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Genome sequencing of the mpox virus 2022 outbreak with amplicon-based Oxford Nanopore MinION sequencing

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ABSTRACT

We present an amplicon-based assay for MinION Nanopore sequencing of mpox virus (MPXV) genomes from clinical specimens, obtaining high-quality results with an average genome coverage of 99% for Ct values of up to 25, and a genome coverage of 97.1% for Ct values from 25 to 30 which are challenging to sequence. This assay is easy to implement in PCR-based workflows and provides accurate genomic data within a short time.

1. Introduction

With the first confirmed cases of the 2022 multi-country outbreak of mpox virus (MPXV), laboratories all over the world have started virus genome sequencing. The first genome was presented by a laboratory in Portugal on 28 May 2022, followed by many countries worldwide (https://virological.org/). As of 08 June 2023, 4272 high-quality genomes have been sequenced from 87,545 known cases (4.9%). In contrast to sequencing efforts at the beginning of and during the current SARS-CoV-2 pandemic, the number of MPXV genomes being uploaded to public databases was quite low, and few laboratories participated regularly in sequencing (https://nextstrain.org/monkeypox/hmpxv1?label=clade:B.1). Although DNA viruses as MPXV evolve much slower compared to RNA viruses as SARS-CoV-2, surveillance of non-synonymous mutations should be of importance as different phenotypes and variants affecting virulence and transmission can occur.

MPXV, a species of the family *Poxviridae* and genus Orthopox virus, which includes the eradicated Variola virus, has been known to be endemic in central (clade I) and western (clade II) African countries. MPXV of the 2022 outbreak have been assigned as clade IIb by the WHO, since they are phylogenetically closest related to MPXV cases which have been imported from Nigeria to non-endemic countries such as the United Kingdom, Israel and Singapore in 2018, 2019 and 2021, respectively (WHO, 2022, Erez et al., 2019, Yong et al., 2020, Vaughan et al., 2020). However, in contrast to previous outbreaks, the comparably high number of genomic sequences has revealed an unexpected high number of mutations in the MPXV genome of the current outbreak, and the mode and length of human transmission chains seem to have changed (Nolen et al., 2016).

To explain these occurrences in the current outbreak, sequencing is inevitable. The requirement of technical laboratory skills and necessities (e.g., availability of biosafety level 3 laboratories for virus and sample handling prior to inactivation, e.g. extraction and propagation) and access to sequencing platforms with high sequencing output, such as Illumina, might impede frequent sequencing for many laboratories. Furthermore, the size (197,000 bp) and complexity of the MPXV genome with many repeat regions and possible deletions and insertions require poxvirus-specific expertise in bioinformatics and make completely automated analysis, as is often being used for SARS-CoV-2, difficult.

Here, we present an amplicon-based assay for MinION Nanopore sequencing, which can scale up sequencing for laboratories already performing shotgun sequencing and can enable sequencing for laboratories without access to Illumina platforms. We could show that, on average, up to 99% of an MPXV genome could be generated with sequencing only 200,000 reads of samples with Ct values < 25, and 97.1% of genomes of samples with Ct values from 25 to 30 which are challenging to sequence on Illumina platforms and require an extensive number of reads. We could prove the accuracy of the approach by comparing the generated genomes with high-quality Illumina-derived genomes from the same samples. We propose that amplicon-based

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Nanopore sequencing can contribute to solving unanswered questions of the 2022 outbreak and to monitoring the frequency and appearance of mutations as the outbreak continues.

2. Methods

2.1. Primer design and evaluation

Using a tilted (overlapping amplicons with primers separated into two pools) approach for primer design, 682 primer pairs for 375 bp amplicons were designed for the amplification of MPXV, using Monkeypox/PT0006/2022|sampling_date_20220515 (accession ON5850 33.1) (https://ampliseq.com/).

2.2. PCR amplification, sequencing and analysis

For this study, three different sample sets were chosen: To evaluate the feasibility, sensitivity and genome coverage of the MinION sequencing approach, 111 patient samples positive for MPXV with Ct values from 14.9 to 33.8 were chosen. Only limited patient metadata was available. Samples were from lesion swabs, throat swabs, rectal swabs, urine and sperm. For Illumina sequencing, 111 patient samples positive for MPXV, which had been sequenced in routine diagnostics, with Ct values from 14.1 to 31.6, were chosen for comparison with the MinION-sequenced samples. Samples were from lesion swabs, throat swabs and rectal swabs. A third sample set of five samples was chosen to directly compare the accuracy of MinION sequencing and Illumina sequencing. The samples were from lesion swabs and rectal swabs. Additionally, a fourth sample set of five samples was chosen to compare the genome coverage and accuracy of our MinION amplicon sequencing approach with an alternative approach with longer amplicons (average length 1977 bp) (https://www.protocols.io/view/monkeypox-virus-multiplexed-pcr-amplicon-sequencin-5qpvob1nbl4o/v4). DNA was extracted by using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). For MinION sequencing, the patient samples were amplified in a single reaction with the following PCR conditions: 3 µl of viral DNA, 5 µl of primer pool, 2 mM dNTP (Invitrogen, Karlsruhe, Germany), 5 µl of 10 × Platinum Taq buffer, 4 mM $MgCl_2$ and 2 µl of Platinum Taq polymerase (Invitrogen) with added water to a final volume of 25 µl. Cycling conditions were 94 °C for 2 min, followed by 35 amplification cycles at 94 °C for 20 s and 60 °C for 4 min

The amount of human DNA in relation to MPXV DNA in each sample was determined with a specific clade II MPXV virus qPCR (MPXV-WA) and a PCR that detects human nucleic acid (*MYC*) (Nitsche et al., 2004, Schroeder, Nitsche, 2010). Amplified samples were processed for Nanopore sequencing on the MinION (Oxford Nanopore Technologies, Oxford, United Kingdom) with the ligation sequencing kit 1D, SQK-LSK109 (Oxford Nanopore Technologies). Samples were sequenced on different flow cells with at least 200,000 reads per sample. The bioinformatics analysis was performed as described elsewhere in detail (Brinkmann et al., 2021). Briefly, reads were aligned to the reference strain Monkeypox/PT0006/2022|sampling_date_20220515 by using Guppy v.3.4.5, and primer sequences were clipped from the alignment. The consensus sequence was called by using custom python scripts with a minimum coverage depth < 10 for each called position. Homopolymers were corrected manually. For each sample, 200,000 reads were analyzed.

For Illumina sequencing, libraries were prepared by using the Nextera XT Kit (Illumina) and sequenced on the NextSeq 2000 by using P2 chemistry with 2 × 150 bp. Human background reads were removed, mapping to H. sapiens GRCh38 with bowtie2 v2.3.0. Genomes were constructed with de novo assemblies by using Spades v3.13.1 and mapping to a reference sequence (Monkeypox/PT0006/2022|sampling_date_20220515, accession number ON585033.1).

3. Results

3.1. Comparison of MPXV genomes sequenced by Illumina and amplicon-based Nanopore sequencing

We compared five MPXV genomes sequenced with the Illumina platform with the amplicon-based Nanopore sequencing. Fig. 1a shows the number and position of mutations (in relation to ON676708 MPXV_USA_2021_MD) and non-covered regions of genomes. All mutations in relation to MPXV ON676708 were called concordant for both methods of the corresponding genome pair, with no mutation missed or added. The overall genome coverage of the Nanopore-sequenced samples was slightly lower than the Illumina-sequenced ones, but not lower than 99.69%. Non-covered regions of all Nanopore-sequenced samples were found at both ends (60 and 180 bp), at 19,156 bp (146 bp) and at 133,056 bp (78 bp) and regions with coverage of fewer than 10 amplicons. We further compared five MPXV genomes sequenced with an assay for long amplicons (average length 1977 bp) with our amplicon-based Nanopore sequencing approach (Fig. 1b). Again, all mutations in relation to MPXV ON676708 were called concordant for both methods of the corresponding genome pair, with no mutation missed or added. However, for the approach using long amplicons, genome coverages of only 95% and 92% could be reached.

3.2. Screening of patient samples

We screened 111 MPXV patient samples with Cts ranging from 14.9 to 33.8 with the amplicon-based Nanopore assay for MPXV. The genome coverage for the sequenced genomes was in correlation with the Ct value (MPXV-WA) of the sample. Almost complete coverage from 97.9 to 99.8% (mean: 99.4) could be reached for Ct values of up to 20, mean coverage of 98.6% for Ct values from 20 to 25 and mean coverage of 97.1% for Ct values from 25 to 30. Above 30, mean coverage of 77.4% could be reached, with coverage from 95.6 to the lowest coverage of 31.4 for a Ct value of 33.8. For each sample, 200,000 reads were evaluated, with higher coverage for 400,000 reads (mean coverage for Ct values up to 25 for 200,000 reads 99%, for 400,000 reads 99.5%).

4. Discussion

Multiple unsolved questions regarding the 2022 MPXV outbreak have been raised, including the mode of transmission, the unexpected high number of mutations in clade IIb and possible ongoing adaption to the human host. Only by sequencing samples from the current outbreak, including multiple samples from the same host, can these questions be resolved (Ulaeto et al., 2022). However, sequencing is often impeded by a poor ratio of MPXV to human DNA in samples. For shotgun sequencing, even for samples with a relatively high concentration of the virus, the concentration of viral reads in the sample is rarely higher than 0.5%, making sequencing output of several million reads necessary.

Here we show that amplicon-based sequencing can be used to include more samples for sequencing, while only 200,000 reads are necessary for full coverage of the genome. With this assay, a mean coverage of 99% can be obtained for samples with Ct values < 25, and even for samples with Ct values from 25 to 30, a coverage of 97.1% can be expected. The highest coverage reached was 99.8% for also one of the lowest Cts of 16.5, with the unavoidable lack of coverage of regions at both ends and short stretches with high GC content and low Tm.

However, one must exercise high caution when reconstructing genomes from amplicon-based sequencing with Nanopore, as uploading genomes with unconfirmed mutations should be avoided. It is necessary to mark the consensus sequences at regions with coverage at least below depth < 10 and to rule out that any called mutations are the outcome of sequencing errors and only upload high-quality genomes with a coverage $> 99\%$ to the databases. Furthermore, MPXV genomes can have large insertions and deletions which in most cases will be missed by

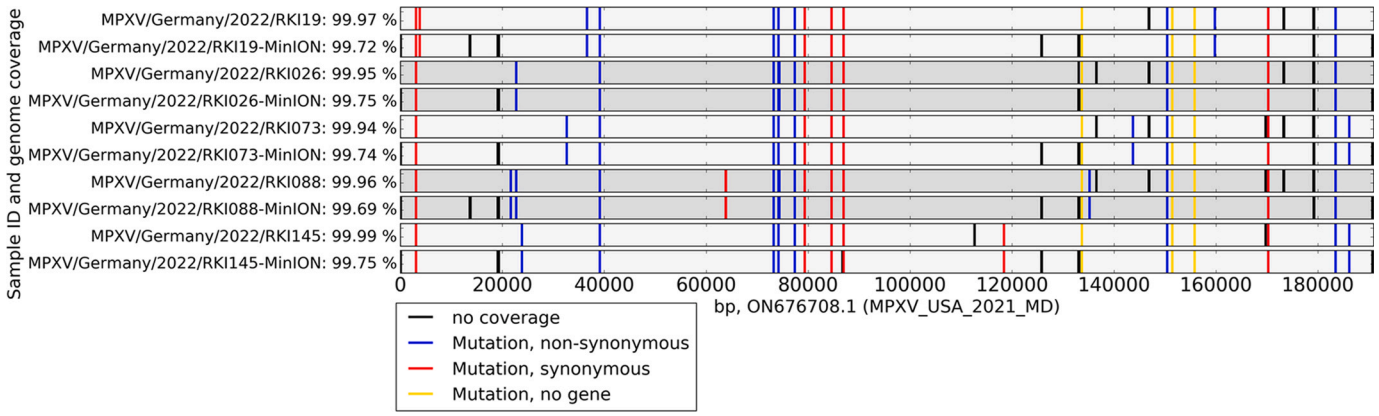

Fig. 1a. The genomes of MPXV samples sequenced by the Nanopore amplicon approach (NCBI MPXV/Germany/2022/RKI19-MinION, MPXV/Germany/2022/RKI026-MinION, MPXV/Germany/2022/RKI073-MinION, MPXV/Germany/2022/RKI088-MinION and MPXV/Germany/2022/RKI145-MinION) and Illumina platform (NCBI MPXV/Germany/2022/RKI19, MPXV/Germany/2022/RKI026, MPXV/Germany/2022/RKI073, MPXV/Germany/2022/RKI088 and MPXV/Germany/2022/RKI145) were compared, including mutations and non-covered regions. Missing coverage: black bars; non-synonymous mutations: blue bars; synonymous mutations within genes: red bars; mutation outside of genes: yellow bars.

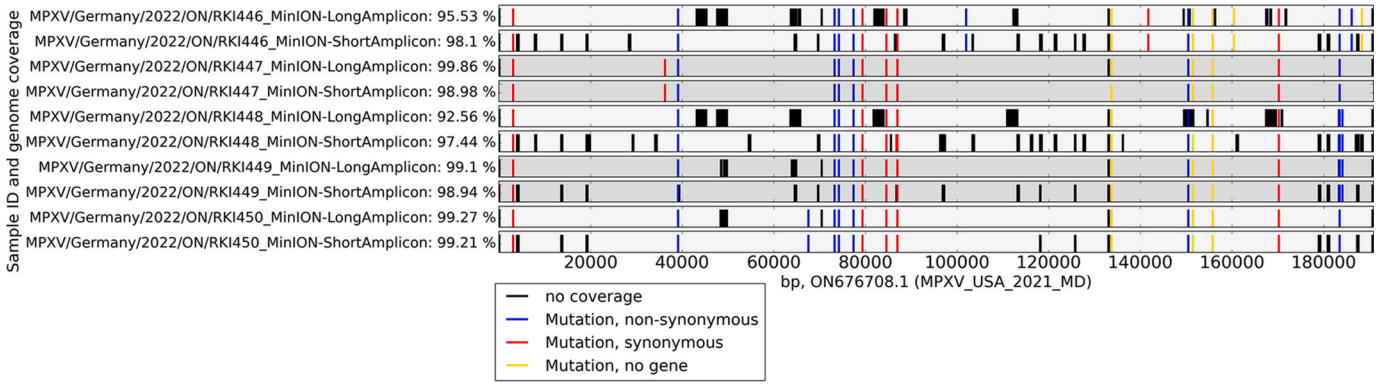

Fig. 1b. The genomes of MPXV samples sequenced by our Nanopore amplicon approach (NCBI MPXV/Germany/2022/ON/RKI446 (MinION ShortAmplicon), MPXV/Germany/2022/ON/RKI447 (MinION ShortAmplicon), MPXV/Germany/2022/ON/RKI448 (MinION ShortAmplicon), MPXV/Germany/2022/ON/RKI449 (MinION ShortAmplicon), and MPXV/Germany/2022/ON/RKI450 (MinION ShortAmplicon)) and an alternative approach with longer amplicons (MPXV/Germany/2022/ON/RKI446 (MinION LongAmplicon), MPXV/Germany/2022/ON/RKI447 (MinION LongAmplicon), MPXV/Germany/2022/ON/RKI448 (MinION LongAmplicon), MPXV/Germany/2022/ON/RKI449 (MinION LongAmplicon), and MPXV/Germany/2022/ON/RKI450 (MinION LongAmplicon)) were compared, including mutations and non-covered regions. Missing coverage: black bars; non-synonymous mutations: blue bars; synonymous mutations within genes: red bars; mutation outside of genes: yellow bars.

amplicon-based sequencing (Pfaff et al., 2022). The MPXV core genome is conserved, whereas the termini contain genes related to host adaption. In Orthopox viruses the deletion of these genes has been shown to be a cause of adaption to the host (Hendrickson et al., 2010). Significant deletions have been shown in the MPXV genomes from the current outbreak, possibly an adaption to the human host (Jones et al., 2022). MPXV genomes generated by amplicon sequencing with a coverage below 99% should therefore be checked for uncommon stretches of Ns, where possible indels could be missed. Often the region of such deletions and insertions lies downstream the left ITR or upstream the right ITR, and generated genomes with stretches of Ns in these regions should then be reconsidered for high-quality shotgun sequencing or capture-based approaches.

5. Conclusion

We have shown that amplicon-based sequencing can be used to reconstruct high-quality, accurate MPXV genomes. Although shotgun sequencing is necessary in some cases, especially to identify insertions and deletions in the MPXV genome, we propose that amplicon-based sequencing can be used to track single nucleotide polymorphisms and to screen samples for possible cases of insertions and deletions.

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Author statement

Annika Brinkmann designed the assay, analyzed the data and wrote the manuscript. Katharina Pape, Steven Uddin, Niklas Woelk and Sophie Förster analyzed the samples and the data. Heiko Jessen provided clinical specimens. Janine Michel and Claudia Kohl analyzed the suspected MPXV specimens. Lars Schaade and Andreas Nitsche conceptualized the approach and wrote the manuscript.

Ethical statement

The studies involving human participants were reviewed and approved by the Ärztekammer Berlin (Berlin Medical Association; #Eth-44/22). The patients/participants provided their written informed consent for sequencing.

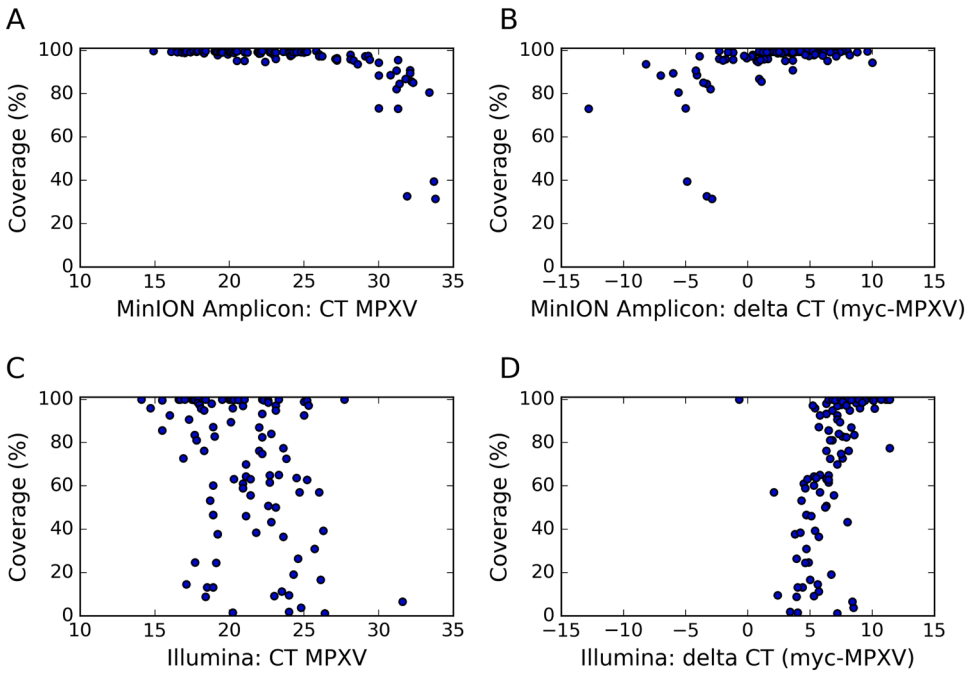

Fig. 2. 111 patient samples were screened for MPXV by qPCR and sequenced with the Nanopore amplicon approach. A mean coverage of 95.1% could be reached for all samples with 200,000 sequenced reads. Up to Ct 25, a mean coverage of 99% could be reached. Unlike Illumina sequencing (shown for a different batch of 11 samples with Cts of up to 31.6 and shown for 10,000,000 reads), where the resulting genome coverage is mostly correlated to the ratio of human (background) and MPXV DNA in each sample, the genome coverage after amplicon sequencing is merely dependent on the MPXV concentration.

CRediT authorship contribution statement

Schaade Lars: Writing – review & editing. **Kohl Claudia:** Formal analysis, Writing – review & editing. **Nitsche Andreas:** Writing – review & editing. **Pape Katharina:** Formal analysis. **Brinkmann Annika:** Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Woelk Niklas:** Formal analysis. **Uddin Steven:** Formal analysis. **Jessen Heiko:** Formal analysis, Resources. **Förster Sophie:** Formal analysis. **Michel Janine:** Formal analysis, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114888.

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