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Molecular epidemiology of the 2022 monkeypox virus outbreak in Slovenia

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Abstract

Introduction: Monkeypox virus (MPXV), typically endemic in West and Central Africa, has raised global concern due to the recent outbreak in several non-endemic countries with human-to-human transmission. Here we present a comprehensive analysis of MPXV genomes from Slovenia.
Methods: Two real-time polymerase chain reaction (RT-PCR) assays for *Orthopoxvirus* (OPV) and MPXV genes were used for laboratory confirmation of mpox. Complete MPXV genomic sequences were obtained using nanopore long reads and Illumina technology. Phylogenetic analyses compared the Slovenian MPXV sequences with the global sequences.
Results: A total of 49 laboratory-confirmed mpox cases were diagnosed in Slovenia in 2022, mainly affecting males under 40. In 48 cases, a complete genome sequence was obtained and phylogenetic analysis revealed five distinct lineages (B.1, B.1.14, B.1.2, B.1.3, and A.2.1), with B.1 and B.1.3 dominating, suggesting multiple introductions into Slovenia. Genome analysis revealed significant divergence from the reference MPXV-M5312_HM12_Rivers.
Conclusions: The genetic diversity observed in the Slovenian MPXV sequences sheds light on the complex dynamics of the 2022 mpox outbreak and highlights the need for further research to understand the impact of mutations on MPXV functional characteristics and their role in the evolution and diversification of current lineages.

Keywords: monkeypox virus, B.1 lineage, phylogenomic analysis, *Orthopoxvirus*, viral genome surveillance

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Introduction

Monkeypox virus (MPXV) belongs to the genus *Orthopoxvirus* (OPXV) of the *Poxviridae* family and causes the disease known as mpox. Until recently, mpox was prevalent only in some regions of West and Central Africa (1). 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 all been associated with recent travel to endemic countries or contact with infected animals (2–5). The current mpox outbreak with high human-to-human transmission in several non-endemic countries in Europe and North America began in May 2022 (6). The outbreak was first recognized in the United Kingdom and, because of the rapid increase in mpox cases worldwide, the WHO declared the multi-country mpox outbreak a public health emergency of international concern (PHEIC; https://www.who.int/news-room/speeches/item/who-director-general-s-statement-on-the-press-conference-following-IHR-emergency-committee-regarding-the-multi-country-outbreak-of-monkeypox-23-july-2022). As of September 11th, 2023, mpox cases have been reported from 114 countries, with 89,752 confirmed cases and 157 deaths (WHO; https://worldhealthorg.shinyapps.io/mpx_global/; 9/11/2023).

The MPXV genome is a double-stranded DNA molecule of approximately 197 kb. Two major viral clades have been described in endemic regions: clade I (former Central African clade), which includes strains from the Democratic Republic of Congo, and clade II (former West African clade) (7). Clade II has two distinct subclades; subclade IIa, which includes the West African strains, and subclade IIb, which contains only genomes associated with the recent outbreak in 2022. Genomic surveillance of the mpox outbreak has shown that the MPXV sequences from 2022 belong together but are phylogenetically distinct from the Nigerian strain, and so they are assigned to a separate subclade (B.1) (8, 9). To date, several MPXV genome sequences have been deposited in public sources and classified into distinct B.1 lineages with unique mutational and evolutionary features (10, 11).

Given the above observations of evidence of microevolution of MPXV and the experience with the COVID-19 pandemic, the importance of real-time genomic surveillance as a tool to track the spread and evolution of viral pathogens becomes clear. Here we report the complete genomes and phylogenetic analysis of all laboratory-confirmed mpox cases confirmed during the 2022 epidemic in Slovenia.

Methods

Samples

The Laboratory for Diagnosis of Zoonoses at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, tested all clinically suspected cases of mpox in Slovenia. Most of the suspected cases were clinically examined and followed up at the Ljubljana University Medical Center (98/129; 76%)—Department of Infectious Diseases (53/129, 41%) and Department of Dermatology (43/129, 33%)—followed by the Maribor University Medical Center (21/129, 16%) and other regional hospitals (10/129, 8%). Suspected cases were those with clinical signs and an epidemiological link to exposure to MPXV. Swabs or biopsy

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specimens were collected in commercial universal transport media (UTM) and sent for testing for MPXV DNA (Table 1).

Table 1 | Samples collected from 129 mpox clinically suspected individuals tested for presence of monkeypox virus (MPXV) DNA in Slovenia in 2022.

Sample	Negative	Positive	Total
Swab, various skin lesions (skin surface, vesicle, ulcer, wound)	80	42	122
Anogenital swab (penis, prepuce, urethra, rectum, anus)	7	2	9
Skin biopsy	1	7	8
Oral and pharyngeal swab	5	0	5
Total	93	51	144

Nucleic acid extraction

Total nucleic acid was extracted from clinical samples using the EZ1 Virus Mini Kit v2.0 according to the manufacturer's instructions (Qiagen, Hillden, Germany).

Real-time polymerase chain reaction

Two different real-time polymerase chain reaction (RT-PCR) tests—LightMix® Modular assays for *Orthopoxvirus* (OPV) and MPXV (Roche, TIB MolBiol, Germany)—were used to confirm the diagnosis. The OPV LightMix® Modular assay amplifies a 113 bp long fragment of the 14 kDa gene specific for orthopoxviruses. The MPXV LightMix® Modular assay amplifies a 106 bp long fragment of the J2L/J2R gene. All RT-PCR reactions were performed using TaqMan®Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Waltham, MA, USA) on the QuantStudio™ 7 Pro Real-Time PCR System (ThermoFisher Scientific). A case was considered confirmed as mpox if both RT-PCR assays were positive.

Complete genome sequencing

From the first confirmed case of mpox in Slovenia, a draft genome sequence was generated on nanopore long reads of the first sample. The long reads were sequenced using an ONT GridION (Oxford Nanopore Technologies, Oxford, UK) instrument with adaptive sequencing. GenBank sequence MT903344.1 was used as a reference. Long-read mapping was performed using minimap2 (v2.20-r1061) (12), samtools (v1.9) (13), and iVar (v1.0) (14). Further refinement was performed using medaka (v1.5.0) (https://github.com/nanoporetech/medaka). All other samples were sequenced on the Illumina NextSeq550 (Illumina, San Diego, CA, USA) instrument. Reads were mapped to the draft genome of the first case sequence using bwa mem v0.7.17-r1188 (15). Consensus sequences were generated using iVar and refined using pilon v1.23 (16). Draft functional annotations were generated using Prokka v1.14.5 (17). The complete MPXV genome sequences were submitted to GenBank and are available under the accession numbers listed in Table 2.

Phylogenetic analysis

In the phylogenetic analysis, we compared Slovenian MPXV sequences with sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST online tool. For each Slovenian MPXV sequence, we added the five most similar MPXV sequences from NCBI to the sequence alignment. In addition, we added MPXV sequences from different Nexclade linages that were not already included in the alignment. MPXV sequences were aligned using Squirrel. A maximum likelihood phylogenetic tree was constructed using IQTree2 (18). Using the same tools, we constructed a phylogenetic tree for Slovenian MPXV sequences only, with MPXV-M5312_HM12_Rivers (NC_063383.1) as the root.

Statistical analysis

A two-tailed nonparametric Mann–Whitney *U* test was used to compare the OPV and MPXV LightMix RT-PCR assays, with statistical significance defined as $p < 0.05$.

Results

Mpox cases

A total of 129 clinically suspected cases were tested for MPXV DNA in 2022, of which 49 (38%) were laboratory confirmed as mpox. The first case of mpox in Slovenia was confirmed on May 23rd, 2022, and the number of cases peaked in July 2022, when 52% (29/56) of individuals tested were positive. The last mpox case was confirmed at the end of September 2022 (Fig. 1).

All confirmed mpox patients were male, and the average age was 38 years, ranging from 20 to 61 years. Most patients were between 31 and 40 years old (17 patients) and between 41 and 50 years old (16 patients; Fig. 2).

As shown in Table 1, a total of 144 different clinical samples were tested for the presence of MPXV DNA, of which 35% tested positive for mpox. Most samples submitted were swabs from the surface of clinically suspected skin lesions (85%); other samples tested included anogenital swabs (6%), skin biopsies (6%), and oral or pharyngeal swabs (3%). The majority of skin biopsies tested positive for mpox (88%), and a similar fraction of skin lesions and anogenital swabs tested positive for mpox (34% and 22%, respectively). All oral specimens tested negative for mpox.

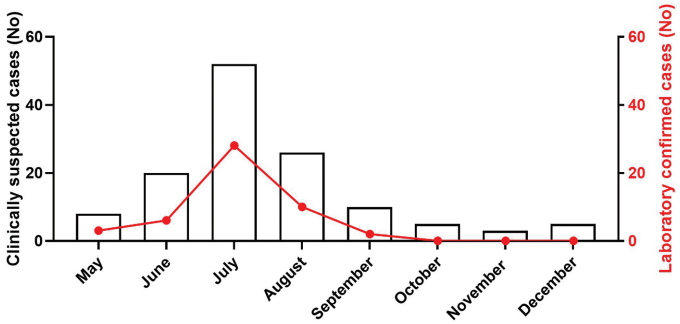

Figure 1 | Number of clinically suspected mpox cases tested (black bars) and number of laboratory-confirmed mpox cases (red line) by month in 2022.

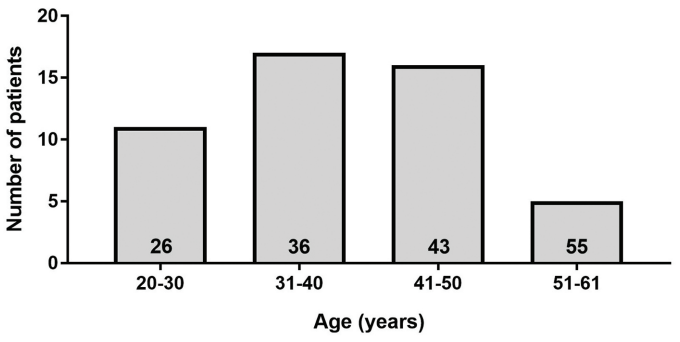

Figure 2 | Age distribution of 49 laboratory-confirmed mpox cases diagnosed in Slovenia in 2022. The mean age of patients in each age group is shown in each column.

RT-PCR diagnostics

Two RT-PCR tests were performed to confirm mpox. Because of a possible deletion in the tumor necrosis factor (TNF) receptor gene, the LightMix Modular Assays for both OPV and MPXV detection were selected as diagnostic approach. All mpox cases were confirmed positive by both RT-PCR assays. MPXV was detected in the patient samples with a median Ct value of 17.4 (range 11.0 to 28.5) by the OPV assay and with a higher median Ct value of 21.1 (range 15.9 to 31.9) by the MPXV assay ($p < 0.0001$; Fig. 3).

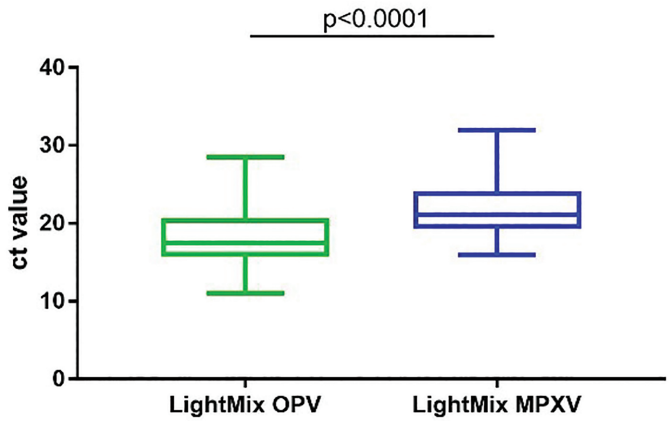

Figure 3 | Distribution of real-time polymerase chain reaction (RT-PCR) Ct values from samples of 49 patients that tested positive for mpox with LightMix Modular OPV and MPXV assays. A two-tailed nonparametric Mann–Whitney test was used, with statistical significance defined as $p < 0.05$.

Phylogenetic analysis

We performed MPXV genome sequencing of all confirmed mpox cases and generated 48 high-quality full-length genome sequences with more than 70% genome coverage. The sequences were deposited at NCBI and the Global Initiative on Sharing All Influenza Data (GISAID) under the accession numbers listed in Table 2. On average, we generated more than 5.5×10^6 pair-end reads per sample and achieved more than 91% genome coverage. The sequencing of MPXV from the last patient in September 2022 was not successful, and the genome was covered at only 21%, but the case had a higher Ct value compared to the samples with successful sequencing.

The phylogenetic analysis included 48 Slovenian MPXV sequences and 73 other MPXV sequences from the 2022 global outbreak. Slovenian MPXV sequences are well positioned in subclade IIb, confirming that all cases are molecularly related to the recent outbreak (Fig. 4A). According to the Nextclade classification, five lineages were detected in Slovenia: B.1, B.1.14, B.1.2, B.1.3, and A.2.1. The majority of sequences belonged to lineages B.1 (50%) and B.1.3 (42%) and were present in the population from May to September. Additional genetic lineages were detected in July 2022 (two B.1.14 and one A.2.1), and one B.1.2 was detected in August 2022 (Fig. 5). The Slovenian sequences were most closely related to 2022 mpox cases from Belgium, the United States, Australia, Spain, Germany, the Czech Republic, and the United Kingdom (Fig. 4A). Different genetic lineages detected at different times suggested multiple unrelated introductions into the country. To detail virus transmission within the country, an additional phylogenetic analysis focusing on Slovenian sequences was performed, and related sequence clusters were observed within the genetic lineages B.1 (Slovenia-MPXV-21 and 32), B.1.3 (Slovenia-MPXV-35, 36, 40, 44), and B.1.14 (Slovenia-MPXV-10 and 12; Fig. 4B). Related clusters could suggest a transmission chain inside the country; however, the bootstrap values were not high enough to confirm this hypothesis.

Because of the high number of mutation events in Slovenian MPXV genomes, the MPXV-M5312_HM12_Rivers (MT903340) strain was analyzed as a reference genome. The Slovenian sequences differ from the reference strain by a mean of 66 single-nucleotide polymorphisms (SNPs) and by a mean of 30 total amino acid substitutions and 94 total nucleotide substitutions (Table 2), which is higher than the expected substitution rate for orthopoxviruses (19).

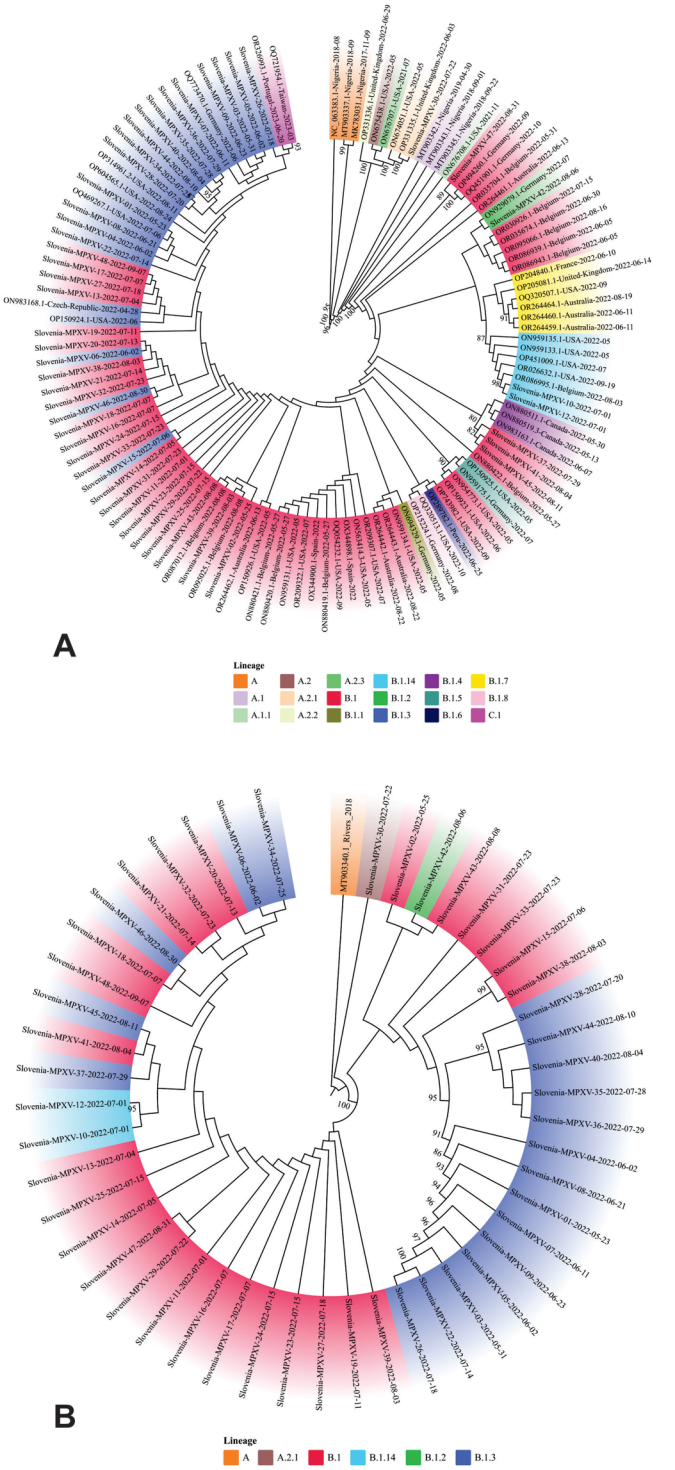

Figure 4 | Phylogenetic relationships among monkeypox virus (MPXV) genomes: (A) phylogenetic tree of the 48 Slovenian complete genome MPXV sequences and 73 complete genome MPXV sequences from the 2022 global outbreak; (B) phylogenetic tree of the complete MPXV genome sequences with emphasis on the 48 Slovenian MPXV sequences. The trees were generated using the Nextstrain tool. The color coding is indicated in the legend. The details of the Slovenian mpox sequences are listed in Table 2.

Table 2 | **Detailed information about monkeypox virus (MPXV) sequences from Slovenia in 2022.**

Patient no.	Sequence name	NCBI	Confirming date	LightMix OPV RT-PCR (Ct value)	LightMix MPXV RT-PCR (Ct value)	Average depth (N)	Coverage ratio (%)	Number of readings (N)	Lineage GISAID	Lineage NEXTCLADE	Single nucleotide polymorphisms relative to NC_063383.1	Nucleotide substitutions relative to NC_063383.1	Amino acids S+A: substitutions relative to NC_063383.1
1	Slovenia-MPXV-01-2022-05-23	ON609725.2	2022-05-23	12.7	16.9	2830	99.9	4543163	B.1.3	B.1.3	66	71	32
2	Slovenia-MPXV-02-2022-05-25	ON631241.1	2022-05-25	11.0	15.9	4734	99.9	7205296	B.1	B.1	62	67	29
3	Slovenia-MPXV-03-2022-05-31	ON754984	2022-05-31	16.1	19.6	144	99.9	261877	B.1.3	B.1.3	65	70	31
4	Slovenia-MPXV-04-2022-06-02	ON754985	2022-06-02	13.5	16.9	3478	99.9	5453014	B.1.3	B.1.3	65	70	31
5	Slovenia-MPXV-05-2022-06-02	ON754986.1	2022-06-02	16.3	19.9	590	99.9	939490	B.1.3	B.1.3	65	70	31
6	Slovenia-MPXV-06-2022-06-02	ON754987	2022-06-02	24.8	28.0	627	80.9	978194	B.1.3	B.1.3	78	485	48
7	Slovenia-MPXV-07-2022-06-11	ON838178	2022-06-11	20.7	24.8	1375	99.9	2463118	B.1.3	B.1.3	65	70	31
8	Slovenia-MPXV-08-2022-06-21	OP605551	2022-06-21	15.8	22.1	7850	96.0	11446301	B.1.3	B.1.3	65	70	31
9	Slovenia-MPXV-09-2022-06-23	OP605552	2022-06-23	21.8	24.0	1602	99.9	2535960	B.1.3	B.1.3	65	70	31
10	Slovenia-MPXV-10-2022-07-01	OP605553	2022-07-01	28.3	29.0	8247	94.8	12524862	B.1.14	B.1.14	63	69	31
11	Slovenia-MPXV-11-2022-07-01	OP605554	2022-07-01	18.2	21.1	8330	94.4	12634217	B.1	B.1	60	65	28
12	Slovenia-MPXV-12-2022-07-01	OP605555	2022-07-01	17.9	22.1	9057	95.6	13730794	B.1.14	B.1.14	104	183	37
13	Slovenia-MPXV-13-2022-07-04	OP605556	2022-07-04	16.5	20.6	10228	96.3	15121088	B.1.13	B.1	63	407	45
14	Slovenia-MPXV-14-2022-07-05	OP605557	2022-07-05	14.0	17.9	9545	92.5	14174482	B.1	B.1	70	132	36
15	Slovenia-MPXV-15-2022-07-06	OR540671	2022-07-06	21.5	25.6	94	80.0	301878	B.1	B.1.3	236	237	79
16	Slovenia-MPXV-16-2022-07-07	OP605558	2022-07-07	15.7	18.7	4526	93.1	6759868	B.1	B.1	59	64	27
17	Slovenia-MPXV-17-2022-07-07	OP605559	2022-07-07	14.7	18.9	4215	93.7	6704353	B.1	B.1	60	65	28
18	Slovenia-MPXV-18-2022-07-07	OP605560	2022-07-07	14.6	18.2	4400	92.5	6599034	B.1	B.1	63	71	28
19	Slovenia-MPXV-19-2022-07-11	OP605561	2022-07-11	16.6	19.3	4687	89.1	6935089	B.1	B.1	57	62	26
20	Slovenia-MPXV-20-2022-07-13	OP605562	2022-07-13	17.4	20.9	1381	92.9	2229516	B.1	B.1	64	73	29

Table 2 | **Continoued.**

Patient no.	Sequence name	NCBI	Confirming date	LightMix OPV RT-PCR (Ct value)	LightMix MPXV RT-PCR (Ct value)	Average depth (N)	Coverage ratio (%)	Number of readings (N)	Lineage GISAID	Lineage NEXTCLADE	Single nucleotide polymorphisms relative to NC_063383.1	Nucleotide substitutions relative to NC_063383.1	Amino acids S+A: substitutions relative to NC_063383.1
21	Slovenia-MPXV-21-2022-07-14	OP605563	2022-07-14	17.6	19.7	767	88.1	1244258	B.1	B.1	58	64	26
22	Slovenia-MPXV-22-2022-07-14	OP605564	2022-07-14	16.5	19.3	3596	94.3	5520666	B.1	B.1.3	64	70	30
23	Slovenia-MPXV-23-2022-07-15	OP605565	2022-07-15	18.4	21.3	4621	93.4	6754455	B.1	B.1	59	64	26
24	Slovenia-MPXV-24-2022-07-15	OP605566	2022-07-15	18.7	21.8	3721	89.9	5517141	B.1	B.1	57	64	26
25	Slovenia-MPXV-25-2022-07-15	OP605567	2022-07-15	23.8	27.0	4513	93.9	6470339	B.1	B.1	62	67	29
26	Slovenia-MPXV-26-2022-07-18	OP605568	2022-07-18	14.0	17.2	4529	95.3	6790603	B.1	B.1.3	66	71	32
27	Slovenia-MPXV-27-2022-07-18	OP605569	2022-07-18	17.3	20.8	4130	95.0	6207919	B.1	B.1	74	92	31
28	Slovenia-MPXV-28-2022-07-20	OP605570	2022-07-20	18.7	22.7	4297	96.1	6480769	B.1.3	B.1.3	67	72	32
29	Slovenia-MPXV-29-2022-07-22	OP605571	2022-07-22	18.1	21.8	2407	94.3	4104266	B.1	B.1	63	66	27
30	Slovenia-MPXV-30-2022-07-22	OP605572	2022-07-22	20.3	23.0	3365	97.9	5836010	A.2.1	A.2.1	39	38	21
31	Slovenia-MPXV-31-2022-07-23	OP605573	2022-07-23	20.7	25.1	4917	98.5	7764869	B.1	B.1	61	66	29
32	Slovenia-MPXV-32-2022-07-23	OP605574	2022-07-23	17.4	22.6	1481	93.4	2341391	B.1	B.1	62	71	28
33	Slovenia-MPXV-33-2022-07-23	OP605575	2022-07-23	14.9	17.9	4373	95.7	6412356	B.1	B.1	60	65	28
34	Slovenia-MPXV-34-2022-07-25	OP605576	2022-07-25	18.2	21.0	1548	93.7	2521552	B.1	B.1	75	nd	nd
35	Slovenia-MPXV-35-2022-07-28	OP605577	2022-07-28	20.7	24.1	536	84.6	793521	B.1	B.1.3	73	78	29
36	Slovenia-MPXV-36-2022-07-29	OP605578	2022-07-29	14.7	18.2	932	99.0	1541677	B.1.3	B.1.3	72	77	34
37	Slovenia-MPXV-37-2022-07-29	OP605579	2022-07-29	20.6	24.2	1411	93.4	2388115	B.1	B.1	70	nd	nd
38	Slovenia-MPXV-38-2022-08-03	OR540672	2022-08-03	17.3	21.5	630	89.9	1126600	B.1	B.1	225	227	87
39	Slovenia-MPXV-39-2022-08-03	OP605580	2022-08-03	18.7	21.6	219	92.5	418794	B.1	B.1	58	63	25
40	Slovenia-MPXV-40-2022-08-04	OP605581	2022-08-04	28.5	31.9	4930	97.4	7518590	B.1.3	B.1.3	68	73	33

Table 2 | Continued.

Patient no.	Sequence name	NCBI	Confirming date	LightMix OPV RT-PCR (Ct value)	LightMix MPXV RT-PCR (Ct value)	Average depth (N)	Coverage ratio (%)	Number of readings (N)	Lineage GISAID	Lineage NEXTCLADE	Single nucleotide polymorphisms relative to NC_063383.1	Nucleotide substitutions relative to NC_063383.1	Amino acids S+A: substitutions relative to NC_063383.1
41	Slovenia-MPXV-41-2022-08-04	OP605582	2022-08-04	15.8	19.4	5156	97.4	7898740	B.1	B.1	70	67	30
42	Slovenia-MPXV-42-2022-08-06	OP605583	2022-08-06	15.7	20.5	1813	99.9	3132195	B.1.2	B.1.2	69	74	34
43	Slovenia-MPXV-43-2022-08-08	OP605584	2022-08-08	23.7	28.1	638	99.9	1088568	B.1	B.1	66	71	30
44	Slovenia-MPXV-44-2022-08-10	OP605585	2022-08-10	21.5	26.4	13603	99.9	20749084	B.1.3	B.1.3	69	74	33
45	Slovenia-MPXV-45-2022-08-11	OP605586	2022-08-11	17.7	22.0	2270	94.7	3377159	B.1	B.1	62	nd	nd
46	Slovenia-MPXV-46-2022-08-30	OP605587	2022-08-30	15.4	19.7	2135	90.6	3806519	B.1	B.1	96	nd	nd
47	Slovenia-MPXV-47-2022-08-31	OP605588	2022-08-31	17.3	20.6	3261	92.9	4998940	B.1	B.1	120	238	31
48	Slovenia-MPXV-48-2022-09-07	OP605589	2022-09-07	15.8	19.1	177	73.3	265443	B.1	B.1	52	53	25

nd = substitutions cannot be determined in the phylogenetic analysis.

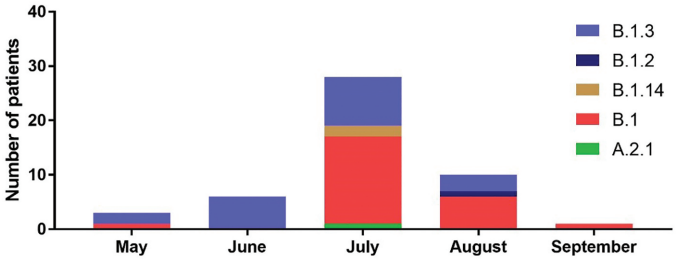

Figure 5 | Number of different genetic lineages detected in Slovenia in 2022 by month of mpox case confirmation.

Discussion

With the recent global mpox outbreak, a previously relatively rare zoonotic disease endemic to Africa has spread worldwide and, as declared by the WHO, poses a threat to global health. The global outbreak had a distinct clinical presentation, and rapid spread was observed with a geographic pattern and clustering of cases in specific sexual networks. The high prevalence of mpox cases in non-endemic countries has contributed to the rapid implementation of genomic surveillance programs and monitoring of MPXV epidemiology and evolution.

In Slovenia, a total of 49 mpox cases were diagnosed during the 2022 outbreak. All patients were male and on average younger than 40 years, which is consistent with previously published observations showing that MPXV is mainly transmitted between younger men who have sex with men (20). Contributing to the young risk group may be the cessation of smallpox vaccination in the early 1980s, which led to a lack of immunity in the younger population.

Although the number of patients in Slovenia is small compared to other European countries such as Spain, France, the United Kingdom, or Germany, where thousands of cases have been confirmed (WHO; https://worldhealthorg.shinyapps.io/mpx_global/; assessed on September 11th, 2023), all suspected patients were molecularly confirmed, allowing in-depth molecular phylogeny of the MPXV in Slovenia. Because of the alarming finding of a significant deletion in the TNF receptor gene of MPXV, which was used as a target region in some RT-PCR assays, LightMix Modular assays were used for diagnosis to avoid potential false negative results. All mpox cases were laboratory confirmed by RT-PCR detection of both OPV and MPXV because such an approach is the gold standard for mpox confirmation (21, 22). All but one case of mpox were fully sequenced, and TNF-alpha deletion was not detected. The MPXV genome from the last case diagnosed in September 2022 was only 21% covered, possibly due to degradation of MPXV nucleic acid while the sample was additionally inoculated into the VERO E6 cell line, and the viability of the virus could not be detected (data not shown).

Phylogenetic analyses revealed that the sequences of Slovenian mpox cases belong to group IIb and are related to other sequences from the 2022 global outbreak. The current outbreak was caused by the B.1 lineage, but microevolution of B.1 lineages occurred through genomic changes in a short period of time (10, 23). Consistent with this observation, the first mpox case in Slovenia occurred in a patient that returned from Gran Canaria in May 2022 and was infected with MPXV, which was assigned to genetic lineage B.1. Of the five MPXV lineages detected in Slovenia, B.1 and B.1.3 were the predominant ones, and only one sequence belonged to the A.2.1 lineage. Such a distribution was expected because the A.2 lineage (predominantly from the United States,

Thailand, and India) has a lower mean nucleotide substitution rate than the B.1 lineage, and the majority of cases had B.1 lineages (24). However, the quality of the sequences has a major impact on the differentiation of B.1 lineages, which is evident in the phylogenetic tree in this study, where some sequences could not be further distinguished beyond B.1. The peak of the cases diagnosed in Slovenia was in July 2022, with four different genetic lineages detected, suggesting multiple individual introductions of MPXV into the country. Additional phylogenetic analyses revealed related clades, suggesting a chain of transmission within the country. However, due to low bootstrap values and lack of epidemiological data, this observation cannot be confirmed.

High divergence was observed between the MPXV from Slovenia and the reference genome from MPXV-Rivers. Although gene losses are not unexpected in orthopoxviruses, in our study the number of detected SNPs and amino acid substitutions is also higher than the expected substitution rate for orthopoxviruses (10, 11, 25). Detailed studies of mutations are expensive and time-consuming because of the difficulty in obtaining a complete genome sequence of good quality due to the size of the MPXV genome. However, further research is needed to assess the impact of mutations on the protein and functional characteristics of MPXV and its role as a driver in the evolution, diversification, and spread of current lineages. Real-time genomic surveillance of MPXV in different countries could provide useful data for explaining the origin and characteristics of the 2022 mpox global outbreak.

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