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SHORT COMMUNICATION

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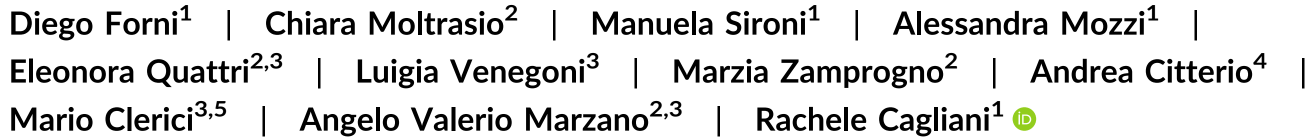
Whole-genome sequencing of hMPXV1 in five Italian cases confirms the occurrence of the predominant epidemic lineage



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Abstract

The ongoing outbreak of monkeypox virus (hMPXV1) is the largest recorded in historically nonendemic countries. Genomic surveillance has emerged as a pivotal tool to track the spread and monitor the evolution of viral pathogens. Therefore, to assess the genetic diversity of circulating hMPXV1 in northern Italy in June to July 2022, we sequenced and analyzed five complete genomes of viruses sampled from patients presenting with a typical course of hMPXV1 infection. Phylogenetic analysis confirmed that all five genomes belong to the predominant epidemic lineage (B.1). Inspection of genetic changes and comparison with the reference sequence showed the presence of 12 nucleotide substitutions. Seven are nonsynonymous mutations leading to amino acid changes in six proteins belonging to different functional classes. Moreover, 11 of these 12 nucleotide mutations involve GA>AA or TC>TT replacements, suggesting that host APOBEC3 enzymes are responsible for the generation of substitutions in circulating viruses. Finally, metagenomic analysis evidenced bacterial superinfection (*Streptococcus pyogenes*) in one patient. Through this study, we contributed to expand the number of complete genomes of viruses circulating in Italy and characterize them as belonging to the predominant outbreak lineage.

KEYWORDS

genomic surveillance, hMPXV1, monkeypox, whole-genome sequencing

1 | INTRODUCTION

Monkeypox is a viral zoonotic disease caused by monkeypox virus (MPXV), a member of the *Orthopoxvirus* genus in the family Poxviridae. Until recently, monkeypox was considered a rare zoonosis endemic to West and Central Africa, although sporadic cases of exportation from Africa were previously described.[1] At the beginning of May 2022, an unexpected monkeypox outbreak, with distinctive clinical and epidemiological characteristics, was first recognized in the United Kingdom and subsequently worldwide. Cases, which apparently had no epidemiological links to West or Central Africa, increased rapidly in number and, on July 23, 2022, the WHO declared the multicountry outbreak of monkeypox a Public Health Emergency of International Concern (PHEIC) (https://www.

Diego Forni and Chiara Moltrasio contributed equally to this study.

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who.int/director-general/speeches/detail/who-director-general-s-statement-on-the-press-conference-following-IHR-emergency-committee-regarding-the-multi–country-outbreak-of-monkeypox–23-july-2022). Up to December 23, 2022, a cumulative total of 83 497 laboratory-confirmed cases of monkeypox and 72 deaths have been reported to the WHO from 110 countries (https://www.who.int). The epidemiological characteristics of the outbreak consist of human-to-human transmission and most infections are reported in young males. Viral spread is thought to be mainly mediated by sexual contact and most patients suffer from a mild disease often characterized by anogenital lesions.[1]

The MPXV genome is a double-stranded DNA molecule of approximately 197 Kbp. Two major viral clades have been described in the endemic regions: clade I, mainly distributed in the Congo Basin, and clade II, prevalent in West Africa. Clade II is further divided into two subclades broadly corresponding to viruses sampled in Nigeria (IIb) and West of Nigeria (IIa).[1] Genomic surveillance of the monkeypox outbreak indicated that the majority of the 2022 sequences cluster together and are phylogenetically related to clade IIb, but differ from the Nigerian strains by 46 single nucleotide substitutions, which place them in a separated subclade (B.1).[2,3] The overwhelming majority of these mutations involve GA>AA or TC>TT substitutions, suggesting the action of host apolipoprotein B mRNA editing catalytic polypeptide-like 3 (APOBEC3) enzymes, which exert an important role in innate antiviral immunity.[2,3]

Based on the distinctive genomic and epidemiological features, the virus causing the 2022 monkeypox outbreak was renamed hMPXV1.[4] To date, several hMPXV1 genome sequences were deposited in public repositories and classified in distinct B.1 lineages (B.1.1–B1.1.14) (https://clades.nextstrain.org/). However, the retrospective analyses of monkeypox cases registered in the United States in 2021 showed that these sequences do not cluster with B.1 genomes, but form a distinct A.2 lineage,[3] suggesting multiple, independent introductions of hMPXV1 in the United States. Interestingly, lineage A viruses also show a substitution pattern consistent with the activity of APOBEC3. Indeed, Gigante and coworkers indicated that the substitution spectrum started to change in 2017, when the virus was still being mainly transmitted in Africa.[3] Considering the above observations and the experience of the COVID-19 pandemic, the importance of genomic surveillance as a tool to track the spread and monitor the evolution of viral pathogens is evident. Herein, we report the complete genomes and phylogenetic analysis of five human monkeypox cases detected in northern Italy in June to July 2022.

2 | MATERIALS AND METHODS

2.1 | Patients

Swab (n = 3) and skin (n = 2) samples were collected from five patients with human monkeypox infection attending the sexually transmitted diseases outpatient service of the Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, from June to July 2022.[5,6] Positivity for monkeypox DNA was confirmed through real-time polymerase chain reaction (RT-PCR) (RTPCR018-LPD-R, Vircell Microbiologists) on both pharyngeal and vesico-pustular fluid swabs for all cases.

All patients provided written informed consent to be included in the study. The study was approved by Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (RC_2022).

2.2 | Sampling and DNA extraction

In three patients (MPX-3, MPX-4, MPX-5), skin swabs at lesional sites were performed; the samples were placed in a culture medium composed of 2-sucrose-phosphate enriched by fetal bovine serum (5%) and gentamicin/streptomycin. The subsequent viral DNA extraction was performed using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

The skin specimens were obtained from 4 mm punch biopsy in two patients (MPX-1, MPX-2). DNA was extracted from skin biopsies with proteinase K and sodium dodecyl sulfate with RNase and phenol/chloroform.

DNA was quantified using Qubit dsDNA High Sensitivity assay (Life Technologies) on the Qubit 3.0 instrument.

2.3 | Library preparation and DNA sequencing

Metagenomic shotgun sequencing was performed for each sample (one lesion for each patient). Sequencing libraries were constructed using the Illumina DNA Prep kit and quantified using high-sensitivity Bioanalyzer reagents (Agilent). Libraries were pooled and sequenced using the Illumina NextSeq. 500 platform with a 150 bp paired-end method. All reagents were used according to the manufacturer's recommendations.

Raw reads were analyzed with Trim Galore (https://github.com/FelixKrueger/TrimGalore) for adapter and quality trimming, by using default parameters. As an initial analysis, trimmed reads were screened using the taxonomic classification tool Kraken2[7] against its extended database k2_PlusPF (https://benlangmead.github.io/aws-indexes/k2) and with the "-report-minimizer-data" option. Human reads were also identified and removed using the "extract_kraken_reads" script. The remaining trimmed fastq files were assembled using the SPAdes genome assembler (v3.15.4)[8] with k-mer length of 21, 33, 55, and 77. The generated contigs were then ordered using abacas (v1.3.1)[9] based on the hMPXV reference strain (MPXV-M5312_HM12_Rivers, NCBI Accession ID: NC_063383).

Nonhuman reads were also mapped with the bwa mem algorithm[10] to the same reference strain, after removing the inverted tandem repeat located at the 3′ end of the genome. Mapping quality was checked using samtools 1.15[11] (mapping quality >20) and a consensus sequence was generated using samtools and iVar 1.3.1.[12] The latter was run with a base minimum quality score threshold of 30,

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a minimum depth to call consensus of 10, and a minimum frequency threshold of 0.5. The two Inverted Terminal Repeats were then manually assembled by copying from one to the other, as previously suggested.[3] Finally, the two consensus sequences were checked for consistency, by generating and alignment with Multiple Alignment using Fast Fourier Transform (MAFFT)[13] and manually inspected.

The mutation pattern was analyzed by the NextClade online tool using as a reference the strain MPXV_USA_2022_MA001 in NC_063383 coordinates (pseudo_ON563414) (https://clades.nextstrain.org/, v2.8.1, last access November 25, 2022). Nextclade was used for clade and lineage assignment, identification of single nucleotide variant mutations, insertions, and deletions.

Complete high-coverage genomes sampled in Italy and Spain were downloaded from the GISAID initiative database (last access November 25, 2022). These genomes, along with the five generated herein, were analyzed with the Nextstrain suite (v5.0.1)[14] to infer phylogenetic relationship.

3 | RESULTS

3.1 | Patients

Clinical and demographic features of the five patients with human monkeypox infection are summarized in Table 1. All patients, with a median age of 34 years, were Men who have Sex with Men and disclosed to have had unprotected sex in the months preceding the infection. One patient had a history of HCV/HIV co-infection. One patient traveled to Spain in the 2 weeks before symptom onset while the others reported that they had not traveled. None had been vaccinated for smallpox. Cutaneous manifestations typically consisted of multiple vesico-pustular lesions mainly located on the pubic region (*n* = 3) and penil shaft (*n* = 3). Genital (*n* = 1) and anal/perianal region (*n* = 1) was also affected, as well as upper (*n* = 1) and lower limbs (*n* = 1) and hands (*n* = 2). Almost all patients (*n* = 4) presented mild systemic symptoms including fever (*n* = 4), headache (*n* = 4), and

TABLE 1 Characteristics of patients and clinical samples

Patient	MPX-1	MPX-2	MPX-3	MPX-4	MPX-5
Sex	M	M	M	M	M
Age	44	40	34	32	27
Nationality	Ukraine	Brazil	Italy	Romania	Italy
Travel history (country)	Na	Na	Spain (trip 2 weeks before onset)	Na	Na
Unprotected sex	Yes	Yes	Yes	Yes	Yes
HIV+	Yes	No	No	No	No
HCV+	Yes	No	No	No	No
Onset of cutaneous manifestations[a]	6/2/2022	7/4/2022	7/10/2022	7/12/2022	7/20/2022
Number of lesions	>10	>5	>10	>2	>2
Localization	Genital region; lower limb; middle finger of the right hand	Pubic region; penil shaft; upper limb; palm of the hand	Anal/perianal region	Penil shaft; pubic region	Penil shaft; pubic region
Swab/biopsy sample lesion site	Genital region	Pubic region	Perianal region	Pubic region	Pubic region
Systemic symptoms	Fever, headache, fatigue	Fever, headache, fatigue	No	Fever, headache	Fever, headache, fatigue
Evolution of skin lesions	Synchronous	Synchronous	Na	Asynchronous	Asynchronous
Lymphadenopathy	No	No	No	No	No
Sample date[a]	6/7/2022	7/6/2022	7/13/2022	7/26/2022	7/26/2022
GISAID strain Name	hMpxV/Italy/LOM-EM-001/2022	hMpxV/Italy/LOM-EM-002/2022	hMpxV/Italy/LOM-EM-003/2022	hMpxV/Italy/LOM-EM-004/2022	hMpxV/Italy/LOM-EM-005/2022
hMPXV genome sequence ID	EPI_ISL_15942296	EPI_ISL_15942885	EPI_ISL_15942886	EPI_ISL_15942637	EPI_ISL_15942638
Reference	6		5		

Abbreviations: MPXV, monkeypox virus; Na, not available.

[a]The date is in the format MM/DD/YYYY.

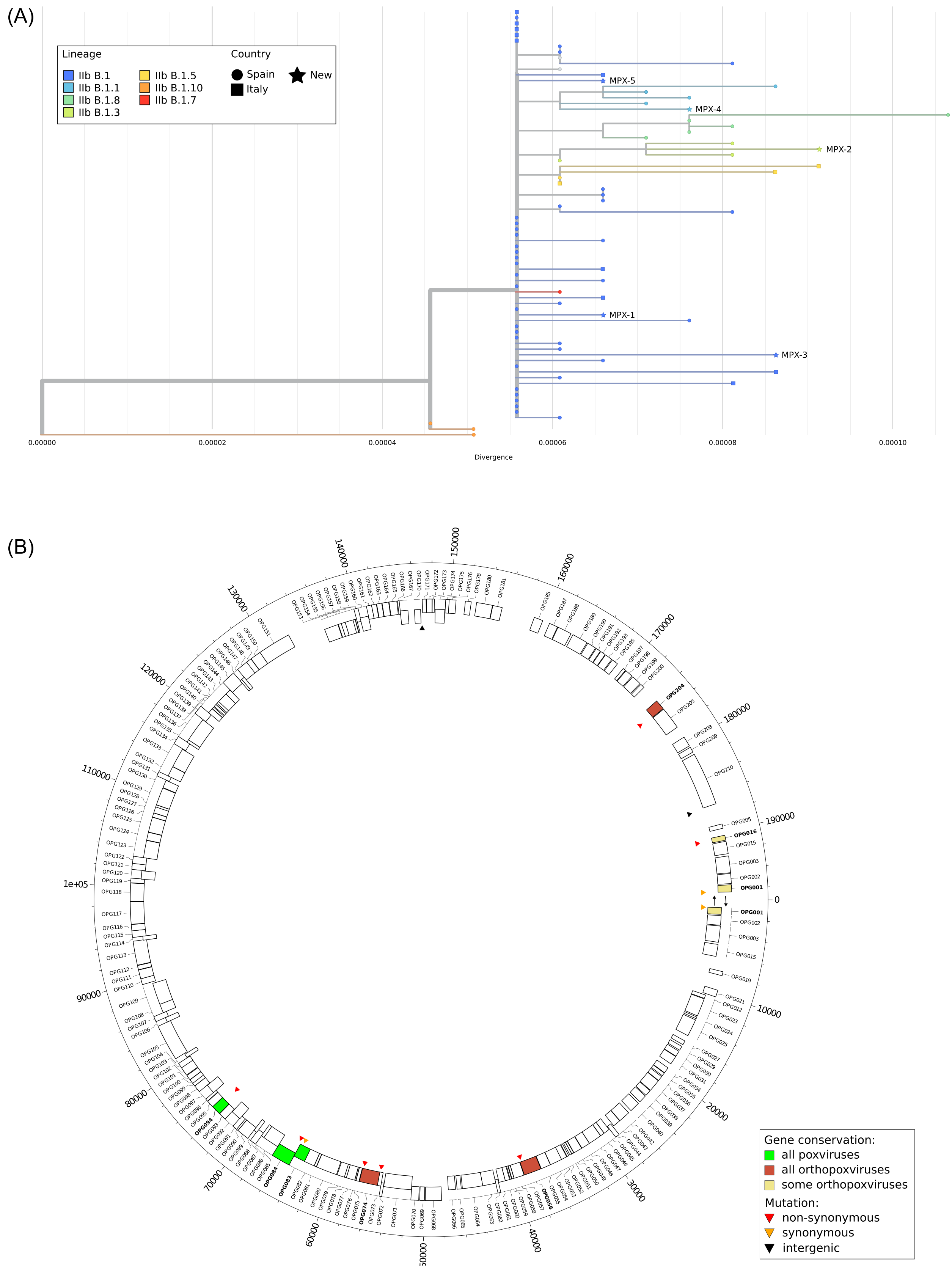

FIGURE 1 Phylogenetic relationship among hMPXV1 genomes. (A) Phylogenetic tree of hMPXV1 complete genome sequences from Italy and Spain. The tree was generated using the Nextstrain tool and lineage and geographic location coding are reported in the legend. Genomes generated in this study are indicated with stars. (B) Circos plot of the hMPXV1 genome. Positions and annotation refers to the MPXV-M5312_HM12_Rivers strain (NCBI ID: NC_063383). Genes are shown according to sense of transcription. The 12 mutations identified in this study are shown as triangles. Genes carrying these mutations are colored by their degree of conservation in poxviruses. Color coding is reported in the legend. MPXV, monkeypox virus.

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asthenia (n = 3). Lymphadenopathy was not observed in any patient. Only one patient required hospitalization for logistical reasons of isolation. Active medical treatment (e.g., tecovirimat) was not required and all patients achieved complete remission.

3.2 | MPXV genome sequences: Mutation patterns and phylogenetic analysis

We obtained and assembled the full hMPXV1 genomes from the five patients (Supporting Information: Table 1). Sequences were deposited in GISAID (https://gisaid.org/) (Table 1). To determine the genetic relationships of the five viruses with other hMPXV1 strains, a phylogenetic tree was constructed using whole genomes. In particular, the 5 newly sequenced viruses were analyzed together with 70 hMPXV1 genomes, 13 of which were from Italy and 57 from Spain (Figure 1A and Supporting Information: Table 2). As reported in Figure 1A and Table 2, three samples (MPX-1, MPX-3, and MPX-5) clustered with lineage B.1, the other two with B.1.1 (MPX-4) and B.1.3 (MPX-2).

Comparison with the reference sequence (MPX_USA_2022_MA001, lineage B.1, NCBI Accession ID: ON563414) showed the presence of 12 nucleotide substitutions. On the contrary, analysis of read depth across the genomes did not suggest the presence of copy number variants. Seven of 12 substitutions were nonsynonymous mutations leading to amino acid changes in 6 proteins (Table 2 and Figure 1B). These mutations affect genes with different degrees of conservation in the poxvirus phylogeny (Figure 1B) and involved in immunomodulation (*B16R* and *OPG16*, aka *N3R*), morphogenesis (*C18L*), cell entry/attachment (*G10R*) and transcription (*I8R*). Two nonsynonymous variants were also identified in the *Q1L* gene, which is poorly characterized but may modulate virulence (Table 2). Regarding the variants, all substitutions have been previously reported, with the exception of the substitution C64984T (in the *I8R* gene), which is not present in any of the 1850 genomic sequences (complete genomes with high coverage) deposited in GISAID (last access November 29, 2022). The nonsynonymous substitutions have varying degrees of conservation in the orthopox phylogeny (Supporting Information: Figure 1).

Moreover, in line with previous analyses of hMPXV1 diversity,[2,3] all nucleotide substitutions were G>A or C>T changes, a feature consistent with the action of host human APOBEC3 enzymes. As the GC-content of hMPXV1 genome is approximately 33%, the mutation bias is evident. Furthermore, 11 of the 12G>A and C>T substitutions were in a 5′-GA-3′ or 5′-TC-3′ dinucleotide context (i.e., changes were GA>AA or TC>TT), which is further evidence of APOBEC3-mediated editing.

TABLE 2 Nucleotide and amino acid changes in hMPXV1 genomes

Patient	hMPXV1 lineage[a]	Nucleotide substitutions[b]	Amino acid substitutions[b]	Gene (NC_063383)	Gene (VACV-WR)[c]	Gene function[d]
MPX-1	B.1	G850A	L242L	OPG001	C23L	Chemokine binding protein
		C196360T	L242L	OPG001	B29R	Chemokine binding protein
MPX-2	B.1.3	G55133A	R665C	OPG074	Q1L	Activates the ERK1/2 signaling pathway and increases virulence
		C64426T	T97T	OPG083	I7L	Serine protease, involved in virion morphogenesis
		G190660A	R84K	OPG016	-	Secreted protein, blocks NKG2D receptor
MPX-3	B.1	G187156A	-	Nongenic	-	-
MPX-4	B.1.1	C38937T	R33Q	OPG056	C18L	Promotes wrapped virion trafficking
		G74360A	R194H	OPG094	G10R	Part of the entry-fusion complex; promotes the virus membrane fusion with host cell membrane
		C146816T	-	Nongenic	-	-
		G175349A	S273N	OPG204	B16R	Secreted IFN type-1 decoy receptor
MPX-5	B.1	G57076A	S17L	OPG074	Q1L	Activates the ERK1/2 signaling pathway and increases virulence
		C64984T	S88L	OPG084	I8R	NTP-dependent helicase; plays an important role during transcription of early mRNAs

Abbreviation: MPXV, monkeypox virus.

[a]Nextclade was used for lineage assignment.

[b]The mutation pattern was derived using as a reference the strain MPXV_USA_2022_MA001 in NC_063383 coordinates (pseudo_ON563414).

[c]Accession for VACV WR is NC_006998.

[d]Functional annotations derive from a previous work[15] or from UniProt (https://www.uniprot.org/).

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3.3 | Metagenomic analysis

Secondary bacterial infections of skin lesions were recognized as complications of monkeypox.[16] Few studies reported associated superimposed bacterial infections requiring intense antibiotic treatments[17,18] and sequence reads of *Staphylococcus aureus* and *Streptococcus pyogenes* were recently identified in MPXV-positive skin lesions.[19]

The metagenomic approach we applied allowed us to obtain the entire monkeypox genome but also to assess the presence of reads attributable to other microorganisms. Using Kraken2 with a cutoff of 1000 reads, we identified a co-infection with *S. pyogenes* in just one sample (MPX-2).

4 | DISCUSSION

Genomic surveillance has proven to be exceptionally useful to monitor the evolution, spread, and diversity of emerging and re-emerging pathogens. In the case of hMPXV1, initial analyses indicated that the vast majority of 2022 monkeypox cases were caused by a predominant lineage (B.1) but also pointed to the presence of other lineages, suggestive of multiple introductions (at least in the United States).[2,3] To date, only 13 hMPXV1 complete genomes from Italian cases were deposited in public repositories. We therefore sequenced the viral genomes from five cases diagnosed in Milan between June and July 2022, although one of them probably acquired the infection in Spain. We confirm that all five genomes belong to the predominant epidemic lineage. Unfortunately, because of limitations in the availability of biological specimens, we only sequenced viral DNA from one lesion in each patient. The recent sequencing of five lesions from the same Scottish case provided evidence of co-infection with two hMPXV1 variants, suggesting that analysis of multiple lesions can provide more exhaustive information for surveillance purposes (https://virological.org/t/monkeypox-virus-genome-sequences-from-multiple-lesions-indicates-co-infection-of-a-uk-returning-traveller/873).

Analysis of genetic changes that differentiate hMPXV1 genomes from MPXV sequences sampled before 2017 suggested the action of APOBEC3 enzymes, which catalyze the deamination of cytidine to uridine in single-stranded DNA substrates, thus playing a key role in innate antiviral immunity.[2,3] The nucleotide substitutions we observed in the five sequenced genomes are consistent with this view and indicate that APOBEC3-mediated mutations have been accumulating in circulating viruses during the epidemic. The genes affected by such mutations belong to different functional class and it is presently unclear whether these substitutions, as well as those that characterize hMPXV1 genomes, have an effect on viral phenotypes or simply reflect neutral changes with no influence on viral fitness.

Concerning the clinical presentation, all patients presented a typical course of hMPXV1 infection, and we found limited evidence of bacterial superinfection. Clinical data of the single HIV-positive patient seem to confirm previous findings that controlled HIV infection is not a risk factor for a severe course of monkeypox disease.[20]

AUTHOR CONTRIBUTIONS

Diego Forni, Chiara Moltrasio, Manuela Sironi, Angelo Valerio Marzano, and Rachele Cagliani contributed to the conception and study design. Eleonora Quattri enrolled the patients and performed collection of biological samples. Chiara Moltrasio, Alessandra Mozzi, Luigia Venegoni, Marzia Zamprogno, Andrea Citterio, and Rachele Cagliani performed the experimental procedure. Diego Forni, Manuela Sironi, and Rachele Cagliani performed formal analysis, validation, and data curation. Chiara Moltrasio, Diego Forni, Manuela Sironi, and Rachele Cagliani contributed to writing – original draft. Eleonora Quattri, Mario Clerici, and Angelo Valerio Marzano revised the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GISAID at https://gisaid.org/, ID lists are reported in Table 1, and Supporting Information: Table 2.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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