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RESEARCH ARTICLE

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Temporal intra-host variability of mpox virus genomes in multiple body tissues

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Abstract

Whole-genome sequencing (WGS) has been widely used for the genomic characterization and the phylogenesis of mpox virus (MPXV) 2022 multi-country outbreak. To date, no evidence has been reported on intra-host evolution within samples collected over time from a single patient with long-term infection. Fifty-one samples were collected from five patients at different time points post-symptom onset. All samples were confirmed as MPXV DNA positive, amplified by a multiplexed PCR amplicon, and sequenced by WGS. Complete MPXV genomes were assembled by reference mapping and then aligned to perform phylogenetic and hierarchical clustering analysis. Large intra-host variability was observed among the MPXV genomes sequenced from samples of two immunocompromised with advanced HIV-1 infection patients with prolonged MPXV shedding. Overall, 20 nucleotide mutations were identified in the 32 genomes from HIV patients, differently distributed in samples collected from different tissues and at different time points. No sequence compartmentalization nor variation was observed in the three patients with rapid viral clearance. MPXV exhibits adaptation to changing environments within the infected host and consequently demonstrates tissue compartmentalization. Further studies are needed to elucidate the role of this adaptation in forming a pool of genetic variability and contributing to viral persistence and its clinical implications.

KEYWORDS

compartmentalization, genetic evolution, intra-host variability, mpox, MPXV

Martina Rueca and Fabio Giovanni Tucci contributed equally to the study.

1 | INTRODUCTION

Mpox (formerly known as monkeypox) is a zoonotic disease that was once widespread in West and Central Africa and is caused by the species mpox virus (MPXV), a member of the *Orthopoxvirus* genus of the *Poxviridae* family.[1] In May 2022, the World Health Organization (WHO) received the first laboratory-confirmed mpox cases with no epidemiological link to endemic countries.[2] The number of cases had increased significantly in the past few months, arousing public concern. On July 23, 2022, the WHO declared the 2022 MPXV outbreak a public health emergency of international concern, and the situation was constantly monitored.[3] In contrast to previously observed cases outside of endemic countries, during this multi-country outbreak, MPXV spread occurred for the first time with sustained human-to-human transmission, with no epidemiological link to historically endemic regions. Thus, the epidemiological differences of the 2022 outbreak suggested the use of the novel nomenclature to better characterize this new clade (defined B.1) and to avoid offense or economic effect for any ethnic, regional, or other groups.[4] Since the beginning of the MPXV outbreak, more than 86 309 laboratory-confirmed cases in 110 countries have been reported with 107 deaths.[2] The cases primarily involved men who had sex with men (MSM), most of whom were between the ages of 20 and 50 years and had occurred in a multi-country outbreak with no epidemiological link to West and/or Central Africa.[5]

The majority of mpox cases have mild symptoms such as skin eruption and rash.[6,7] Although most patients recover without medical treatment, antiviral agents are strongly advised in cases of severe symptoms, immunocompromised status, or lesions in an unusual anatomical area.[8] Smallpox antiviral drugs such as cidofovir, brincidofovir, and tecovirimat are used in treatment.[9] This latter has been shown to be effective in vitro against the MPXV lineage B.1, which is responsible for the 2022 multi-country outbreak.[10]

The Centers for Disease Control and Prevention reported that lesions on the genital tract were the most common type of damage in MPXV-infected patients, implying that this area was involved in the primary infection.[11] The secondary spread of lesions could result in ulceration in other permissive body districts, as described in an Australian patient who had been followed since the beginning of the infection.[12]

Ulaeto et al. previously published a comment recommending multiple lesions sequencing, particularly after secondary dissemination, to investigate the intra-patient evolution and adaptation of MPXV during a multi-country outbreak.[13] The replicative virus was discovered in a variety of body districts and lesions. Despite previous proposals for compartmentalized replication of MPXV,[13] no evidence of intra-patient variability of MPXV has been reported to date.

MPXV has a 197-kb double-stranded DNA genome that encodes for approximately 190 open reading frames with a central conserved coding region and two variable ends that contain inverted terminal repeats.[14] Although the mutational rate was calculated to be around 10^{-6} nucleotide substitutions per year, 50 nucleotides substitutions were found in the MPXV clade with respect to the previously sequenced MPXV genome, indicating a significantly higher evolutionary rate than other *Orthopoxviruses*.[15] Biologic mechanisms underlying this increased diversity have yet to be identified, although APOBEC3 deaminase editing has been proposed as an important source of variation in MPXV.[16,17]

We present the molecular characterization of MPXV in various body districts and lesions in five male patients diagnosed at the National Institute for Infectious Diseases "Lazzaro Spallanzani" (INMI) between May 2022 and November 2022. The overall goal of whole-genome sequencing (WGS) was to investigate the wide-spectrum tropism of MPXV by studying genetic intra-host variability in multiple tissues.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

Five patients (pts) with MPXV infection diagnosed at the INMI were enrolled in the study (pts #1–5). All were men between 27 and 59 years of age (median age: 39; interquartile range: 6; range: 27–59), three were immune-competent individuals (pts #1–3), while two (pts #4 and #5) were immune-compromised with advanced HIV-1 infection and prolonged MPXV shedding. Overall, 51 samples were collected from May 2022 to January 2023. Specimens were retrieved from different body districts (skin lesions, oropharyngeal swabs-OPS, saliva samples, broncho alveolar lavages-BAL, anal swabs, plasma samples, semen, and ocular swabs), and at different time points post symptom onset (PSO; range: 3–141 days). When referring to skin lesions, swabs from the same lesion were collected over time. Table 1 shows the demographic and virologic information of the enrolled patients and collected samples at different time points from each patient.

The study was included in the MonkeyCohort protocol: "Studio di coorte osservazionale monocentrica su soggetti che afferiscono per sospetto clinico o epidemiologico di malattia del vaiolo delle scimmie (Monkeypox)"; approval number 40z, Register of Non-Covid Trials 2022. The patients provided written informed consent to participate in this study.

2.2 | MPXV DNA detection and WGS analysis

MPXV DNA was detected by using two different commercially available real-time (rt) PCR assays: the Real-Star Orthopoxvirus PCR kit (Altona Diagnostics GmbH) and the Elite InGenius Monkeypox virus rtPCR assay (ELITech Group). Amplification protocols were carried out according to the manufacturer's instructions.

The full genome of MPXV was amplified by a multiplexed PCR amplicon approach by using two pools of primers (totaling 163 overlapping amplicons with a medium length of 2000 bps) according to Chen et al.[18] Libraries were then prepared to start from 10 to 100 ng of DNA using Ion Xpress Plus Fragment Library Kit (Thermo

TABLE 1 Demographic and virologic data of enrolled patients and collected samples.

Patient	Sex	Age	Date of symptoms onset	Treatment	Collection date (PSO)	Sample type	PCR Ct value
Pt #1	Male	39	May 15th, 2022	None	3 days	Shoulder crust	21
						Perianal lesion swab	15
					4 days	Forehead lesion swab	19
						Feces	26
					5 days	OPS	23
					6 days	Saliva	25
					9 days	Penile rod lesion swab[a]	ND
						Anal lesion swab	22
						Semen[a]	ND
Pt #2	Male	27	August 8th, 2022	None	8 days	OPS	16
					11 days	OPS	17
Pt #3	Male	39	September 10th, 2022	Tecovirimat from 9 days PSO	4 days	Chin lesion swab	25
					6 days	Saliva[a]	18
					8 days	Left eye swab	25
Pt #4	Male	59	August 15th, 2022	Tecovirimat from 65 to 79 days PSO Cidofovir from 114 to 122 days PSO	43 days	Perianal lesion swab	18
						Back lesion swab	18
						OPS	25
					49 days	Plasma[a]	29
					62 days	Plasma	27
						Back lesion swab	20
						Tongue lesion swab	19
					67 days	OPS	28
						Saliva	20
					79 days	Plasma[a]	27
					92 days	Perianal lesion swab	24
					94 days	Tongue lesion swab	20
						OPS	26
						Chest lesion swab	25
					95 days	Semen	26
						Plasma	27
					105 days	Perianal lesion swab	14
						Tongue lesion swab	18
						Right buttock lesion swab	18
						OPS	25
					107 days	Plasma	26
					113 days	BAL	16
					120 days	Trachea biopsy	16
					141 days	Anal swab	29
						Chest lesion swab	25

(Continues)

TABLE 1 (Continued)

Patient	Sex	Age	Date of symptoms onset	Treatment	Collection date (PSO)	Sample type	PCR Ct value
Pt #5	Male	33	19th November 2022	Tecovirimat from 8 days PSO	6 days	Plasma[a]	25
					11 days	Plasma	23
						Perianal lesion swab	16
						OPS	21
						Saliva	16
					16 days	Plasma[a]	27
						Anal swab	17
						Anal swab	16
						OPS	30
					33 days	Plasma[a]	24
						OPS	22
						Anal swab	14

Abbreviations: BAL, bronchoalveolar lavage; ND, not determined; OPS, oropharyngeal swab; PSO, post symptoms onset.
[a]Samples that were amplified but did not yield a full-length MPXV sequence.

Fisher Scientific) according to the manufacturer's instructions and sequencing was performed on Gene Studio S5 Prime Sequencer (Thermo Fisher Scientific) to obtain approximately 1 million reads per sample.

2.3 | Genome reconstruction and sequences analysis

Reads longer than 50 nucleotides and with a mean Phred score of at least 20 were mapped on the MPXV genome USA-2022-MA001 (GenBank accession number: ON563414.3) using the bwa-mem aligner software.[19] To obtain the frequency of nucleotides for each position, the successfully aligned reads were processed with Samtools software.[20] Consensus sequences were then reconstructed by using a homemade script, calling a major mutation with a minimum coverage of 50 reads and a minimum frequency of 50%. MPXV WGS collected in September 2022 in Europe were selected from Nextstrain and aligned with the obtained consensus sequences using MAFFT software. Phylogenetic analysis was performed with IQ-TREE; HKY and FreeRate models with empirical base frequencies were selected with ModelFinder. The best tree was found after performing 5000 bootstrap ultrafast replicates.

2.4 | Hierarchical clustering

The hierarchical clustering analysis (HCA) was performed using all positions found mutated in at least one consensus sequence, including all minority variants present in the other samples of the same patient (with a minimum coverage of 50 reads). HCA and graphic elaborations were made using custom script with the Matplotlib library.[21] The Euclidean distance was calculated as a metric distance in conjunction with the ward criterion linkage. Only positions present with sufficient coverage (50×) in at least 50% of the samples were considered.

3 | RESULTS

A total of 51 samples from five patients were amplified by a multiplexed PCR amplicon and sequenced (Table 1). Forty-three (84%) full-length MPXV genomes were obtained: 7 genomes from pt #1 (4 from skin lesions, 1 from OPS, feces, and saliva), 2 from pt #2 (2 OPS), 2 from pt #3 (1 OPS, 1 ocular swab), 23 from pt #4 (8 skin lesions, 4 anal swabs, 3 plasma, 4 OPS, 1 saliva, 1 semen, 1 BAL, 1 trachea biopsy), and 9 from pt #5 (4 anal swabs, 3 OPS, 1 from plasma, 1 saliva). Intra-patient genomes were identical in pt #1 (7 sequences), pt #2 (2 sequences), and pt #3 (2 sequences) regardless of sample type and collection time; thus, for these patients, one genome sequence was selected as representative of the patient and retained for phylogenetic analysis. On the contrary, MPXV genomes sequenced from different samples of pts #4 and 5 presented differences in consensus sequences obtained from analyzed samples of a single patient. In each patient, two mutations are shared among all samples; in contrast, 14 and 3 substitutions (pt #4 and #5, respectively) are distributed among different samples over time.

3.1 | Phylogenetic analysis

As shown in Figure 1 and Supporting Information: Figure 1, all the MPXV sequences belong to the B.1 lineage, with those infecting pt #3 clustered in the B.1.7 sub-lineage. Interestingly, MPXV sequences from each patient clustered separately from those of the others and were interspersed among the foreign and Italian reference strains. Again, pts #4 and #5, which contained the greatest number of MPXV genomes, revealed distinct phylogenetic clusters of their sequences that branched off from a specific initial node with significant bootstrap and demonstrated clear intra-patient evolution.

3.2 | Genetic characterization and hierarchical clustering analysis

As stated above, no intra-host genetic difference among MPXV sequences collected over time from different samples of pts #–3 were seen. On the contrary, high intra-host variability was found in pts #4 and #5. Tables 2A and 2B list the mutations revealed in pts #1–3 (Table 2A), and pts #4 and 5 (Table 2B) as compared to the reference genome. As shown, while the seven MPXV genomes infecting pt #1 were genetically identical to the MPXV-USA-2022-MA001 strain, the sequences from the two OPS of pt #2 were identical to each other, but they differed from the reference strain for

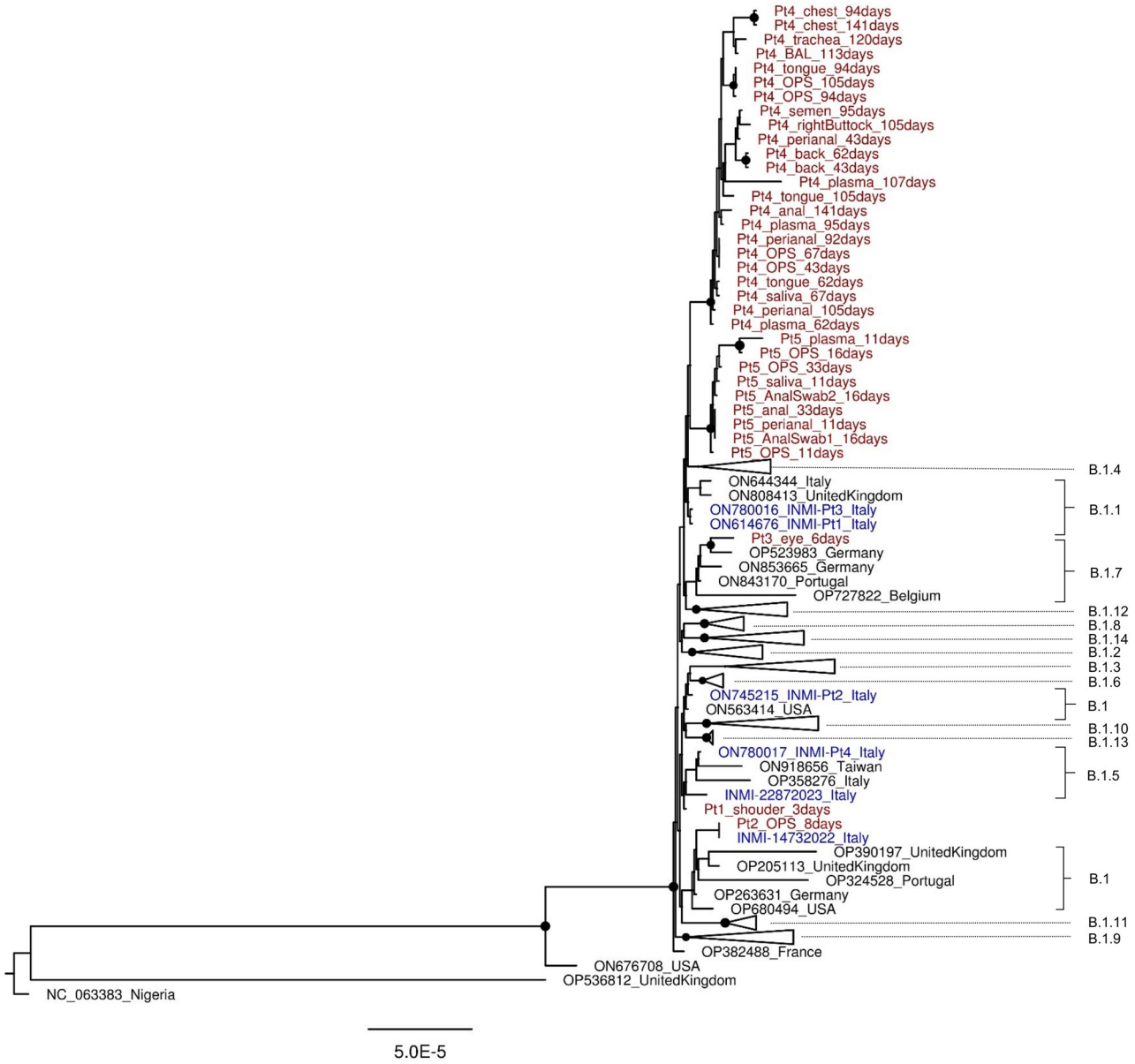
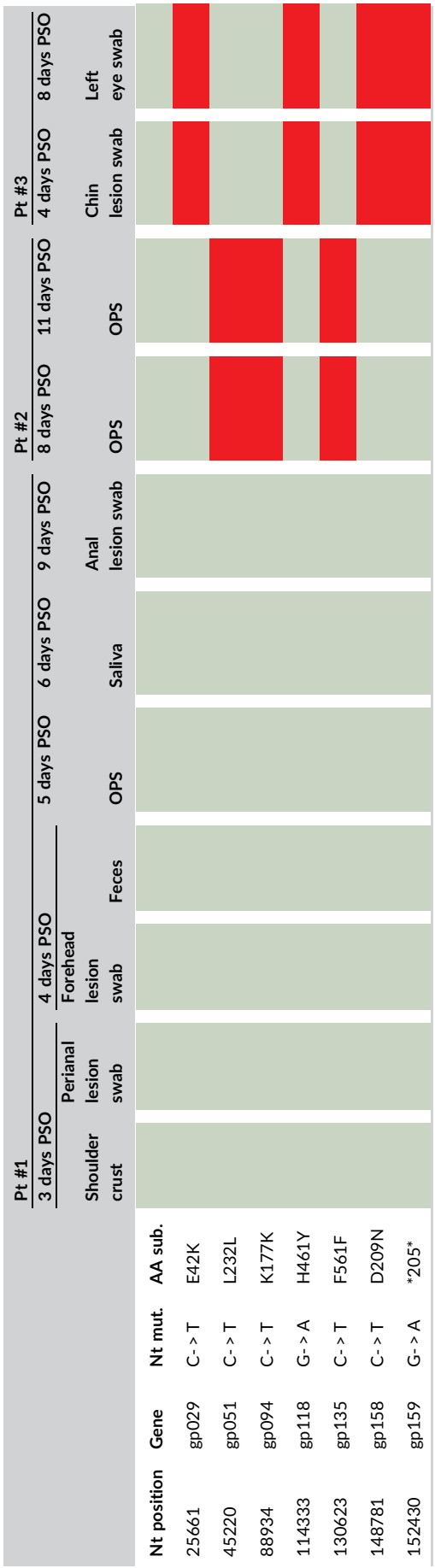


FIGURE 1 Phylogenetic analysis of full-length MPXV genomes. Nodes with a bootstrap value of at least 80 are marked with a black dot. Sequences from the study patients (pts #1–5) are marked in red; other full-length MPXVs sequenced at INMI are marked in blue. Phylogenetic clusters of other MPXV lineages are collapsed. MPXV, mpox virus.

TABLE 2a Differences in consensus sequences from samples of pts #1–3 as compared to MPVX-USA-2022-MA001 reference genome.



Note: In red and in green, presence or absence of the listed mutations as compared to reference genome, respectively.
Abbreviations: AA sub, aminoacidic substitution; Nt mut, nucleotide mutation.

three mutations located in the gp051, gp094, and gp135 genes of the MPXV genome. Also, the two sequences from pt #3 differed from the MPXV-USA-2022-MA001 strain for four mutations involving the gp029, gp118, gp158, and gp159 genes.

The largest intra-host variability was observed among the MPXV genomes sequenced over time in different body samples from pts #4 and #5. Overall, 16 nucleotide mutations (7 synonymous and 9 non-synonymous) were identified in the 23 MPXV genomes from pt #4, with two of them being synonymous (i.e., *205* and I1158I localized in the gp159 and gp182 genes, respectively), and present in all samples. The remaining 14 mutations were differently distributed among samples collected at different time points and located in nine genes: one mutation each in the gp002, gp003, gp044, gp049, gp115, gp118, gp121, and gp179 genes, and 6 mutations in the gp045 gene, encoding for F13L protein, a palmitoylated membrane glycoprotein, that is involved in the wrapping of viral vesicles.

Similar circumstances were seen for pt #5. Overall, his MPXV genomes revealed five mutations (two synonymous and three non-synonymous), two of them (the synonymous T139T mutation in the gp079 gene and the non-synonymous E640K mutation in the gp120 gene) found in all samples, and the remaining three (gp003, gp026, and gp153 genes) in different body specimens.

HCA was performed using MPXV sequences from pts #4 and #5 with the goal of better characterizing the observed genetic intra-host variability and clustering the sequences based on mutational patterns (Figure 2A,B).

As shown in Figure 2A, HCA separated MPXV sequences of pt #4 according to the tissue origin, thus suggesting that, despite some overlaps, genome mutations could be discriminatory among the tissues. MPXV sequences from skin lesions (i.e., chest, back, and right buttock) revealed different sets of synonymous mutations that tended to remain constant over time when considering the specific lesion localization: the chest lesion showed a set characterized by P137P, I82I, L258L, *205*, and I1158I mutations, while the mutational pattern of the back lesion evidenced the presence of S628F, L100L, *205*, and I1158I mutations. The only sequence from the right buttock lesion clustered near those from the back lesion, distinguishing itself from the latter by the presence of non-synonymous Y258C and the absence of synonymous L100L substitutions.

MPXV sequences from the lower respiratory tract specimens (i.e., trachea biopsy and BAL) shared the P137P, *205*, and I1158I mutations, with the sequence from the trachea sample harboring the D294V mutation (also present in BAL as a minority variant).

MPXV genomes found in the upper respiratory tract (i.e., tongue, OPS, and saliva) revealed two distinct mutational patterns: the first one observed in a tongue sample and OPS collected at 94–105 days PSO and characterized by A295E, N267D, *205*, and I1158I mutations; the second observed in respiratory samples (including saliva) collected at 43–67 days PSO and characterized by no majority variant except *205* and I1158I mutation (shared by all pt #4 samples).

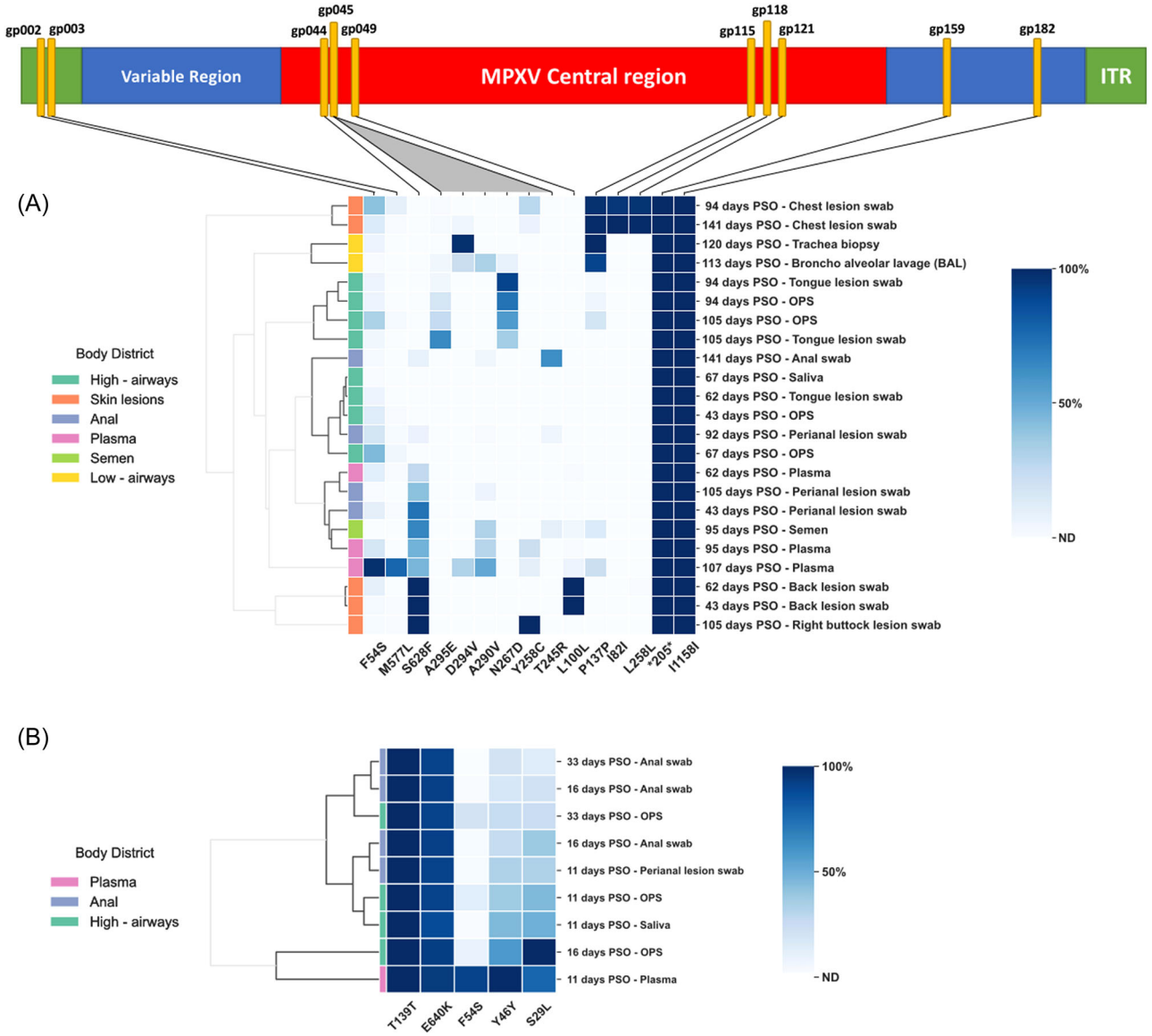


FIGURE 2 Hierarchical clustering analyses of the MPXV sequences from pt #4 (A) and #5 (B). Because the coverage was insufficient for all obtained sequences, the *85* position was excluded from analysis in pt #4. Based on their tissue origin, samples were grouped into six categories: high airways, low airways, skin lesions, anal, plasma, and semen. The heat map represents the percentage of reads supporting the nucleotide mutation. Amino acid mutations were sorted by genomic position as represented in the upper part of the figure. MPXV, mpox virus.

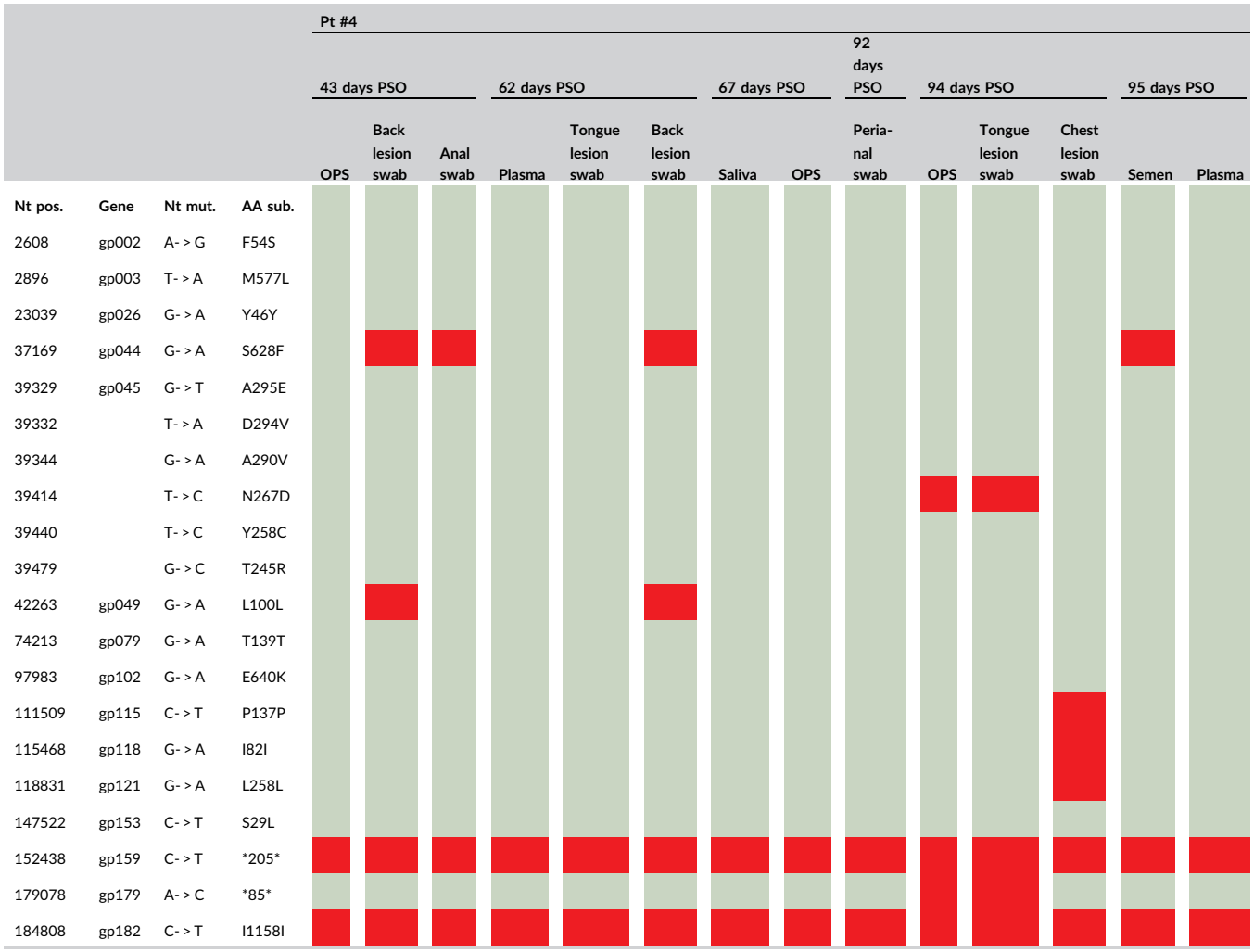
Sequences from plasma (at Days 62 and 95 PSO), semen (at Day 95 PSO), and perianal lesions (at Days 43 and 105 PSO) clustered together, while the sequence from plasma collected at Day 107 PSO harbored a unique set of mutations (i.e., F54S, M577L, A290V, *205*, and I1158I). Moreover, as shown in Figure 3, the frequency of the various mutations varied with some increase over time (i.e., A295E in tongue lesion swabs; T245R in perianal and anal lesion swabs samples; F54S and A290V in plasma samples) while others considerably decreased (i.e., S628F in perianal and anal lesion swabs samples; N267D in tongue lesion swabs).

Figure 2B depicts the HCA of nine pt #5 sequences. Also, in this case, the analysis revealed genetic clusters based on the tissue compartmentalization of the sequences and their time of collection. Interestingly, the sequence from the plasma sample collected at 11-day PSO contained all the mutations (the synonymous T139T, Y46Y, S29S, and the non-synonymous E640K, F54S) that were present in all the other samples, even if at a lower frequency.

4 | DISCUSSION

Long-term follow-up studies in patients with MPXV infection are scarce, and the intra-host evolution of the virus has never been extensively evaluated before. Here, we focused on the analysis of complete viral sequences obtained from multiple body tissues collected over time from five infected patients, three of whom were

TABLE 2b Differences in consensus sequences from samples of pts #4 and 5 as compared to MPVX-USA-2022-MA001 reference genome



In red and in green, presence or absence of the listed mutations as compared to reference genome, respectively.
Abbreviations: AA sub, aminoacidic substitution; Nt mut, nucleotide mutation.

immunocompetent (pts #1–3) and two immunocompromised due to advanced HIV-1 infection (pts #4 and #5), belonging to a population that represents 38%–50% of 2022 multi-country outbreak affected individuals.[22] These latter patients exhibit prolonged MPXV shedding with viral DNA detected in the different clinical samples up to 141 and 33 days PSO for pts #4 (Supporting Information: Figure 2) and #5, respectively. On the contrary, pts #1–3 rapidly cleared the virus, and the samples characterized by viral load values and residual volumes sufficient for WGS analysis ranged between 3 and 11 days PSO (Table 1). Overall, our findings demonstrate intra-host evolutionary dynamics and tissue compartmentalization during MPXV infection. Intra-host MPXV sequences were found with different mutations as compared to the reference genome in different samples from immunocompromised HIV-infected patients that exhibit prolonged viral shedding. Recent data showed that in patients with advanced HIV infection and poor $CD4^+$T cell count, a severe necrotizing form of mpox could occur, also characterized by a prolonged course.[22,23] The very protracted (over 6 months) course of mpox in our pt #4, with an exceptionally long-lasting viral detection from different sites, due to the permissive effect of severe immunodeficiency, was likely the driver of the observed MPXV genomic variability. Recently immune signature of MPXV infection in patients during the acute phase was described.[24] In immunocompetent mpox cases, even regardless of HIV infection, an early expansion of activated effector CD4+ and $CD8^+$T cells and a poxvirus specific Th1 cell response persisted over time. It is conceivable that in our pts #4 and #5, the advanced stage of HIV disease may have conditioned an impaired MPXV-specific immune response, favoring viral persistence, compartmentalization, and the development of genomic mutations.

Such conditions lead to independent MPXV evolution in different body districts, contributing to compartmentalized replication. Some mutations are common to all samples and others are located in MPXV genomes infecting specific body tissues and/or dynamically evolving over time. Additionally, the existence of a mix of minority variants with mutations present at very low frequency was highlighted, thus

suggesting that intra-host evolution of MPXV can occur in the form of a quasi-species, as already seen for other DNA viruses,[25,26] and also some evidence of intra-host MPXV genome variations was observed.[27] Although the triggers inducing these mutations are still to be identified, our results illustrate MPXV adaptation to a continuously changing environment within the infected host and consequently demonstrate that tissue compartmentalization of MPXV can exist, mainly in patients with prolonged infections. It will be important to understand if this adaptation plays a significant role in forming a pool of genetic variability, thus contributing to viral persistence. These novel concepts in MPXV infection mainly derive from the analysis of pt #4 sequences, which have been widely sampled for a long time. MPXV genomes found in differently located skin lesions exhibit different mutations that remain stable over time when multiple time points of the same lesion are examined. This finding is particularly intriguing because the evidence of specific mutations strongly supports the hypothesis that single skin lesions sustain clonal viral replication, as previously suggested.[13] Additional evidence about the MPXV compartmentalization can be inferred from mucosal samples taken from the upper (tongue, saliva, and oropharyngeal) and lower (trachea biopsy and BAL) respiratory tracts, and the anal region. In fact, the presence of a specific mutational pattern was observed in each specimen source, resulting in distinct phylogenetic clusters and thus again suggesting that forces driving MPXV evolution can be compartment dependent. This idea is reinforced by the observation that the evolution of some mutations in terms of an increase in frequency over time occurs only in certain tissues.

Although less numerous and collected in a shorter period, the analysis of viral sequences from pt #5 confirms the above findings, thus suggesting that MPXV compartmentalization may be a general phenomenon. Even if no specific tissue mutation is observed, the variability of viral genomes in different districts from pt #5 is clear, as is tissue clustering by cluster analysis.

Again, interestingly, our results reveal that the intrasample variability is generally higher in plasma than in other samples, both in pts #4 and #5. This evidence supports the fact that a continuous genetic flow between body compartments can commonly exist and

FIGURE 3 Frequency of the mutations observed over time in samples from OPS, tongue lesion swabs, perianal, and anal lesion swabs of pt #4.

therefore suggests that blood may act as a collector of the viral quasi-species circulating in the infected host.

Finally, the possible role of antiviral therapy in increasing genomic variability must be considered. The gp045 gene, encoding the F13L protein that is the target of the antiviral agent tecovirimat,[28,29] harbors more substitutions than the other genes (6; 37.5% vs. 1; 7% for the other genes) in genomes obtained from pt #4. Mutations in the gp045 gene were discovered in pt #4 samples from Day 94 PSO (OPS, tongue lesion swabs, anal swabs, and trachea biopsy). Since the patient was treated with tecovirimat from Days 65 to 79 PSO, this evidence could lead to the hypothesis that the emergence of such substitutions is driven by the selective pressure induced by treatment. However, very little is known about tecovirimat's mode of action and interaction with the F13L protein, and no evidence about the in vivo emergence of the MPXV resistance mutation exists so far, even though recently FDA raised the concern that small changes to the VP37 protein (F13L homolog) could have a large impact on the antiviral activity of tecovirimat, suggesting for this drug a low genetic barrier to the development of viral resistance.[30] According to this observation, previous studies have described in-vitro and in-vivo tecovirimat resistance substitutions in the F13L protein-coding region in other *Orthopoxvirus* genomes.[31]

5 | CONCLUSIONS

Taken together, our results support the notion of structured compartmentalization of the viral populations circulating within an MPXV-infected host and they point out the role of the immunological status of the patient in favoring the evolution of such phenomenon. Immunocompromised status and prolonged viral shedding led to a different evolution of MPXV in different body districts, and compartmentalized replication may be observed with the presence of specific mutations in lesion samples collected over time. This observation suggests that a clonal viral replication occurs in skin lesions. The virus also appears prone to mutate in the other body districts, and the appearance of specific genetic mutations and common minority variants suggests a compartmentalized replication. Of note, different factors external to the virus should be taken into consideration and could influence its evolution in different districts over time. Antiviral therapy may have a role in increasing the selective pressure; also, the viral interaction with the microbiome of the different districts under examination should also be considered in this regard. Moreover, the possible role of APOBEC3, upregulated in HIV-infected patients, should be elucidated in favoring intra-host evolution in immunocompromised status due to HIV advanced infection with poor $CD4^{+}$T cell count.[16] Overall, these findings open perspectives for further studies which are of extreme importance to more extensively investigate MPXV infection, its genomic intra-host variability, and possible clinical and therapeutic implications of this genomic variability.

AUTHOR CONTRIBUTIONS

Martina Rueca, Emanuela Giombini, Giulia Matusali, and Fabrizio Maggi conceived the study and developed the study protocol. Enrico Girardi and Francesco Vaia provided oversight and supervision. Martina Rueca, Giulia Gramigna, Lavinia Fabeni, Ornella Butera, Eliana Specchiarello, and Fabrizio Carletti did the molecular assays and sequencing. Valentina Mazzotta, Carmela Pinnetti, Andrea Mariano, Simone Lanini, and Annalisa Mondi collected the clinical data. Fabio Giovanni Tucci, Cesare Ernesto Maria Gruber, and Emanuela Giombini did the bioinformatic analyses of the data. Martina Rueca, Cesare Ernesto Maria Gruber, and Fabrizio Maggi wrote the original draft of the manuscript. Enrico Girardi, Emanuele Nicastri, and Andrea Antinori critically reviewed the manuscript. All authors had access to the data in the study and had final responsibility for the decision to submit for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All sequences generated and analyzed during the current study are available in the NCBI repository. The complete MPXV genome sequences from pts #1 to 3 were deposited on GenBank under the Accession numbers OQ627926, OQ627927, and OQ627928, respectively; while the raw sequencing data of the samples collected from pts #4 and 5 were deposited on Sequence Read Archive (SRA) under the BioProject's ID PRJNA941173. Deidentified data that underlie the results reported in this article will be made available on request. Proposals should be directed to the corresponding author.

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REFERENCES

1. Focosi D, Novazzi F, Baj A, Maggi F. Monkeypox: an international epidemic. *Rev Med Virol*. 2022;32:e2392.
2. WHO. 2022-23 Mpox (Monkeypox) Outbreak: Global Trends. https://worldhealthorg.shinyapps.io/mpx_global/
3. WHO. WHO Director-General Declares the Ongoing Monkeypox Outbreak A Public Health Emergency of International Concern. July 23, 2022. https://www.who.int/europe/news/item/23-07-2022-who-director-general-declares-the-ongoing-monkeypox-outbreak-a-public-health-event-of-international-concern
4. Ulaeto D, Agafonov A, Burchfield J, et al. New nomenclature for mpox (monkeypox) and monkeypox virus clades. *Lancet Infect Dis*. 2023;23(3):273-275.
5. CDC. Mpox Cases by Age and Gender and Race/Ethnicity. 2023. https://www.cdc.gov/poxvirus/monkeypox/response/2022/demographics.html
6. Thornhill JP, Barkati S, Walmsley S, et al. Monkeypox virus infection in humans across 16 countries – April-June 2022. *N Engl J Med*. 2022;387:679-691.
7. Antinori A, Mazzotta V, Vita S, et al. Epidemiological, clinical and virological characteristics of four cases of monkeypox support transmission through sexual contact, Italy, May 2022. *Euro Surveill*. 2022;27(22):2200421.
8. Rizk JG, Lippi G, Henry BM, Forthal DN, Rizk Y. Prevention and treatment of monkeypox. *Drugs*. 2022;82(9):957-963.
9. Khani E, Afsharirad B, Entezari-Maleki T. Monkeypox treatment: current evidence and future perspectives. *J Med Virol*. 2023;95(1):e28229.
10. Huang Y, Mu L, Wang W. Monkeypox: epidemiology, pathogenesis, treatment and prevention. *Signal Transduct Target Ther*. 2022;7(1):373.
11. CDC. Monkeypox Virus Infection in the United States and Other Non-endemic Countries—2022. https://emergency.cdc.gov/han/2022/han00466.asp
12. Hammerschlag Y, MacLeod G, Papadakis G, et al. Monkeypox infection presenting as genital rash, Australia, May 2022. *Euro Surveill*. 2022;27(22):2200411.
13. Ulaeto DO, Dunning J, Carroll MW. Evolutionary implications of human transmission of monkeypox: the importance of sequencing multiple lesions. *The Lancet Microbe*. 2022;3(9):e639-e640.
14. Kugelman JR, Johnston SC, Mulembakani PM, et al. Genomic variability of monkeypox virus among humans, Democratic Republic of the Congo. *Emerging Infect Dis*. 2014;20(2):232-239.

15. Vaughan AM, Cenciarelli O, Colombe S, et al. A large multi-country outbreak of monkeypox across 41 countries in the WHO European Region, 7 March to 23 August 2022. *Euro Surveill*. 2022;27(36):1-9.
16. Dumonteil E, Herrera C, Sabino-Santos G. Monkeypox virus evolution before 2022 outbreak. *Emerging Infect Dis*. 2023;29(2): 451-453.
17. Wang L, Shang J, Weng S, et al. Genomic annotation and molecular evolution of monkeypox virus outbreak in 2022. *J Med Virol*. 2023;95(1):e28036.
18. Chen NFG, Gagne L, Doucette M, et al. Monkeypox Virus Multiplexed PCR Amplicon Sequencing (PrimalSeq) V.4. https://www.protocols.io/view/monkeypox-virus-multiplexed-pcr-amplicon-sequencin-5qpvob1nbl4o/v4
19. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14): 1754-1760.
20. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079.
21. Hunter JD. Matplotlib. A 2D graphics environment. *Comput Sci Eng*. 2007;9:90-95.
22. Mitjà O, Alemany A, Marks M, et al. Mpox in people with advanced HIV infection: a global case series. *Lancet*. 2023;401(10380): 939-949.
23. Nunez I, Valdez-Ferrer SI. Fulminant mpox as an AIDS-defining condition: useful for stigmatising? *Lancet*. 2023;401(10380): 881-884.
24. Agrati C, Cossarizza A, Mazzotta V, et al. Immunological signature in human cases of monkeypox infection in 2022 outbreak: an observational study. *Lancet Infect Dis*. 2023;23:320-330.
25. Trimpert J, Groenke N, Kunec D, et al. A proofreading-impaired herpesvirus generates populations with quasispecies-like structure. *Nat Microbiol*. 2019;4:2175-2183.
26. Nishizawa T, Okamoto H, Tsuda F, et al. Quasispecies of TT virus (TTV) with sequence divergence in hypervariable regions of the capsid protein in chronic TTV infection. *J Virol*. 1999;73:9604-9608.
27. Vauhkonen H, Kallio-Kokko H, Hiltunen-Back E, et al. Intrahost monkeypox virus genome variation in patient with early infection, Finland, 2022. *Emerging Infect Dis*. 2023;29(3):649-652.
28. Frenois-Veyrat G, Gallardo F, Gorgé O, et al. Tecovirimat is effective against human monkeypox virus in vitro at nanomolar concentrations. *Nat Microbiol*. 2022;7:1951-1955.
29. Mondi A, Gagliardini R, Mazzotta V, et al. Clinical experience with use of oral Tecovirimat or intravenous Cidofovir for the treatment of monkeypox in an Italian reference hospital. *J Infect*. 2023;86:66-117.
30. U.S. FDA. FDA Mpox Response. https://www.fda.gov/emergency-preparedness-and-response/mcm-issues/fda-mpox-response
31. Duraffour S, Lorenzo MM, Zöller G, et al. ST-246 is a key antiviral to inhibit the viral F13L phospholipase, one of the essential proteins for orthopoxvirus wrapping. *J Antimicrob Chemother*. 2015;70(5): 1367-1380.

SUPPORTING INFORMATION

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

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