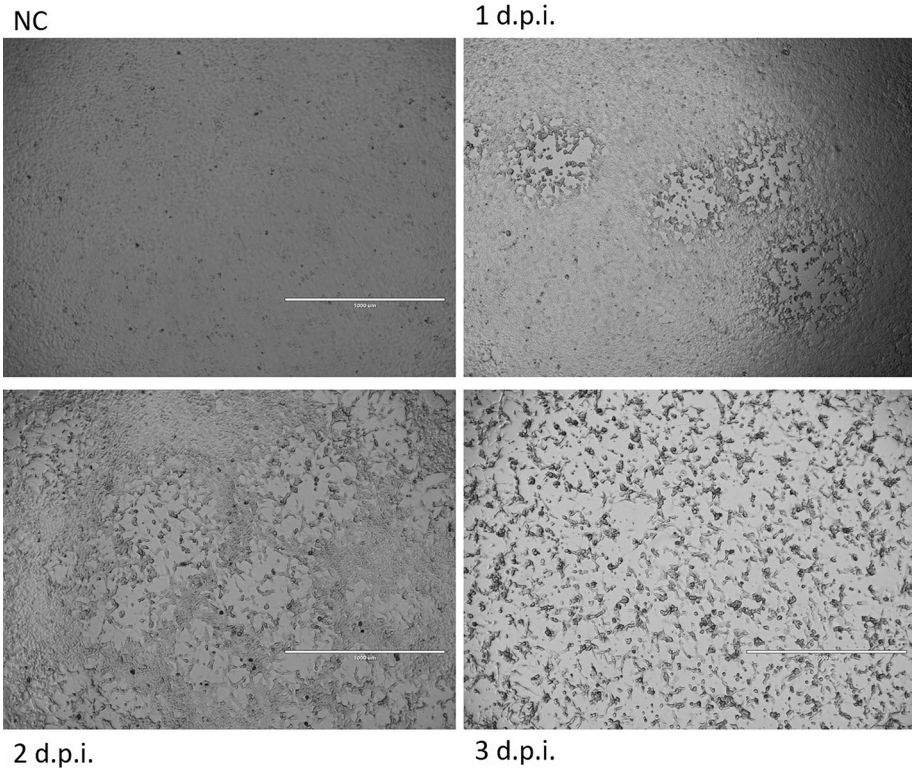


Figure 7 | Isolation of viable MPXV in cell culture. Onset of cytopathic effect on the VERO E6 cell line 1, 2, and 3 days after inoculation (d.p.i.) of a swab sample from an Mpox patient compared to the negative control (NC). The images were acquired with the EVOS FL Imaging System (4×; Life Technologies), and the scale is 1,000 nm.

network of laboratories capable of performing molecular testing. Thus, after virus inactivation, stability of isolated DNA was monitored in our laboratory through 5 days of storage at room temperature by daily aliquot testing using the MPXV generic assay (28). The results showed that the MPXV DNA was stable over 5 days of storage with no significant drop in Ct values observed, even when the DNA aliquot was exposed to temperatures above 30 °C for several hours on day 3. This stability is consistent with the results of other studies (97, 99). In addition, the DNA stability after MPXV inactivation using an autoclave (121 °C, 30 min) was investigated. Despite successful virus inactivation (no CPE on VERO E6 cells were observed), viral DNA was still detectable by MPXV generic assay (28) without a significant decrease in Ct values (isolated virus Ct value: 14.2; autoclaved virus Ct value: 15.0).

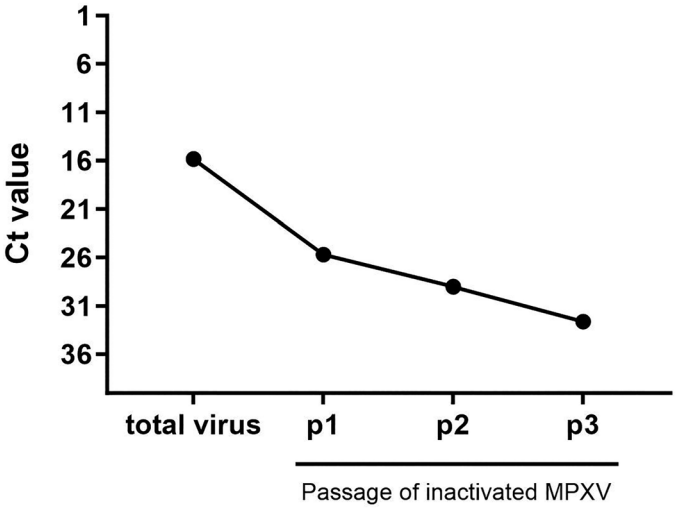


Figure 8 | Ct values measured using MPXV generic PCR after heat inactivation of an MPXV sample suspended in cell culture medium at 60 °C for 1 hour (total virus) and after three passages (p1, p2, and p3) on the VERO E6 cell line.

### Detection and quantification of MPXV viability and infectivity

Although qPCR can detect viral DNA quickly and accurately and provide an estimate of its concentration, it cannot detect and quantify viral viability and infectivity. This limitation is particularly notable in the case of MPXV because it is a very stable DNA virus. Instead, the $TCID_{50}$ assay (median tissue culture infectious dose) and the plaque assay are used to quantify infectious viruses (Fig. 9).

The plaque assay is considered the gold standard for the quantification of replication-capable lytic virions. In this method, the virus is diluted and 10-fold dilutions applied to susceptible cells. After a certain incubation period, the liquid medium is replaced by a solid or semi-solid medium to prevent the virus from spreading, resulting in visible plaques within the cell monolayer. The virus titer, expressed as PFU, is determined by staining and counting the plaques (100).

In contrast, the TCID50 assay determines the point at which 50% of the cultured cells are infected and thus provides an approximate virus titer. In contrast to the plaque assay, in the TCID50 assay the viral inoculum is mixed with a cell medium and inoculated into the cells, which are usually seeded on 96-well plates. The wells are observed and analyzed for the presence or absence of CPE, and the virus dilution at which 50% of the wells show infection is calculated. TCID50 titers are determined using the Reed–Muench or Spearman–Kärber methods (100–102).

**MPXV plaque assay protocol.** During the 2022 Mpox outbreak, a plaque assay was developed in our laboratory to quantify the infectious virus titer of MPXV isolates on cell cultures. Although MPXV forms plaques on VERO E6 cell monolayers, an overlay medium with agarose was used for the assay. VERO E6 cells were seeded overnight in DMEM cell growth medium supplemented with 10% FBS at a concentration of $1 \times 10^5$ cells / 500 µl / well on a 24-well plate. The next day, the cells were washed with 2× Medium 199 (Gibco, Thermo Fisher Scientific), and 200 µl of a 10-fold serial dilution of the virus in 2× Medium 199 was inoculated into the cells in four replicates. Uninfected wells served as negative controls. After 1 hour of incubation at 37 °C and 5% $CO_2$, the inoculum was removed and the wells were covered with 500 µl of overlay medium. The overlay medium consisted of supplemented medium (2× Medium 199 with 6% $NaHCO_3$ and 4% FBS) and 2.5% CMC agarose (Sigma Mo512-100G methylcellulose) at a ratio of 1:1. The plates were then incubated at 37 °C and 5% $CO_2$ for 5 days. The infected cells were fixed with a 4% formaldehyde solution for 30 minutes. The formaldehyde and overlay medium were removed prior to staining. Plaque formation was visualized with a 1% crystal violet solution. PFU per ml were calculated based on the number of plaques multiplied by the dilution factor. MPXV isolated on VERO E6 cells in Slovenia during the 2022 outbreak reached a mean titer of $3 \times 10^7$ PFU/ml (between $6.9 \times 10^4$ and $1.6 \times 10^8$ PFU/ml) after three passages on cells (Fig. 9B).

**MPXV $TCID_{50}$ method protocol.** During the 2022 Mpox outbreak, we also implemented the TCID50 method for MPXV quantification because it is faster and easier to perform than the plaque

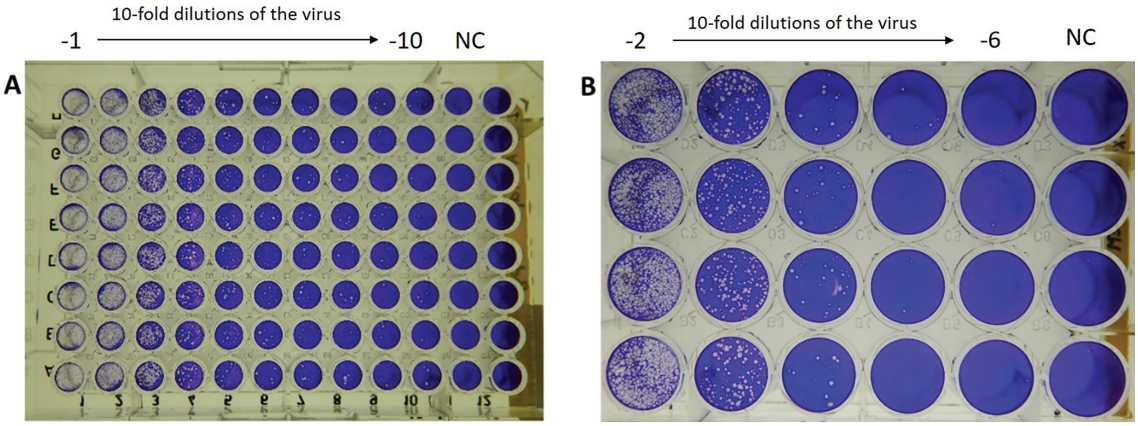


Figure 9 | Quantification of viable MPXV using the $TCID_{50}$ method and plaque assay. In the $TCID_{50}$ method (Panel A), 10-fold dilutions of the virus in DMEM medium from $10^{-1}$ to $10^{-10}$ were inoculated into columns 1 to 10 of 96-well plates. Columns 11 and 12 served as negative controls (NC), where only DMEM medium without virus was added. For the plaque assay (Panel B), 10-fold virus dilutions were inoculated, from $10^{-2}$ (column 1) to $10^{-6}$ dilutions (up to column 5). Column 6 serves as a negative control (NC), in which only virus-free medium is added to the cells. The $TCID_{50}$ and plaque assay plates are stained with crystal violet.

assay. This method closely follows our previously established protocol for SARS-CoV-2 quantification (103). Tenfold dilutions of the virus in DMEM containing 10% FBS were inoculated onto 24-hour-old VERO E6 cells seeded on a 96-well plate at a concentration of $1 \times 10^5$ cells / 100 µl / well in eight replicates. The plates were incubated at 37 °C and 5% $CO_2$ for 3 days. At the end of incubation, the cells were fixed with a 4% formaldehyde solution for 20 minutes and stained with a 1% crystal violet solution. Wells in which CPE were observed in at least 50% of the cells per well were identified as MPXV positive. Virus concentration was calculated using the Spearman–Kärber algorithm (102). Slovenian MPXV 2022 isolates on VERO E6 were quantified with a median of $4 \times 10^8$ $TCID_{50}$/ml (between $1 \times 10^6$ and $2 \times 10^9$ $TCID_{50}$/ml; Fig. 9A).

## Serological assays

Serologic methods measuring immune response against MPXV are not vital for diagnostic confirmation of individual Mpox cases, but they are essential for assessing population seroprevalence and immunity, and for conducting epidemiologic and vaccine-related studies. In the past, the lack of commercial anti-MPXV serologic tests was due to the high cross-reactivity between OPVs as a result of > 90% similarity at the amino acid level between MPXV and VARV (40). Consequently, there are no commercially available serologic tests that could reliably distinguish MPXV from other OPVs such as VACV, VARV, and cowpox virus (CPXV) (104). As a result, such tests may not have been useful in OPV-endemic areas such as Europe (CPXV) (105, 106) and Africa (MPXV) (107, 108). In the traditional endemic regions of MPXV, only limited serological surveillance data are still available (109).

Despite these challenges, various in-house immunoassays—immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA)—have been developed for surveillance, seroprevalence studies, and differentiation between vaccination-induced and naturally acquired immunity (14, 17, 20, 110—112). Following the global Mpox 2022 outbreak, a range of innovative serological methods based on different platforms and technologies were developed, such as the multi-antigen ELISA, which included 27 poxvirus antigens (24 MPXV and three VACV antigens) (111), a chemiluminescence-based serological assay (113), a robust peptide-based IgG ELISA suitable for serosurveys in endemic areas (104), and a multiplex bead-based microsphere immunoassay with high throughput and high sensitivity (93%) for the detection of anti-MPXV IgG antibodies using a combination of MPXV peptides and OPV protein (114, 115).

A study conducted during the 2003 Mpox outbreak in the United States provided important insights into the timing of the immune response against MPXV. An IgM response was detected in serum collected more than 4 days after infection, and IgG response was observed in serum collected more than 14 days after MPXV infection. Of note, previously unvaccinated Mpox cases showed a more rapid and sustained IgM response that lasted up to 18 weeks post-infection, and in individuals previously vaccinated against smallpox the immune response lasted 11 weeks post-infection. Conversely, the IgG immune response was long-lasting in previously vaccinated individuals, up to 21 weeks after rash onset, compared to 19 weeks in previously unvaccinated individuals. This study suggests that MPXV infection induces a strong immune response in the acute phase even in individuals that have been previously vaccinated against smallpox (116). Further serological studies supported these initial results and indicated that IgM antibodies can persist for several weeks (peak 2 to 3 weeks after OPV exposure) and IgG antibodies for several years after infection (14, 117–120).

Recently, particularly after the COVID-19 pandemic, the importance of serological tests for the detection of functional antibodies, especially neutralizing antibodies (N-Ab), has become evident. Vaccination with VACV is thought to provide functional cross-protection against MPXV infection, with polyclonal N-Ab playing a crucial role. However, a limited number of studies have investigated the correlation between N-Ab levels and clinical protection (121–124). Traditionally, the detection of N-Ab against OPV has relied on the plaque reduction neutralization test (PRNT). However, recently accumulated evidence suggests that a microneutralization assay supplemented with an external complement source increases sensitivity of N-Ab detection (125). Studies demonstrated that patients develop a robust immune response producing IgM, IgG, and N-Abs approximately 2 weeks after the onset of symptoms (112). Of note, N-Ab and IgG titers show a significant negative correlation with the MPXV isolation success rate and a positive correlation with time from symptom onset (112). This suggests that patients are effectively controlling the infection and reducing virus shedding (112). In addition, antibodies that neutralize MPXV have also been detected decades after historical smallpox vaccination. However, the exact role of MPXV N-Ab in disease protection and transmissibility is still under investigation (125, 126), especially considering that they are also detected in asymptomatic individuals (127).

### In-house immunofluorescence assay protocol

Although ELISA-based assays have higher sensitivity compared to indirect immunofluorescence assays (IFA), an in-house IFA was initially used in Slovenia to characterize individual Mpox cases (128). After confirmation of the first cases in 2022 and successful isolation of the virus, we soon developed an in-house IFA for determination of anti-OPV antibodies in serum samples. In the BSL-3 facility, VERO E6 cells were cultured for 48 hours before inoculation with MPXV at a concentration of $10^4$ $TCID_{50}$/ml. Five days after inoculation, infected cells were washed twice with saline containing 5% FBS. The cells were removed from the flask with glass beads and the pellet was resuspended in freezing medium consisting of DMEM with 10% FBS and 10% dimethyl sulfoxide (DMSO). The DMSO-treated cells containing MPXV antigen were frozen at –80 °C until further use. To prepare IFT slides, the frozen cells were thawed and centrifuged for 5 minutes at 1,500 rpm and 4 °C. The infected cells were washed twice with PBS and resuspended in saline containing 5% FBS. For IFT slides, infected cells were mixed with uninfected VERO E6 cells in a 1:3 ratio, and 7 µl of the cell suspension per well was added to the 15-well slides. The slides were fixed in ice-cold acetone for 15 minutes and frozen at –30 °C until further use.

For validation of the in-house IFA developed, serum samples were collected from the first three confirmed Mpox Slovenian cases, one sample each per patient: 7, 21, and 40 days after PCR-confirmation, respectively. The smallpox vaccination status of these patients was not available. Serum samples were diluted twofold from 1:16 to 1:1,024 in phosphate-buffered saline (PBS), applied to prepared slides and incubated for 30 minutes at room temperature. After washing, a fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Vircell, Granada, Spain) was used for detection. After incubation at room temperature for 30 minutes,

the slides were washed in water and mounted in 3% glycerol/water. Positive FITC staining was detected in infected cells with a fluorescence microscope at ×20 magnification.

The highest IgG antibody titer (1:512) against MPXV was detected in the serum collected 21 days after PCR confirmation of the disease. IgG antibodies were also found in the serum collected 40 days after PCR confirmation (1:64), whereas the serum collected 7 days after PCR confirmation was anti-MPXV IgG antibody negative (Fig. 10). These results are consistent with recent studies in which OPV-directed IgG antibodies are typically detected 2 to 4 weeks after the onset of symptoms in titers between 1:40 and 1:320 (17, 20, 110).

### Neutralization test protocol

The neutralization test (NT) is considered the serological gold standard in virology. The test detects N-Abs, which are central players in humoral immunity. N-Abs block the entry of viruses into the host cells and neutralize their biological effect. In NT, virus and patient serum are mixed and inoculated into the cells. The N-Abs present in the serum bind to the free virus and prevent the virus from binding to the receptors of the host cell and infecting the cells (129, 130).

During the 2022 Mpox outbreak, the NT was performed at our BSL-3 facility following a previously established procedure with slight modifications (103). In brief, Vero E6 cells were propagated at a density of $10^5$ cells per well in a 96-well plate 1 day before the start of the NT assay. Heat-inactivated (60 °C, 30 min) serum samples from Mpox patients were used in a twofold serial dilution starting at 1:10. The diluted samples (100 µl) were then incubated with an equal volume of MPXV (at a concentration of $10^3$ $TCID_{50}$/ml). After 1 hour of incubation at 37 °C, 50 µl of each plasma dilution–virus mixture was added to the cells in triplicate and incubated at 37 °C and 5% $CO_2$ for 5 days. After fixing the plates with 4% paraformaldehyde for 30 minutes, crystal violet was used to detect CPE. The neutralizing endpoint titer was determined as the highest dilution causing a 90% reduction in CPE in at least two of three parallels. An NT titer ≥ 1:10 was considered positive.

Serum samples from the same patients used for validation of IFT were also tested with NT, and a serum sample from a healthy smallpox-unvaccinated person was included as a negative control. With the NT, relatively low N-Ab titers were detected in two patients, 1:20 and 1:40, in samples collected 21 and 40 days after Mpox confirmation, respectively. Specific N-Abs were absent in the serum sample collected 7 days after Mpox confirmation as well as in the healthy unvaccinated control (Fig. 11). The NT results confirmed the IFA results and showed that anti-MPXV IgG and N-Abs can be detected at least 2 weeks after Mpox confirmation, which is consistent with previously reported results (112).

## Conclusions

In the last 2 years, MPXV has become a public health problem worldwide. At present, real-time PCR is considered the diagnostic gold standard and ensures accurate and timely confirmation of suspected Mpox cases based on suspicious skin lesions, and digital PCR improves the precision of MPXV DNA quantification. Whole genome sequencing reveals the diversity within the Clade IIb outbreak and highlights the role of microevolution in the adaptation of the virus to the human host. Traditional virological methods such as EM and virus isolation remain essential for comprehensive virus characterization. Despite the current challenges, serological tests detecting a range of anti-MPXV antibodies are important adjunct diagnostic and research tools for confirmation of late-presenting or asymptomatic MPXV cases, contact tracing, epidemiological studies, and seroepidemiological surveys. A multidisciplinary approach combining advanced molecular tech-

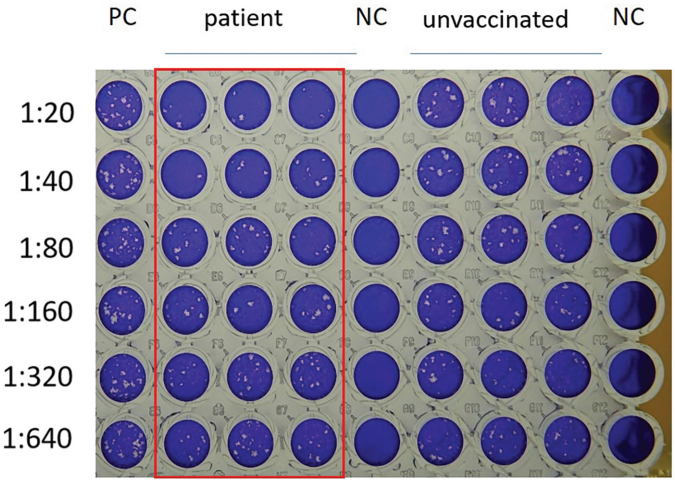


Figure 11 | Neutralization test (NT) for the detection of neutralizing antibodies (NT-Ab) against MPXV in the serum of an Mpox patient. Serum dilutions mixed with MPXV were inoculated in triplicate. A serum sample from an individual that had not previously been vaccinated against smallpox and in whom no OPV infection had previously been detected was used as a negative control. In the positive control (PC) column, only the virus was inoculated without serum, and, in the negative control (NC) columns, only growth medium was added to the cells. NT-Abs were detected in the patient's serum at a titer of 1:40 (columns marked with a red square), whereby an inhibition of the cytopathic effect was observed in comparison to the unvaccinated control.

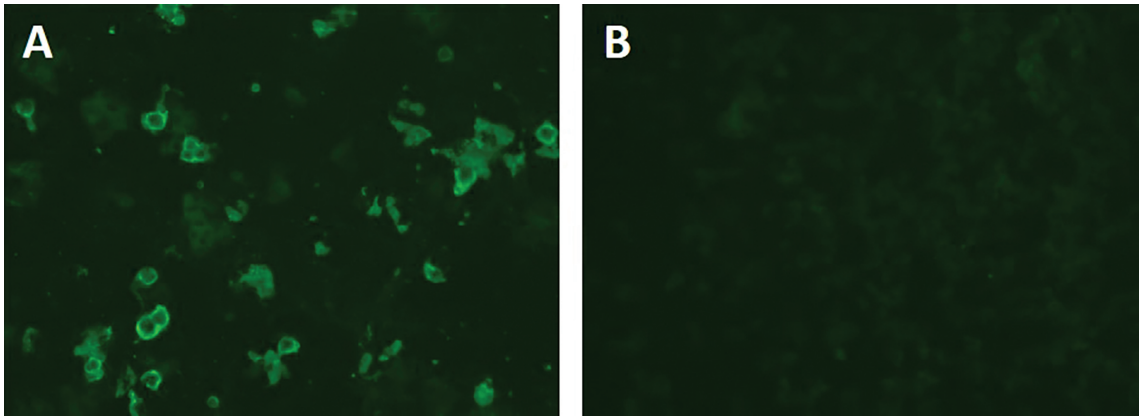


Figure 10 | Immunofluorescence assay (IFA) for the detection of IgG antibodies against MPXV in the serum of an MPXV-positive patient. VERO E6 cells infected with MPXV were used as antigen in the IFA. Panel A shows a positive immunofluorescence signal for anti-MPXV IgG antibody detected in a serum sample collected 21 days after PCR-confirmation of the disease, and panel B shows a negative control. The figure shows the immunofluorescence of 1:64 serum dilution.

niques with traditional virological methods is important for rapid and reliable diagnosis, surveillance, and control of the outbreak.

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