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Epidemiology and phylogenomic characterization of the Clade IIb C.1 Mpox outbreak in Phnom Penh, Cambodia (2023–2024)

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ABSTRACT

Mpox is an infectious disease caused by the Monkeypox virus, which is divided into two main genetic clades: Clade I and Clade II. A large-scale outbreak linked to Clade IIb emerged in 2022 and rapidly spread to more than 100 countries worldwide. Here, we describe the first and only documented Mpox outbreak in Cambodia (2023-2024), the public health outbreak response efforts and analysis, and integrating epidemiological and genomic approaches.

To investigate the outbreak, samples from suspect cases were confirmed using qPCR before virus whole genome sequences were obtained for phylogenomic analyses.

Epidemiological investigation revealed transmission primarily through intimate contact within socially connected networks, exclusively among men who have sex with men. None of the confirmed cases reported recent international travel or zoonotic exposure. Phylogenomic analysis showed that all Cambodian Mpox genomes belonged to lineage C.1, nested within Clade IIb. Bayesian analysis of publicly available C.1 genomes indicated that the most closely related sequence was from Thailand. Monophyletic clustering of Cambodian sequences, alongside a high proportion of APOBEC3 mutations, indicates localized human-to-human transmission after introduction.

Altogether, these results illustrate the risk of regional lineages like C.1 introducing Mpox into previously unaffected countries, where socially connected human networks can sustain outbreaks despite control efforts.

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Introduction

Mpox is a viral infectious disease caused by the Monkeypox virus (MPXV), a member of the *Orthopoxvirus* genus within the *Poxviridae* family [1, 2]. First detected in 1958 in imported laboratory monkeys in Denmark, the virus was later found to infect humans, with the first human case reported in 1970 in the Democratic Republic of Congo (DRC) [3–5]. For most of its history, Mpox emerged sporadically through zoonotic spillover events, jumping from animals to humans and causing localized outbreaks in West and Central Africa [6–9]. Human-to-human transmission was rare and short-lived [10]. However, this changed in 2022 when a global outbreak, originating from a West African lineage, enabled the virus to spread beyond its historical boundaries. Unlike previous outbreaks, this one was driven by sustained human-to-human transmission, particularly within sexual networks of men who have sex with men (MSM) [10]. Comparable patterns of sustained transmission have since been observed in Central Africa, reflecting a broader shift in the virus's epidemiology [11, 12].

Genomic analysis has shown that MPXV diverged into two main clades: Clade I (historically, Congo Basin clade) and Clade II (historically, West African clade), with both Clades further divided into subclades a and b [12, 13]. The 2022 global outbreak was caused

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by subclade IIb viruses characterized by accelerated evolution driven by APOBEC3 (apolipoprotein B mRNA editing enzyme) deaminases [14, 15]. This group of conserved cytidine deaminases plays an essential role in the innate immune system by targeting single-stranded DNA and causing TC-to-TT mutations [15]. By mid-2023, lineage C.1 and lineage B.1.20 emerged as the dominant strains within subclade IIb and have continued to be the dominating circulating lineages since then [16]. Lineage B.1.20 is primarily found in the United States, whereas C.1, which includes the C.1.1 sublineage, is mostly identified in East Asia (South Korea and Japan) and parts of Europe (Portugal and Germany) [16, 17]. Many C.1 strains, however, are not classified as part of the C.1.1 sublineage [17].

Despite these global insights, genomic and epidemiological data from Southeast Asia remain limited, with Cambodia lacking any historical record of Mpox infection. This gap highlights a critical need to understand how MPXV is introduced, transmitted, and evolves in newly affected populations. The recent emergence of 20 local cases in Cambodia, reported between December 11, 2023, and May 13, 2024, provides a unique opportunity to address this gap and generate locally relevant data. In doing so, this study aims to characterize the demographic and clinical features of these cases and analyse the transmission dynamics of the outbreak, thereby providing critical insights into MPXV evolution and public health implications in the region.

Material and methods

Ethical approval

The study was reviewed and approved by the Cambodian National Ethics Committee for Health Research (NECHR) (reference number: N° 203 NECHR) and conducted in accordance with the Declaration of Helsinki. All virus genetic sequences and associated information included in this study were obtained as part of national outbreak response and public health surveillance activities. All analyses were conducted using pseudonymized data and complied with Cambodian ethical standard allowing exemption from obtaining informed consent requirement.

Study design and population

This retrospective observational study included all laboratory-confirmed MPXV cases reported in Cambodia between December 11, 2023, and May 13, 2024. While the total number of reported cases was small (n = 20), these data capture the full epidemiological extent of this locally sustained outbreak in the country.

Sample collection, metadata collection and Mpox diagnosis

As part of a national effort to monitor Mpox, samples from suspected Mpox cases and symptomatic close contacts were collected and transported to the National Institute of Public Health (NIPH) for diagnostic testing and genomic surveillance. Sample types included swabs from skin lesions (crusts, pustules, vesicles and fluids) and the oropharynx. All samples of positive cases were referred to the Virology Unit, Institut Pasteur du Cambodge (IPC) for confirmation and virus whole genome sequencing. At IPC, viral DNA was extracted using the QIAamp DNA Mini kit (Qiagen) as per manufacturer's instructions. Diagnosis was carried out using real-time polymerase chain reaction (PCR) with MPXV-specific primers and probes, following previously described methods [18]. Samples with a cycle threshold (Ct) value under 40 were considered Mpox-positive and stored at – 20°C for further viral whole genome sequencing (*vide infra*). A total of 20 Mpox cases were confirmed, from which 27 distinct samples were collected. The national surveillance initiative also included standardized metadata collection via a national case investigation form. Data on confirmed cases were retrieved from the "go.Data" outbreak database maintained by the Cambodian Ministry of Health, spanning December 2023 to May 2024, when the first locally acquired Mpox cases were reported. Metadata included demographics, clinical information, sexual behaviour history (past three months), travel history and close contact information. Additional clinical details were sourced from Techo Santepheap Hospital. All these data are available in Supplementary Table 1.

Viral whole genome sequencing and bioinformatic processing

MPXV whole genome sequencing was conducted using an amplicon-based enrichment approach based on the primer scheme described by Welker et al., followed by library preparation [19]. The final library was loaded on a R10.4.1 Flow Cell (FLO-MIN114, Oxford Nanopore Technology) and sequenced on a GridION sequencer (Oxford Nanopore Technology). Post-sequencing, bioinformatic analysis was performed using the nextflow-driven pipeline developed by the ARTIC network, specifically designed for Mpox, to construct reference-based consensus genomes (https://github.com/artic-network/artic-mpxv-nf). In summary, the raw signals ("squiggles") from the GridION sequencer were converted into raw reads using Guppy v7.1.4 (available on https://community.nanoporetech.com), with basecalling model r1041_e82_400bps_hac_v4.2, and these reads were assigned to their respective samples. The raw reads

were subsequently processed with fastp v0.20.1, trimming them to >200 bp and removing adapters and low-quality bases (Phred quality score < 7) [20]. Trimmed reads were then aligned to the Clade II reference genomes (accession number: NC_063383) using minimap2 v2.28 [21]. Primer sequences were soft-clipped based on the provided BED file, producing primer-trimmed BAM files that served as the basis for both variant calling and consensus generation. Variant calling was performed using Medaka v1.12.0, and the final consensus genomes were generated with bcftools v1.17 [22]. Mutations were kept only if they met stringent quality thresholds: a minimum base quality of 30, read depth of at least 30x and variant agreement of at least 80%. A total of 27 high-quality Mpox consensus genomes were recovered. Lineage assignment using Nextclade v3.10.0 classified all genomes as subclade IIb.C.1 MPXV; however, no sublineage was assigned [23]. For further phylogenomic analysis, one genome per patient was selected based on the highest horizontal coverage, forming the Cambodian C.1 MPXV dataset ($n = 20$) used for further analyses. Finally, sequencing results for the Cambodian MPXV outbreak samples, such as vertical and horizontal coverage, read counts and clade assignments, can be found in Supplementary Table 2.

Phylogenomic analysis of subclade IIb MPXV genomes

Published subclade IIb MPXV reference genomes were sourced from NCBI GenBank on November 11, 2024. To build a high-quality (HQ) reference dataset, we removed duplicates, along with genomes with horizontal coverage below 90% (more than 10% ambiguous bases), those flagged by IQ-TREE v2.3.6, and genomes lacking metadata for location and collection date [24]. HQ genomes representing the three major lineages (A, B, and C) were subsampled to 46 genomes per lineage using seqkit v2.8.2, allowing us to trim the data while still capturing the broad diversity of subclade IIb genomes [25]. The final dataset, consisting of 158 genomes (138 curated references and 20 Cambodian C.1 MPXV genomes) was used to generate a high-quality MPXV alignment using Squirrel v1.0.12 (https://github.com/aineniamh/squirrel). This process began by aligning the genome to the Clade II reference genome (accession number: NC_063383) to establish a single coordinate system using minimap v2.17 [21]. This alignment was further refined by trimming the 3' terminal repeat regions and masking repetitive and low-complexity regions. The maximum likelihood (ML) phylogeny was then constructed using IQ-TREE v2.3.6, with ModelFinder selecting the 'K3Pu + F + I + R5' substitution model as best fit [24]. An MPXV subclade IIa reference genome (accession number: KJ642615) served as the outgroup. Branch support was calculated through ultrafast bootstrap approximation with 10,000 replicates [26]. The final tree was visualized in FigTree v1.4.4 [27].

APOBEC3 reconstruction

Apolipoprotein B mRNA-editing enzyme (APOBEC3) are cytosine deaminases that play a crucial role in the human immune response, with APOBEC3-associated mutations serving as a hallmark signature of sustained human-to-human viral transmission [15]. To identify this mutational signature, the APOBEC3 module of Squirrel v1.0.12 was run to construct a phylogeny of the final MPXV dataset and perform ancestral state reconstruction. This allowed the mapping of each single nucleotide polymorphisms (SNP) to its predicted branch on the phylogeny, where nucleotide changes were classified as APOBEC3 (TC-to-TT or its reverse complement, GA-to-AA) or non-APOBEC3 (resulting from polymerase errors during replication).

Temporal signal and Bayesian spatiotemporal analysis of C.1 MPXV genomes

For in-depth spatiotemporal analysis of the C.1 outbreak, all published C.1 MPXV reference genomes were retrieved from NCBI GenBank on November 11, 2024. These references were polished using the same quality metrics applied earlier, and no subsampling was performed, ensuring that all available published genomes were included in the Bayesian analyses. These were then combined with Cambodian consensus genomes, resulting in a final dataset of 328 genomes (Supplementary Table 3). An alignment was generated using Squirrel v1.0.12, as described previously. Following this, an APOBEC3 partition was extracted using a3partitioner v0.1.0, focusing on sites with APOBEC3 modifications [28]. This approach helped minimize false positive SNPs (due to sequencing or bioinformatic errors), as most mutations over short timescales should be consistent with APOBEC3 activity, as noted by O'Toole and colleagues [15]. Temporal signal in the APOBEC3 partition was established by supplementing the conventional root-to-tip distance analysis with Bayesian Evaluation of Temporal Signal (BETS) [29]. Log marginal likelihoods were estimated using generalized stepping-stone sampling (GSS), with an initial Markov chain length of 10 million, 50 path steps, and a chain length of 100,000 iterations (Supplementary Table 4) [30]. Bayesian spatiotemporal dynamics of the (Cambodian) C.1 outbreak were investigated in BEAST v1.10.4 under a strict clock (GTR + G substitution model) and a skyline coalescence model [31–34]. Three chains of 100 million states were run (sampling every 10,000 states) to ensure convergence. The

independent chains were combined using LogCombiner v1.10.4, and convergence was assessed with Tracer v1.7.2 [35]. A maximum clade credibility (MCC) tree was generated from the posterior tree distribution using TreeAnnotator v1.10.4 (https://beast.community/treeannotator), with node heights scaled to the median values and a 10% burnin fraction (Supplementary Table 4).

Mutational profiling

Phylodynamic analysis indicated that Cambodian C.1 MPXV genomes were most closely related to Thai reference genomes. Mutational profiling involved aligning the Cambodian genomes with the Thai genomes using Squirrel v1.0.12, followed by the identification of two uniquely conserved SNPs (coverage > 100x, agreement > 95%) with snipit v1.6. Squirrel maps each genome against the RefSeq reference (accession number: NC_063383), ensuring that all alignment coordinates are relative to this reference. Conserved SNPs were defined as those present in Cambodian but absent in closely related Thai genomes. Squirrel also determines the coding position of each reconstructed mutation, and when applicable, the resulting non-synonymous amino-acid change. Next, Grantham scores were then assigned to estimate the potential biochemical impact of each change. A detailed list of conserved SNPs, including the positions, annotation and gene function are provided in Supplementary Table 5. Finally, mutational profiling of the identified lineages (C.1 and its C.1.1 sublineage) was performed using the B1.3 reference (accession number: ON983168) genomes (Supplementary Table 6).

Code and data availability

Bioinformatic processing of raw reads was performed using the artic-mpxv-nf pipeline v1.4.0, available at https://github.com/artic-network/artic-mpxv-nf. The Python script used to generate the APOBEC3 partition, along with a comprehensive documentation that guides its usage, is accessible at https://github.com/DaanJansen94/a3partitioner.16,28 This partition served as input for temporal signal evaluation and Bayesian spatiotemporal analysis.

Data availability

Supplementary Table 1 contains the clinical metadata. The raw sequence data have been submitted to the NCBI Sequence Read Archive (SRA) and can be accessed with the BioProject accession number PRJNA1270390. The Cambodian MPXV consensus genomes have been deposited to Pathoplexus and the International Nucleotide Sequence Database Collaboration (INSDC) and are accessible with BioProject accession number PRJEB90079.

Results

Public health responses and rapid mobilization following the emergence of Mpox in Cambodia

On December 11, 2023, Cambodia was confronted with its first confirmed case of Mpox, setting in motion a rapid and coordinated public health response to mitigate the risk of further transmission. Within 48 h, the Ministry of Health issued a public statement confirming the case and initiating national-level response measures. Following this announcement, the Communicable Disease Control Department (CDC) held two virtual meetings with national and sub-national Rapid Response Teams (RRTs) to review the clinical presentation of Mpox, transmission routes, protective measures, surveillance protocols, sample collection and case investigation procedures.

As part of the continued national response, the CDC organized an educational campaign on December 28, 2023, targeting healthcare providers from Men's Health Cambodia, Family Clinic and RRTs at the Phnom Penh Municipal Health Department. During this session, reporting procedures for suspected and probable Mpox cases were introduced, alongside the establishment of an Incident Management Structure for Mpox and standardized protocols for sample collection and transportation. Additionally, the risk communication team developed and disseminated educational material, including posters, to raise awareness in high-risk areas. With these preparedness measures in place, the response transitioned to active case management and containment. All confirmed cases were isolated and received supportive care at a designated hospital in Phnom Penh. Patients were interviewed for contact tracing. Many cases did not report contacts within the 21-day incubation window. Close contacts of the confirmed cases were monitored at home for symptoms over 21 days post-exposure and tested if symptomatic. No post-exposure prophylaxis was administered.

To support case detection and management, NIPH was designated as the national Mpox testing centre, while IPC conducted viral genomic sequencing. Hospital departments implemented Mpox-specific protocols for case management and infections control. Collaborative efforts among the CDC, non-governmental organizations, private sectors and the National Centers for HIV/AIDS, Dermatology and STD (NCHADS) strengthened activate case detection and facilitated educational outreach targeting high-risk populations. The national surveillance initiative also incorporated standardized metadata collection

through a national case investigation form, facilitating coordinated epidemiological analysis.

Epidemiological and clinical characteristics of the 2023–2024 Cambodian Mpox outbreak

Building on coordinated national efforts, the epidemiological and clinical profile of the 2023–2024 Cambodian Mpox outbreak was examined. During this period, 48 suspected cases were tested at the NIPH, with 20 confirmed positive. The first confirmed case was identified late December, from a sample collected on December 11, although symptoms began in early November. All confirmed cases were males and identified as MSM, with a median age of 31.6 years (range 20–42). All were single and most cases (95%) resided in Phnom Penh, with one case reported in Kampot Province.

All patients reported intimate or sexual activities within three weeks prior to symptom onset, with an average of 2.8 partners (range 1–10). None reported contact with confirmed Mpox cases. In the three weeks following symptom onset, patients recalled an average of 4.3 close contacts (range 1–10), including 1.7 intimate or sexual contacts. Indirect contacts consisted of an average of 1.5 household contacts (range 0–8), 0.5 friends (range 0–10) and 0.4 co-workers (range 1–4).

Over the preceding three months, all patients reported similar sexual activity levels, averaging 2.8 partners, involving oral-penile and penile-anal exposure. One individual (5%) had participated in group sexual activity. Dating app usage was reported by nearly half the cases, with Grindr (45%), Messenger (10%), Instagram (5%) and other platforms (33%) cited. None of the cases reported animal contact or international travel within three weeks prior to symptom onset. Suspected exposure sites included private residences (31%), spas (23%), guesthouses (7%) and unknown locations (46%).

Following exposure, all cases went on to develop symptoms. The clinical presentation was consistent across patients, with every individual experiencing skin lesions and rash. Other common symptoms included fever (80%), lymphadenopathy (75%), chills and pruritus (60%), headache (50%) and myalgia (45%). Less frequent manifestations were rectal pain (25%), rectal bleeding (15%), cough (10%), back pain (10%), tenesmus (10%), conjunctivitis (5%), abdominal pain (5%) and sore throat (5%). Initial lesion sites appeared most often on the genital area (50%), mouth and lips (20%), perianal region (10%) and hands (10%).

As the illness progressed, lesions spread to other body areas: genitals (85%), legs (30%), trunk (25%), arms (20%), perianal area (25%), palm (25%), face (20%), mouth and lips (25%), neck (15%), and head (15%). Lesion counts varied widely, with 55% of patients presenting 1–9 lesions, 40% having 10–49, and one patient (5%) experiencing over 100 lesions.

Patient management consisted exclusively of supportive care, including symptom relief, wound care, hydration, and monitoring of complications, as no Mpox-specific antiviral treatments were administered. One individual (5%) was on HIV pre-exposure prophylaxis. Fourteen patients (70%) were HIV positive, 12 previously diagnosed and received antiretroviral therapy (ART), while 2 were newly diagnosed during their Mpox infection and had not yet started treatment. Among those on ART, 2 had suppressed viral loads (<1,000 copies/mL), 4 had undetectable viral loads (<40 copies/ml), and 6 were unaware of their current viral load status.

Coinfections with other sexually transmitted diseases were also noted, with syphilis diagnosed in 6 patients (31%) and gonorrhea in 1 patient (5%). The median time from symptom onset to diagnosis was 13.9 days (range 3–56). None of the patients had received smallpox vaccination or post-exposure prophylaxis. All patients were hospitalized, with 95% recovering and one death (5%) reported. The average hospital stay was 19 days (range 10–31).

Single introduction event drives sustained local MPXV outbreak in Cambodia

Sequencing of the Cambodian local outbreak enabled the production of 20 high-quality outbreak consensus genomes, which were all subsequently classified as subclade IIb C.1 MPXV (C.1 lineage). The first case of the outbreak was identified on December 11, 2023 in Phnom Penh, and clustered most closely with a viral sequence (accession number: PQ368488.1) from Thailand (Figure 1, left). This sequence also shows the closest percentage average nucleotide identity (% ANI) to the most ancestral Cambodian consensus genome (NPHL231211017_SL) (Supplementary Table 3). Following this initial case, the Cambodian outbreak genomes collectively clustered into a single monophyletic group within the phylogenetic tree (Figure 1, left) and exhibited a high proportion of APOBEC3 mutations (Figure 1, right). Among the 19 mutations found exclusively within the outbreak genomes (excluding the outgroup), 18 (94.7%) were APOBEC3-driven, while only one (5.3%) was linked to polymerase replication errors (Figure 1, right). These findings point to a single introduction event into Phnom Penh, followed by sustained human-to-human transmission, enabling local circulation of the C.1 lineage until at least May 13, 2024.

Ancestral split of C.1 MPXV and the emergence of sublineage C.1.1

Similar to the Cambodian outbreak, other outbreaks (e.g. Vietnam, Thailand, Indonesia) featured C.1 MPXV, yet none of the cases belonged to the C.1.1

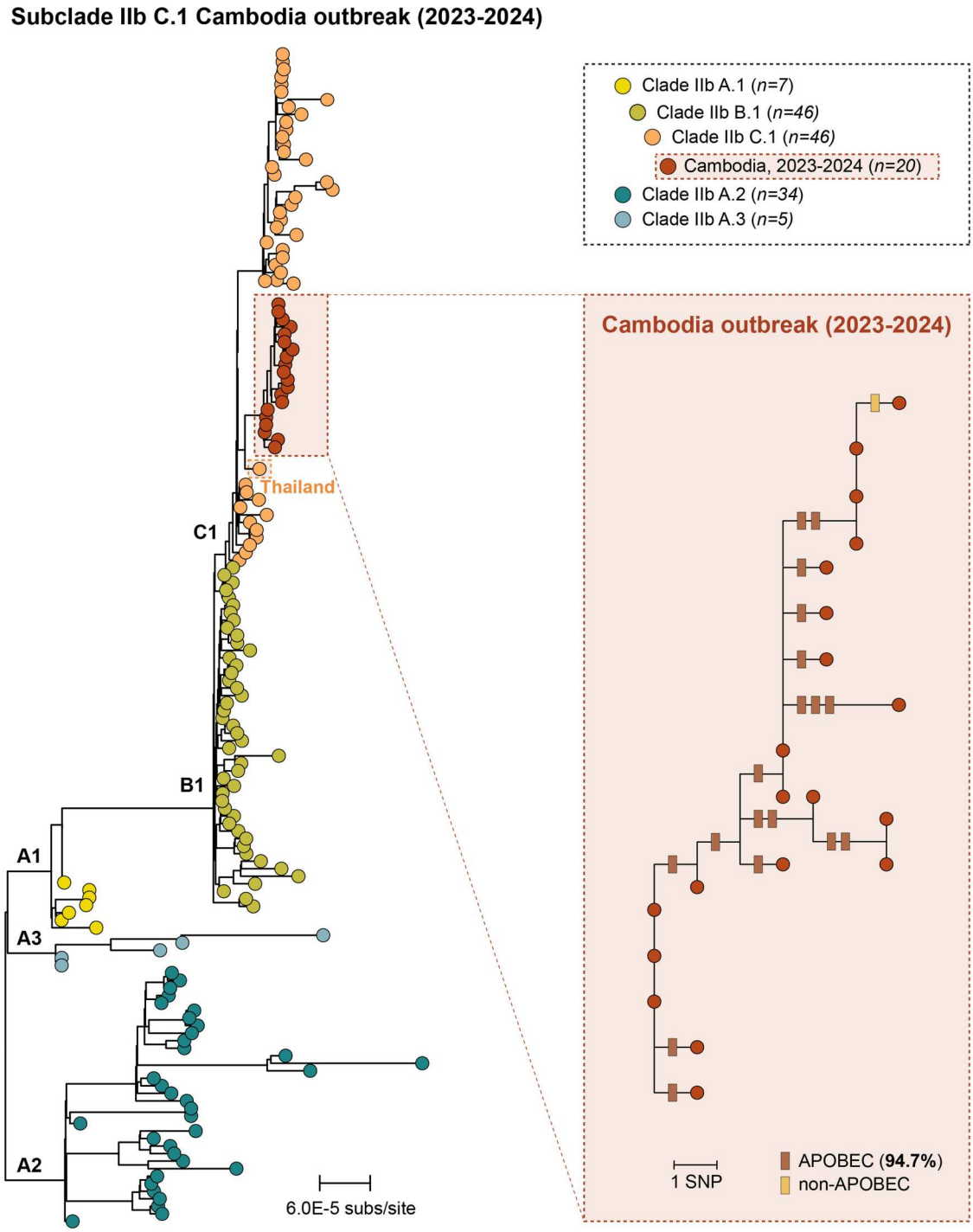

Figure 1. Maximum likelihood phylogeny of subclade IIb MPXV: Lineage C.1 introduction to Cambodia caused a 2023–2024 outbreak characterized by a dominance of APOBEC3-type mutations. In the left panel, the maximum likelihood phylogeny shows the placement of Cambodia sequences (Red) within the subclade IIb C.1 MPXV (Orange). The viral genome most closely related to the Cambodian outbreak (December 11, 2023, to May 13, 2024, n = 20) originates from a sample collected in Bangkok, Thailand, on July 4, 2023. The right panel shows an APOBEC3 analysis identifying 19 SNPs, of which 18 (94.7%) align with known APOBEC3-type deaminase editing signatures (TC-to-TT or its reverse complement, GA-to-AA). Other subclade IIb lineages are indicated as follows: A1 (light yellow), A2 (dark blue), A3 (light blue) and B1 (dark yellow).

sublineage previously reported in China, Portugal, amongst other regions (Supplementary Table 3) [17, 36, 37]. To study the evolutionary dynamics of C.1 MPXV, we gathered all publicly available reference genomes and combined them with the Cambodian consensus genomes (*n = 328;* Supplementary Table 3). This dataset was then analyzed using root-to-tip distance analysis (R-squared: 0.67) and Bayesian evolution of temporal signal (BETS) (Log Bayes factor: 298.40), both of which confirmed a strong temporal signal (Extended Data Figure 1). Once the temporal signal was established, we moved forward with Bayesian spatiotemporal modelling, integrating an agnostic skyline coalescence model with a strict molecular clock (Methods). From this, we found that lineage C.1 has been circulating in East Asia since November

25, 2022 (Figure 2). Following this, the analysis uncovered an ancestral split within the C.1 MPXV lineage, resulting in the emergence of the C.1.1 sublineage (*n = 230*) and a residual cluster of C.1 sequences (*n = 98)*. The ancestral split was inferred to have taken place in South Korea on January 23, 2023 (Supplementary Table 4). It is within this residual C.1 cluster that the Cambodian outbreak can be placed, along with previously documented local outbreaks in Vietnam and Thailand. The Cambodian outbreak; however, did not form a separate sublineage, as it showed conserved downstream mutations but lacks international spread detected as of yet, both essential criteria for classification as a sublineage (github.com/mpxv-lineages/lineage-designation). Phylodynamic analysis further supports the introduction of the C.1 lineage to Cambodia on November 10, 2023 (tMRCA), 31 days prior to the first detected Mpox case and roughly nine and a half months after the ancestral split with C.1.1 (Figure 2). However, since the first sampled patient was symptomatic in early November, it is likely that the actual introduction occurred slightly earlier than the inferred tMRCA.

Sublineage C.1.1 as a globally circulating variant, while residual C.1 remains regionally bound

Following the ancestral split, a distinct spatial pattern developed, with sublineage C.1.1 emerging as a globally circulating variant, while the residual C.1 sequences remained mostly confined to East and Southeast Asia (Figure 2). The spatial backbone of sublineage C.1.1 progressed from South Korea through China before reaching Portugal and other Western nations, with the exception of one case in the USA that was directly tied to Chinese sequences. The sequences in China were all linked to Mpox cases in Guangdong province, particularly Shenzhen, Foshan and Guangzhou. In contrast, the spatial backbone of the residual C.1 sequences, which drove the Cambodian outbreak, traced its path through South Korea, and extended further into Southeast Asia, including Vietnam, Thailand and Cambodia. Among the 98 genomes classified as residual C.1, the vast majority (94.9%) were identified in East or Southeast Asia. The remaining five cases (5.1%) were found in Western countries, with four reported in the USA and one in Germany; however, no travel history information was available for these cases.

Unique mutational signature of the Cambodian outbreak attributed to APOBEC3 activity

The mutational profile of the Cambodian outbreak was thoroughly analyzed to assess its distinct features, revealing an APOBEC3-driven signature that set the Cambodian C.1 lineage apart from prior local outbreaks (Supplementary Table 5). More specifically, Cambodian genomes accumulated two conserved APOBEC3 mutations, present in all viral consensus sequences, serving as hallmark SNPs of the outbreak. Our analysis identified that both hallmark mutations were non-synonymous and were observed in viral proteins: G71290A in DNA-directed RNA polymerase (G5.5R) and G95499A in Uracil-DNA glycosylase (MPXVgp099).

Discussion

This study presents a detailed epidemiological and phylogenomic analysis of the first documented Mpox outbreak in Cambodia (2023-2024), caused by lineage C.1 of subclade IIb MPXV. Indeed, genomic analyses showed that the earliest Cambodian case, identified on December 11, 2023, shared the closest genetic relationship with a subclade IIb.C1 MPXV sequence from Thailand (accession PQ368488.1). The phylogenetic clustering of all Cambodian genomes into a monophyletic group, alongside a high burden of APOBEC3-mediated mutations, supports a single introduction event followed by localized human-human transmission within socially connected networks in Phnom Penh.

This outbreak, occurring in a previously non-endemic region, highlights the capacity for Mpox to establish transmission chains in new settings, particularly among MSM, mirroring patterns observed globally during the 2022–2024 period [16]. Epidemiological investigation revealed that transmission occurred primarily through intimate contact, supported by presence of skin lesions primarily in anogenital areas, and with no evidence of zoonotic exposure or recent international travel among confirmed cases. The median age of individuals with Mpox was 31.6 years, which is consistent with previous reports showing that MPXV subclade IIb infections occur mostly among young adults [17, 37, 38]. Despite the implementation of contact tracing, isolation, and public health communication, incomplete information limited the ability to fully reconstruct transmission chains. These findings underscore the need for integrating Mpox surveillance with broader sexual health services, particularly HIV and STI programmes, given the high rates of co-infection were observed.

The genomic epidemiology of the Cambodian Mpox outbreak situates it within the broader context of lineage C.1 circulation in East and Southeast Asia [16, 17, 37, 39]. While sublineage C.1.1 has demonstrated widespread dissemination beyond Asia, including introductions into Europe and North America, available data indicate that the residual C.1 lineage, including the Cambodian genomes, has largely remained regionally confined [16]. The spatial

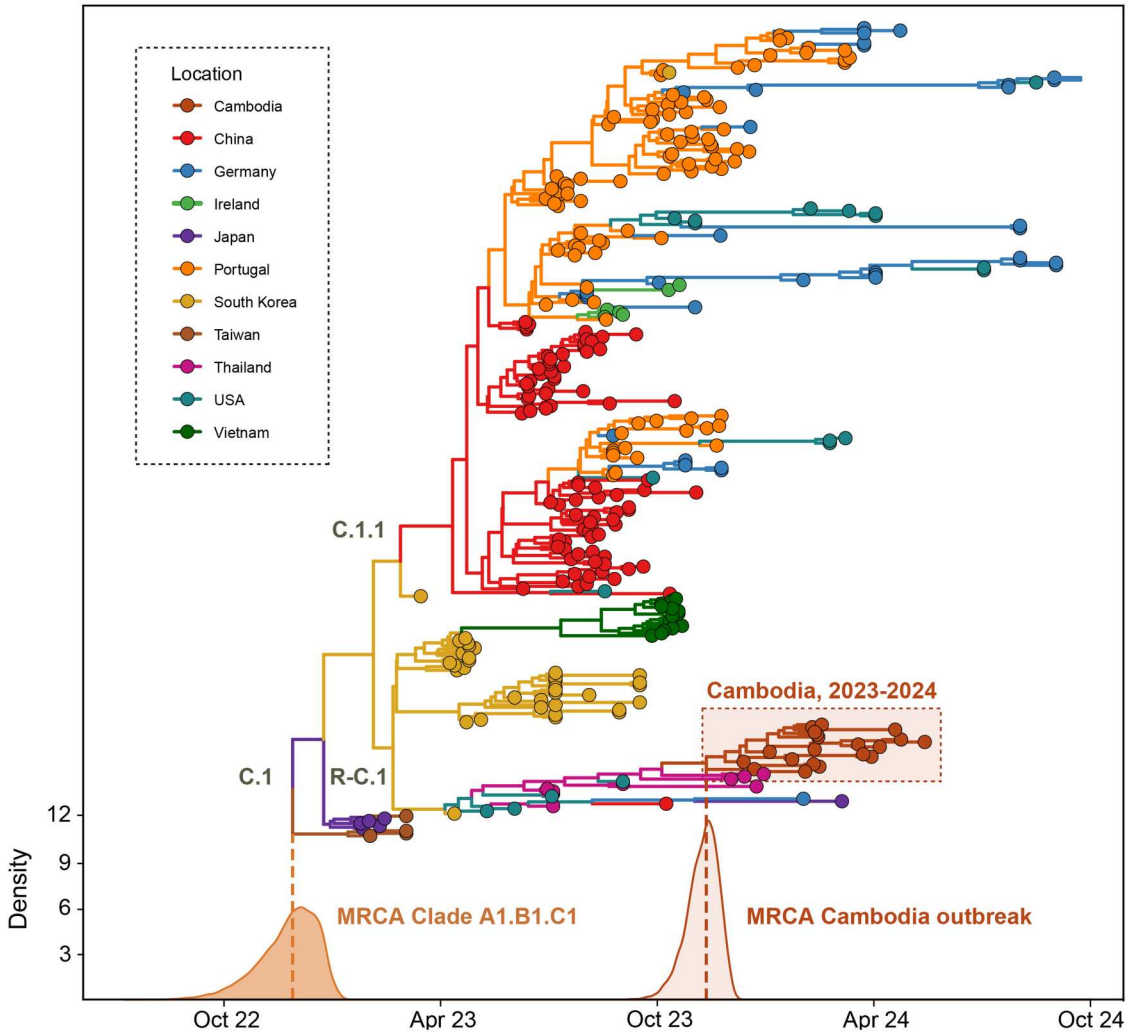

Figure 2. Bayesian spatiotemporal modelling of C.1 MPXV outbreaks uncovers the emergence of sublineage C.1.1 and a residual (R-C.1) cluster of C.1 sequences including the Cambodian outbreak in 2023–2024. This analysis integrated all published C.1 MPXV reference genomes with the Cambodian consensus genomes (n = 328), from which a maximum clade credibility (MCC) time tree was constructed (Supplementary table 3,4). Bayesian analysis using a skyline coalescence model and a strict molecular clock revealed that lineage C.1 has been circulating in East Asia since November 25, 2022 (median: 2022-11-25, 95% high posterior density (HDP) interval: 2022-09-23–2023-01-09), marking the most recent common ancestor (MRCA). A split within the ancestral C.1 lineage resulted in the emergence of C.1.1 and a residual C.1 (R-C.1) lineage. This split is estimated to have occurred on January 23, 2023 (median: 2023-01-23, 95% HDP interval: 2022-12-27–2023-03-01), illustrated as the MRCA (not shown on figure. Based on current data, residual lineage C.1 primarily circulated in East and Southeast Asia and was likely introduced into Cambodia on November 10, 2023 (median: 2023-11-10, 95% HDP interval: 2023-10-12–2023-12-02), illustrated as the MRCA.

confinement of C.1 is consistent with the lack of broader dissemination and the absence of international travel links among Cambodian cases. A mutational signature defined the Cambodian C.1 genomes, characterized by two conserved, non-synonymous APOBEC3-associated mutations: G71290A in DNA-directed RNA polymerase (G5.5R) and G95499A in Uracil-DNA glycosylase (MPXVgp099). These proteins are involved in viral replication and genome integrity, with G5.5R facilitating replication within the host cytoplasm and MPXVgp099 responsible for DNA repair [40]. Although the functional consequences of the observed mutations remain unknown, they are predicted to represent (moderately) conservative amino acid change based on their Grantham score. While awaiting additional insight into APOBEC3-driven mutations on monkeypox virus transmission, these mutations should be interpreted as markers of sustained human-to-human mpox transmission rather than changes expected to affect viral fitness and transmissibility.

The precise origin of the virus in Cambodia cannot be definitively established due to incomplete regional genomic data. While currently available sequences provide a provisional point of comparison, the lack of published genomes from other countries in the region may conceal important links in transmission chains. This limit of comprehensive genomic coverage introduces uncertainty and highlights the critical importance of timely data sharing for effective outbreak investigation and control. Furthermore, epidemiological data may underrepresent the true extent

of the outbreak due to potential underreporting within affected populations.

In conclusion, the 2023–2024 Cambodian Mpox outbreak exemplifies the regional transmission potential of lineage C.1 and the role of localized human networks in sustaining viral spread. The identification of a putative single introduction event, combined with a distinct APOBEC3-mediated mutational profile, contributes to the understanding of Mpox evolution in Southeast Asia. These findings emphasize the need for strengthened regional clinical and genomic surveillance. The absence of publicly available genomes from neighbouring countries constrained the reconstruction of potential cross-border transmission pathways, highlighting an urgent need for enhanced genomic data sharing across Southeast Asia. Additionally, high rates of HIV and STI co-infections among confirmed cases underscore the necessity of integrating Mpox response efforts into existing sexual health programmes to effectively mitigate outbreak impact. Overall, our study not only elucidates the evolution and transmission dynamics of MPXV lineage C.1 in Southeast Asia but also provides actionable evidence for public health strategy: targeted surveillance, rapid genomic sequencing, functional characterization of key mutations, and incorporation of Mpox within broader sexual health frameworks, and regional collaboration are essential to prevent and control future Mpox outbreaks.

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