Exploration of Oxford Nanopore Long-Read Sequencing to Contribute to Influenza Surveillance at the State Level

DEPARTMENT OF HEALTH

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References

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Acknowledgements & Sources

The authors would like to thank the Virology Unit of the Minnesota Public Health Lab for receiving, extracting, and preforming the PCR subtyping/genotyping of the isolates used in this study. We would also like to thank the Sequencing and Bioinformatics Unit for conducting the preparation and sequencing of the DNA libraries for these isolates. The authors acknowledge the Minnesota Supercomputing Institute for providing resources that contributed to this research.

Work noted in this poster was supported by Cooperative Agreement Number NU60OE000104, funded by the US Centers for Disease Control and Prevention (CDC) through the Association of Public Health Laboratories. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC or the Association of Public Health Laboratories

Abstract

Background: Influenza (flu) is a rapidly mutating virus which can cause severe respiratory disease and is responsible for tens of thousands of deaths and millions of illnesses annually in the United States. Influenza virus circulation and genetic diversity were severely reduced starting in fall and winter of 2020/2021, likely due to measures that were taken to mitigate the transmission of SARS-CoV-2. This dramatic decline in genetic diversity has resulted in reduced detection across different influenza subtypes and no reported isolations of influenza B/Yamagata. As travel and other social behavioral changes trend towards a pre-pandemic state, we observed changes in Minnesota's seasonal flu trends at Minnesota Department of Health Public Health Laboratory (MDH-PHL). Influenza detections were lower than years prior due to the COVID-19 pandemic. The public health response to the ongoing SARS-CoV-2 pandemic has included significant update of respiratory pathogen genomic surveillance utilizing next-generation sequencing including ONT. We piloted a method using Oxford Nanopore sequencing platform and existing CDC-developed wet laboratory methods to characterize influenza virus found in specimens collected for influenza surveillance. 46 specimens (26 flu A and 20 flu B) submitted to the MDH-PHL between 2014 and 2022 were included in this study. We analyzed the sequencing data using IRMA (iterative refinement meta-assembler) pipeline, Nextclade (Nextstrain), and Geneious Prime 2019.2.1. In future studies we will continue to assess the public health merit of expanded state-level influenza genomic surveillance from the perspective of cost, turn-around-time, genomic resolution, and external stakeholder needs.

Figure 1: Current PCR-based influenza typing workflow

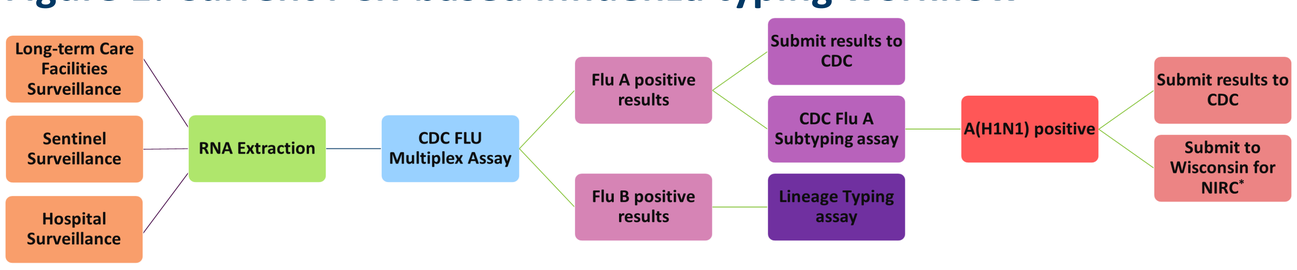

Fig. 1. Routine turn-around-time is two days for PCR and seven days for genotyping/subtyping. Isolates are shipped to CDC or Wisconsin State Laboratory of Hygiene for sequencing.

Figure 2: Pilot workflow to sequence influenza samples

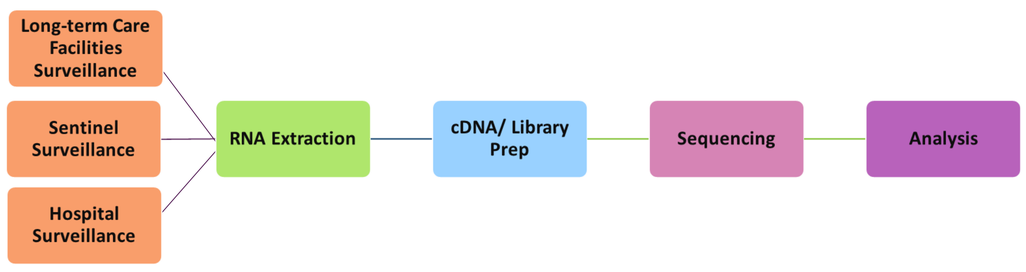

Fig. 2. Following extraction, the pilot workflow including cDNA generation, library preparation, and sequencer loading taking approximately 4 hours of hands-on time plus 3.5 hours of thermocycling. Sequencers were run overnight and read data analyzed as soon as the following morning.

Methods

RNA Extractions

RNA extraction is performed using FDA authorized methods for the CDC Flu and SARS-CoV-2 (SC2) Multiplex Assay.

PCR & Genotyping

PCR is performed using CDC Flu SC2 Multiplex Assay. Influenza A positive results are reported to CDC and the sample submitter. The CDC Flu A Subtyping Assay is performed in house as research use only (RUO).

Whole Genome Sequencing

cDNA libraries for influenza isolates were generated with the Fast Multi-segment Reverse Transcription PCR protocol using SuperScript™ IV One-Step RT-PCR System kit. Libraries were prepared following Nanopore library preparation of influenza virus or SARS-CoV-2 S-gene amplicon protocol and using ONT SQK-LSK109 kit and Native Barcoding Expansion kit. Libraries were loaded onto ONT R9.4.1 flow cell and sequenced using GridION platform.

Bioinformatic Analysis

Basecalling and demultiplexing was carried out using Guppy. Assembly was performed using the Iterative refinement meta- assembler (IRMA) pipeline. Exploratory analysis was conducted using Geneious Prime, Nextclade, Python, and R Studio.

Figure 3: Pilot testing results

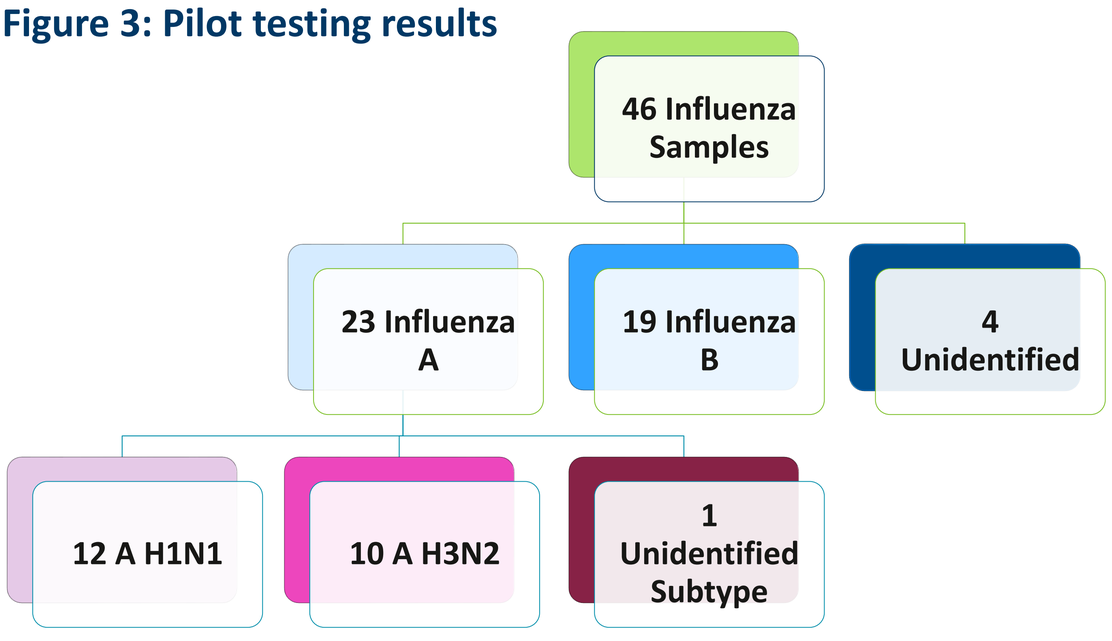

Fig. 3. This chart displays the number of samples that were correctly able to characterized by strain and subtype. Samples classified as Unidentified, failed to assemble. The sample classified as Unidentified Subtype, failed to assemble the gene segment(s) necessary for subtype identification.

Figure 4: National summary displaying trends of outpatient visits for respiratory illnesses over 6 years

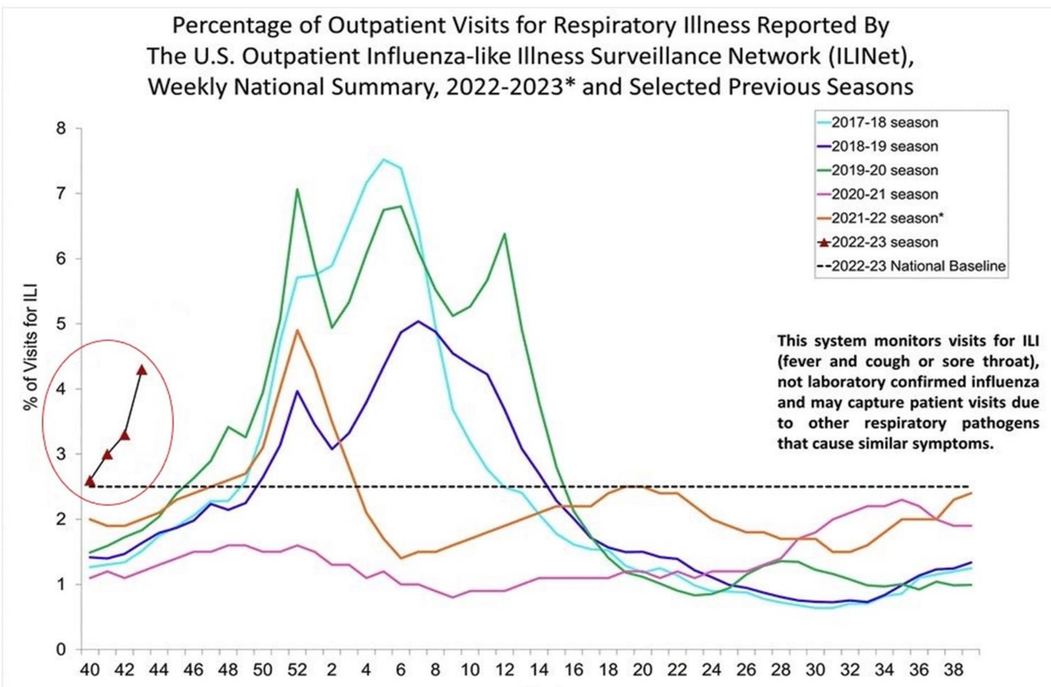

Fig. 4. Influenza seasonal trends were disrupted by the COVID-19 pandemic. 2020-2021 season (pink line) indicates a drop in overall Influenza-like illnesses, compared to previous seasons. Since the 2020 season, Influenza-like illnesses are overall less predicable. Circled in red is the current season, which is trending higher than any year listed on this chart.

Figure 5: Cost analysis for sequencing workflow

cDNA RT-PCR

Item	Price	Uses	Per run cost	Per sample cost
IDT Flu A & B primers				negligible
Invitrogen SuperScript™ IV One-Step	$958.00	100		$9.58
AMPure XP beads				negligible
Ethanol				negligible

Library prep (48 samples)

Item	Price	Uses	Per run cost	Per sample cost
NEB Blunt/TA Ligase Master Mix	$410.00	250		$1.64
NEB NEBNext Ultra II End Repair/dA-Tailing Module	$795.00	96		$8.28
NEB NEBNext Quick Ligation Module	$1,310.00	100		$13.10
ONT Sequencing Auxiliary Vials	$99.00	12	$8.25	$0.17
ONT SFB Expansion	$29.40	4	$7.35	$0.15
ONT Flow Cell Priming Kit	$34.30	6	$5.72	$0.12
ONT Native Barcoding Expansion 96	$1,176.00	12	$98.00	$2.04
ONT Adapter Mix II Expansion	$199.00	12	$16.58	$0.35
ONT Flow Cell (R9)	$475.00	1	$475.00	$9.90
Qubit dsDNA HS assay kit				negligible

Total Per Sample Cost = 45.33

Fig. 5. Cost analysis including the cost for cDNA synthesis, library preparation, and sequencing excluding labor and plasticware. Costs were determined negligible based on their contributing cost to the workflow.

Additional Findings

- A mutation that confers amantadine drug resistance (S31N) was found in all influenza A samples.
- A mutation related to increased virulence (E627k) was found on a subset of the influenza A samples collected in 2022.

Conclusion

Using long-read sequencing on the GridION platform, we were able to conduct influenza surveillance with 91% test sensitivity with 42/46 samples being accurately characterized according to their respective strain. We identified influenza strains A and B and did further characterization to determine the subtypes for influenza A. We were able to capture two mutations, E627K and S31N, that confer increased virulence and a decrease in inhibition by amantadine, respectively. The ability to identify mutations is significant due to the effect of antigenic drift on vaccine efficacy. Additionally, sequencing drastically decreased the hands-on laboratory time (4 hours) compared to the in-use method. Current workflows include sending samples out to CDC or WI for sequencing, however, there is a lagtime in delivery of summary results, and detailed sequencing data is not returned to individual state labs. In the future, a distributed sequencing approach may provide the opportunity for faster, higher throughput surveillance compared to the current nationally centralized sequencing approach for influenza surveillance.