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Perspective

Perspective on the application of genome sequencing for monkeypox virus surveillance

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Monkeypox virus (MPXV), a DNA virus belonging to the *Orthopoxvirus* genus, causes a self-limiting zoonotic disease known as mpox. The human monkeypox infection was first recorded in a 9-month-old child in the Republic of Congo in the 1970s (Ladnyj et al., 1972). For a long time, mpox was mainly prevalent in the humid forests of Central Africa and parts of West Africa (Kabugae and El Zowalaty, 2019). In recent years, MPXV has been spread to other countries through trade and travel. In 2003, a group of rare pets carrying MPXV was exported from Africa to the United States, and an outbreak of animal-to-human transmission occurred (Di Giulio and Eckburg, 2004). In 2018, two cases of mpox were confirmed in travelers from Nigeria to the United Kingdom and one case was confirmed in Israel (Vaughan et al., 2018; Erez et al., 2019). Then, in 2019, one case was confirmed in Singapore (Ng et al., 2019). The outbreak of mpox in multiple non-endemic countries in North America and Europe started in May 2022 (WHO, 2022b). On July 23, 2022, the WHO declared MPXV a Public Health Emergency of International Concern (PHEIC) (WHO, 2022a). As of March 5, 2023, 86,309 confirmed mpox cases have been reported from 107 countries/regions worldwide (WHO, 2023). In the mainland of China, the first imported case was confirmed by the Chinese Center for Disease Control and Prevention (Zhao et al., 2022), making this the fifth confirmed mpox infection in China. Neglected zoonotic mpox has been restricted in Africa but now it is back in the spotlight worldwide (Tan and Gao, 2022).

Metagenomic next-generation sequencing (mNGS) has played an important role in discovering SARS-CoV-2 and is widely used during the global mpox outbreak. Using the next- and third-generation sequencing platforms, the Chinese Center for Disease Control and Prevention obtained the full-length MPXV genome within 12 h. The molecular tracing of the imported mpox is in line with the epidemiological report. Here, we present a brief perspective on the application of genome sequencing for MPXV surveillance with an emphasis on both mNGS and amplicon strategies.

1. MPXV genome and phylogeny

The MPXV possesses a linear double-stranded DNA genome of approximately 197 kb, which contains a central core region and two inverted terminal repeats (ITRs) on both sides (Fig. 1A). The core region encodes essential enzymes and structural proteins, which are highly conserved among Orthopoxviruses (Shchelkunov et al., 2001; Alakunle et al., 2020). The ITRs, which constitute less than 10% of the full genome, exhibit hairpin structures, tandem repeats, and a few coding genes (Fig. 1B), and may play significant roles in virus-host interactions (Lefkowitz et al., 2006).

Several genes from the genome have been used as targets for molecular diagnosis, therapeutic drugs, and vaccine development (Fig. 1B). *D14L* (VACV-Cop *C3L* ortholog) (Li et al., 2010), *F3L* (*E3L* ortholog) (Kulesh et al., 2004; Zhou et al., 2006; Huo et al., 2022), *F8L* (*E9L* ortholog) (Li et al., 2006), *E7R* (*D7R* ortholog) (Orba et al., 2015), *B6R* (*B5R* ortholog) (Li et al., 2006), *B7R* (*B6R* ortholog) (Shchelkunov et al., 2011), *N3R* (Kulesh et al., 2004) and *B21R* (Huo et al., 2022) are commonly used targets for nucleic acid diagnosis. Tecovirimat is a therapeutic drug against MPXV VP37 protein (MPXV-*C19L, F13L* ortholog) (Yang et al., 2005). *F8L* (*E9L* ortholog), *A22R* (*A20R* ortholog), and *E4R* (*D4R* ortholog) constitute the holoenzyme of MPXV genome replication (Peng et al., 2023). The structure of this enzyme has been determined, which provides guidance for the development of new antiviral drugs. Moreover, *E8L*(*D8L* ortholog), *M1R* (*L1R* ortholog), *H3L* (*H3L* ortholog), *A29L* (*A27L* ortholog), *A35R* (*A33R* ortholog), and *B6R* (*B5R* ortholog) have been identified as protective antigens and are considered hot genes in vaccine research (Yang et al., 2022).

Based on their geographic distribution and genomic features, MPXV has been classified into two major evolutionary clades, namely the Congo and West African clades (Nakazawa et al., 2015). Previous studies reveal that the virulence of the Congo clade is higher than that

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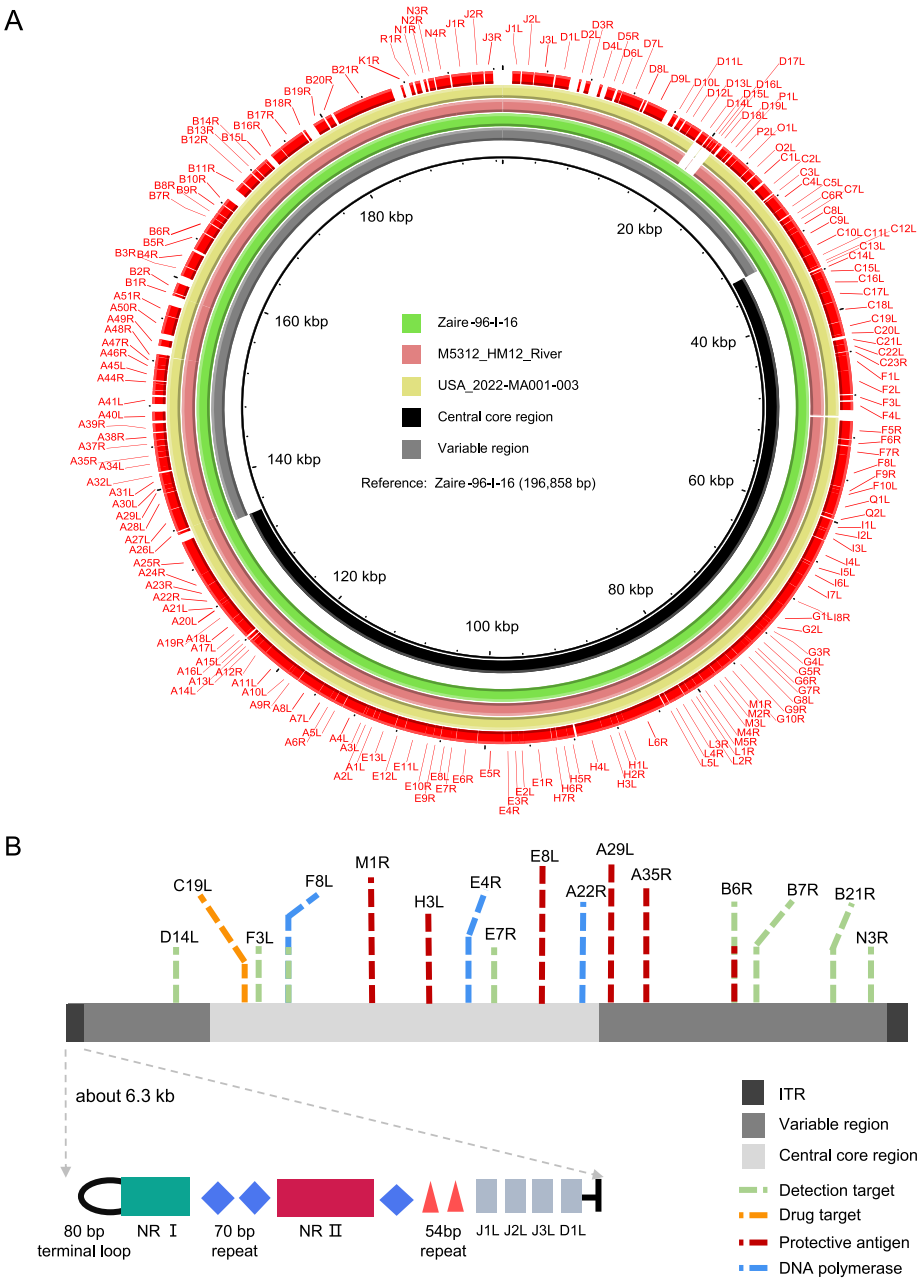

Fig. 1. Genomic annotation of MPXV. **A** Protein coding gene in three major clades of MPXV. The black and gray rings are core and variable regions of the genome. The green, light red, and dark-yellow rings are genomes of Zaire-96-I-16 (NC_003310.1), M5312_HM12_River (NC_063383.1), and USA_2022-MA001-003 (ON563414.3), respectively. **B** Schematic of the monkeypox genome. The green, orange, red, and blue dash lines indicate detection targets, drug targets, protective antigens, and DNA polymerase genes, respectively. ITR, inverted terminal repeat; NR, nonrepeating sequence.

of the West African clade (Chen et al., 2005). The virus, causing the mpox outbreak in May 2022, belongs to the West African clade, and the genome of this virus is similar to strains found in outbreaks in the United Kingdom, Israel, and Singapore in 2018 and 2019, respectively (Isidro et al., 2022b). Considering the appropriate, non-discriminatory, and non-stigmatizing nomenclature of mpox clades proposed by Happi et al., the Congo clade is defined as clade I, and the West African clade is classified into IIa and IIb, with lineage IIb being responsible for most of the human-to-human transmissions between 2017 and 2022 (Happi et al., 2022). The current international outbreak lineages are classified as B.1, B.1.1, B.1.2, B.1.3, B.1.4, and B.1.5. Otherwise, a few A.2 cases have been reported in the United States (Gigante et al., 2022).

2. mNGS and its application for MPXV genome sequencing

Compared to traditional methods such as pathogen isolation, mNGS can significantly reduce the time required for pathogen determination and has benefited pathogen genome analysis for understanding molecular characteristics and molecular tracing (Chiu and Miller, 2019).

During the early period of the current mpox outbreak, Illumina was the mainstream NGS platform because of its higher sequencing accuracy

Table 1
Summary of current platforms for monkeypox genome sequencing.

Platform	Sample	Nucleic acid extraction method	Library Preparation	Data analysis tools	Data description		Reference
					Depth (×)	Coverage (%)	
Oxford/Nanopore (Third Generation Sequencing)	Skin exudate samples	QIAamp DNA Mini Kit	SQK-RBK004	INSaFLU online platform	7	92	(Isidro et al., 2022a)
	A skin lesion swab	High Pure PCR Template Preparation Kit	SQK-LSK109	Medaka, BCFtools, MAFFT, IQ-TREE	78		(Galán-Huerta et al., 2022)
	A skin lesion swab	The automatic EMAG[a]	SQK-RBK004	Guppy, NanoFilt, Kraken, Minimap2, SAMtools, iVar	15–237		(Buenestado-Serrano et al., 2022)
	Skin swab	QiAmp Viral RNA Mini Kit[a]	SQK-LSK109[c]	Kranken2, Minimap2, IQ-TREE	77	98.6	(Martínez-Puchol et al., 2022)
	Swab sample	QIAamp DNA blood kit	SQK-LSK110 kit	Minimap2, SAMtools, iVar	38		(Croville et al., 2022)
	Undefined	Macherey Nagel pathogen kit[a]	SQK-LSK109[d]	Kraken, Bracken, Minimap2, Nanopolish, BCFtools, BWA, Geneious	620–1368		(Gorgé et al., 2022)
	Nasopharyngeal swab, lesion crust, vesicles	eMAGsystem	SQK-RBK004	Minimap2, Kraken2, mosdepth, Picard	14.9		(Alcoba-Florez et al., 2022)
	Vesicle fluid	DNeasy Blood and Tissue kit	SQK-RAD004	Minimap2, Geneious Prime, MAFFT	71–77		(Hoz-Sánchez et al., 2022)
	A skin swab of the lesions (vesicle and crust)	QIAamp Viral DNA Mini Kit	SQK-RPB004	Guppy, Minimap2, SAMtools, NanoStat, Medaka	277.7	100	(Claro et al., 2022)
Illumina (Next Generation Sequencing)	Pustule swab	QIAamp DNA mini kit	Nextera XT paired-end library	TrimGalore, Bowtie2, SAMtools, SPAdes, MAFFT, RAxML	1300		(Israeli et al., 2022)
	Viruses were isolated from swabs of skin lesions on Vero E6 cell	Monarch Nucleic Acid Purification Kits, DNA Blood Mini Kit	NEBNext Ultra II DNA Library Prep Kit	Fastp, BWA, snpEFF, BCFtools, MAFFT, IQ-TREE	75 (Ct 22) and 66 (Ct 25)		(Fuchs et al., 2022)
	Lesions (CT:15–16)	easyMAG[b]	KAPA LTP library preparation Kit	Trim Galore, BWA, Ivar	240.5–427.6	99.2–99.99	(Filipe et al., 2022)
	Nasopharyngeal swab, lesion crust, vesicles	eMAGsystem	Nextera XT DNA Library Preparation Kit	Minimap2, Kraken2, mosdepth Picard	~38	99.91	(Alcoba-Florez et al., 2022)
	Swabs of fluid from lesions throat swab	undefine	Illumina DNA prep kit[e]	BEDtools, BWA, SAMtools, iVar, Nextalign, BioPython	1000 (< Ct 18) 300-800 (> Ct 18)	97	(Chen et al., 2023)
IonTorrent (ThermoFisher) (Next Generation Sequencing)	Vesicle fluid	DNeasy Blood and Tissue kit	NEBNext Fast DNA Library Prep Set for Ion	Minimap2, Geneious Prime	71–77		(Hoz-Sánchez et al., 2022)

[a] DNase and RNase treatment before extraction.
[b] Samples underwent host depletion by using the NEBNext Microbiome.
[c] Sequence-independent single primer amplification (SISPA) was performed on the extracted DNA.
[d] Libraries were prepared after whole genome amplification (Cytiva kit, FisherScientific, Illkirch, France).
[e] Libraries were prepared after multiplexed PCR amplicon.

and larger output of reads than the third-generation sequencing platform (Table 1). However, its performance was limited due to GC content and read length (Pop and Salzberg, 2008). Third-generation sequencing technology can generate longer reads and is less susceptible to GC bias, which benefits the high-quality assembly of tandem repeats in ITR (Saud et al., 2021; Vandenbogaert et al., 2022). Due to the simple and rapid library preparation workflow and real-time output manner, Oxford/Nanopore has become one of the most preferred sequencing platforms, especially in the early outbreak of mpox.

Augmenting the viral loads of the collected samples is advantageous for mNGS. Nevertheless, the viral loads of MPXV is disparate, contingent on the anatomical site, the fluid type retrieved (Palich et al., 2023), and the stage of the disease (Suner et al., 2022). Therefore, a standard operating protocol for sample collection is also needed. Four critical steps of mNGS, including nucleic acid extraction, library preparation, sequencing, and data analysis are briefly described as follows (Fig. 2).

2.1. Nucleic acid extraction

Clinical specimens of mpox cases mainly include skin swabs of the lesions, vesicle fluid, and skin crust (Table 1). All of them could be processed using commercial kits, which are superior to traditional methods and can be combined with automatic nucleic acid extraction machines. MPXV abundance and specimen quality are crucial factors determining the assembly quality. Specimens with low viral loads may fail to yield a complete MPXV genome. Virus isolation is an important method to enrich MPXV. Fuchs et al. has shown that sequencing after virus isolation and culture had a higher coverage of the MPXV genome compared to direct sequencing of skin swabs (Fuchs et al., 2022). When virus isolation is not feasible and total DNA is low, whole-genome amplification (WGA) techniques, which amplify nucleic acid concentration, could be employed.

The proportion of MPXV reads without the removal of human DNA ranged from 0.16% to 6.5% (Table 1). The proportion of human DNA can be reduced using host DNA depletion reagents (Filipe et al., 2022). For instance, physical or chemical methods can be used to disrupt tissues or cells and release host DNA and viral particles. As the MPXV nucleic acid is protected by the envelope or other structures, adding DNase and RNase only degrades the host nucleic acid, thereby increasing the proportion of MPXV nucleic acid (Gorgé et al., 2022).

The purity of nucleic acids is important to judge whether the nucleic acid is contaminated by protein and phenolic substances. Traditional nucleic acid extraction methods are usually susceptible to contamination by impurities due to improper operations, which can further affect downstream experiments. Commercial kits typically use magnetic beads or silica-based membranes for nucleic acid extraction, and the risk of contamination is lower. Magnetic beads can be used for the secondary purification of contaminated nucleic acids. The size selection of nucleic acid fragments depends on the sequencing platform. Nucleic

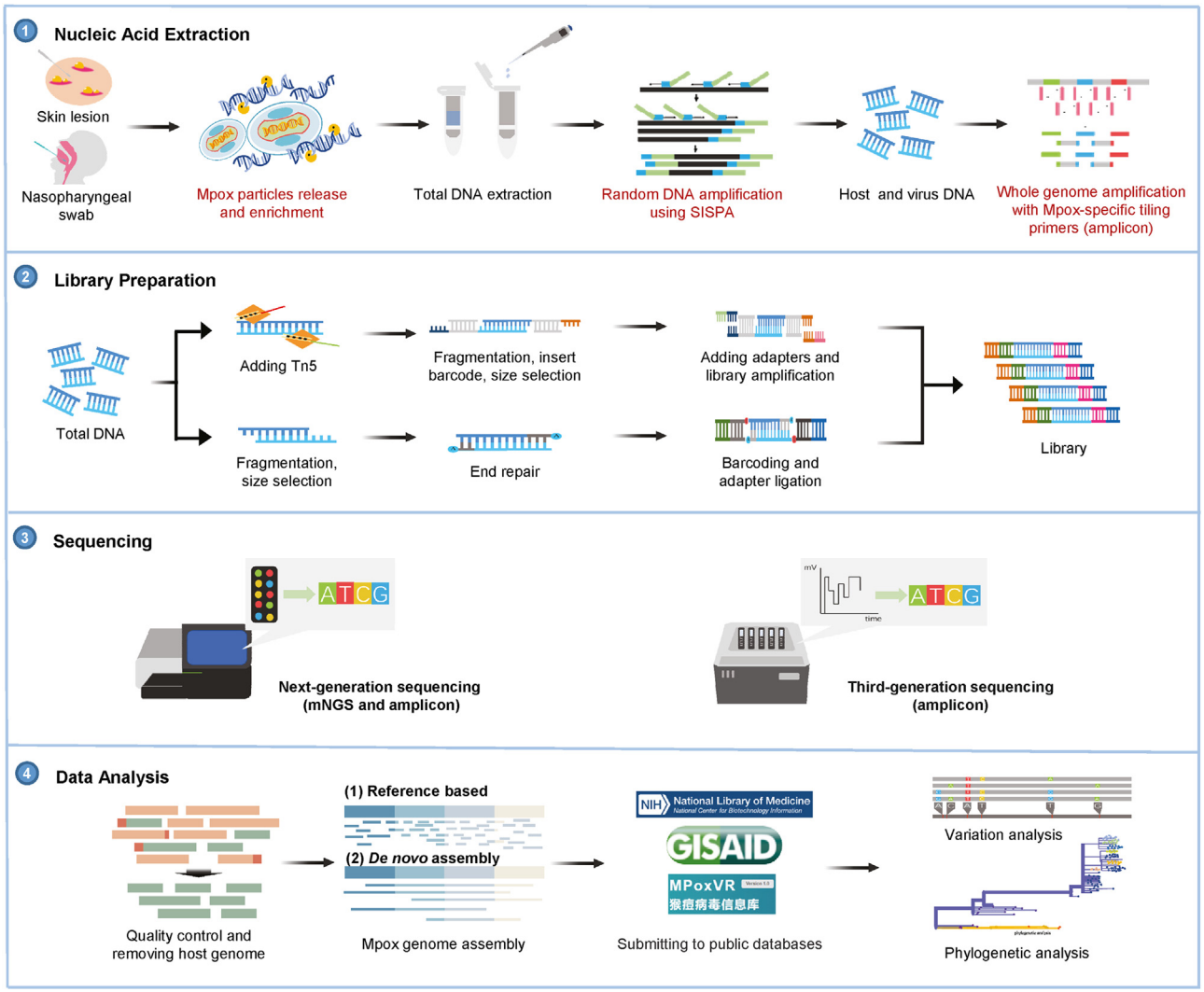

Figure 2. MPXV genome sequencing workflow. Optimization strategies to enriching mpox particles or genome DNA are highlighted in dark red.

acids usually need to be fragmented to meet the requirements of next-generation sequencing, which can be achieved using sonication or Tn5 transposases. Third-generation sequencing usually does not require fragmentation.

In short, the loads of MPXV in clinical specimens determine whether mNGS can yield high-quality MPXV genome sequences. Several optional strategies in nucleic acid extraction had been developed to improve the assembly quality. Although clinical specimens with low cycle threshold (Ct) values can be directly sequenced using mNGS without special treatment (Chen et al., 2023), the criteria of Ct still need to be clearly defined due to the lack of experimental data.

2.2. Library preparation

Adapters and barcodes are commonly ligated to both ends of DNA fragments for library preparation. The NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA) uses TA ligase and polymerase chain reaction (PCR) to ligate adapters and barcodes to both ends of the inserts (*i.e.,* DNA fragments to be sequenced). On the other hand, the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) uses the cut-and-paste feature of transposase to break nucleic acid fragments and insert adapters (Li et al., 2020). The products are then amplified through PCR for sequencing. The library preparation process using the transposase method is advantageous over the ligation method due to its shorter time requirement. For example, the Rapid Barcoding Kit (Oxford Nanopore Technologies, UK) is capable of completing the library preparation process in just approximately 10 min.

The amount of nucleic acid input is crucial in library preparation. WGA technology employs random amplification to increase the amount of nucleic acid and ensure sufficient input for library preparation. Various methods have been developed for WGA, such as degenerate oligonucleotide-primer polymerase chain reaction, multiply-primed rolling circle amplification, and multiple annealing and looping-based amplification cycles (Telenius et al., 1992; Dean et al., 2001; Zong et al., 2012). The optimal technique for WGA should be chosen based on the sample type. For instance, Gorgé et al. utilized WGA for MPXV sequencing and observed a significant increase in the proportion of MPXV-related reads (Gorgé et al., 2022). Sequence-independent single-primer amplification (SISPA) is an effective technique for nucleic acid enrichment in metagenomic next-generation sequencing (mNGS), which increases the concentration of target nucleic acids via single-primer amplification. Martínez-Puchol et al. employed SISPA for nucleic acid enrichment in mNGS of MPXV, utilizing DNase to digest the host DNA prior to nucleic acid extraction to improve the proportion of viral nucleic acid. Their sequencing results demonstrated a high genome coverage of 98.6% for MPXV, with an average coverage of approximately 77× (Martínez-Puchol et al., 2022). Furthermore, Magnetic beads are commonly used during library preparation to purify and enrich nucleic acid fragments. By adjusting the concentration of the magnetic beads, libraries can be size-selected. Lower concentration beads are ideal for enriching longer fragments, while higher concentration beads are better suited for shorter fragments (Bronner and Quail, 2019). Therefore, selecting appropriate concentrations of magnetic beads for purification and size selection is crucial to prevent any potential adverse effects on sequencing quality.

Besides, an amplification-based technique has been developed for clinical specimens with low viral loads (Chen et al., 2023). It has demonstrated superior performance compared to metagenomic next-generation sequencing (mNGS) in samples with a viral cycle threshold (Ct) value exceeding 18. While this method has been employed for MPXV genome sequencing, additional experiments are necessary to confirm its stability and dependability.

2.3. Data analysis

Currently, two commonly used assembly strategies are *de novo* and reference genome-based assembly. As nearly complete reference genome sequences for MPXV are available, reference-based assembly is mostly used. Quality control of mNGS datasets is typically performed using tools like Fastp (Chen et al., 2018), and reads are mapped to the MPXV reference genome sequence using Burrows-Wheeler-Alignment tool (BWA) (Li and Durbin, 2009). Sequencing coverage is calculated using SAMtools (Li et al., 2009), and differences between sequenced samples and reference gene sequences are analyzed using Genome Analysis Toolkit (GATK) (Mckenna et al., 2010). Consensus sequences are generated using BCFtools (Li, 2011). NGS data can also be analyzed using the CLC Genomics Workbench (Qiagen, Hilden, Germany).

For the bioinformatics analysis of amplicon sequencing data, a clearer process is needed. Many researchers use tools like Fastp or SAMtools to remove primer regions at the ends of reads for analysis. For third-generation sequencing data, we recommend using the wf-mpx workflow bioinformatics analysis process developed by the EPI2ME laboratory (EPI2ME, 2022). This pipeline is designed for the Oxford/Nanopore sequencing platform and can easily assemble and analyze mutations in the MPXV genome. Based on our experience with sequencing the genome of the first imported case of mpox in Chinese mainland, we have developed a bioinformatics pipeline that is suitable for analyzing amplicon sequencing data obtained from both next- and third-generation sequencing platforms (Zhao et al., 2022). The pipeline can be found at https://github.com/BioWu/Monkeypox-genome-assembly. Variant analysis and molecular typing can be performed using Nextclade on the MPXV genome sequence (Aksamentov et al., 2021). Additionally, multiple sequence alignments can be performed using MAFFT (Katoh and Standley, 2013) on the sequenced genome and published MPXV sequences, and a maximum likelihood evolutionary tree of these sequences can be built using IQ-TREE (Nguyen et al., 2015).

3. Concluding remarks

As the number of mpox cases continues to rise and the importation pressure of multiple countries grows, the ability to surveil mpox is rapidly advancing. Genome sequencing is crucial for evidence-based epidemiological interventions and outbreak tracing of mpox. Many Centers for Disease Control and Prevention and laboratories have installed multi-sequencing platforms capable of pathogen identification and genome sequencing. However, different sequencing platforms have their advantages and disadvantages in accuracy, sequencing time, and read length. Third-generation sequencing can rapidly generate the draft genome of MPXV, and next-generation sequencing with high accuracy can help polish the preliminary assembly. Simultaneous next- and third-generation sequencing is recommended where conditions permit to obtain a high-quality MPXV genome. In addition, applying appropriate pretreatment and extraction methods according to the types of clinical samples can improve the subsequent library preparation and high-quality sequencing data. For clinical specimens with low viral nucleic acid, using MPXV-specific amplicons is highly recommended to improve the coverage and accuracy of the MPXV genome. Standardization of the formulation of sample collection and optimization of the genome sequencing workflow is necessary to avoid nucleic acid degradation and improve the assembly quality. Furthermore, the state-of-art bioinformatics analysis pipelines need to be easily accessed and released. Lastly, virulence and infectivity assessment based on the MPXV genome warrant further attention. International efforts are needed to explore novel or optimized current platforms for MPXV genome sequencing and its application strategies.

Footnotes

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