Original Article

Evaluation of A Single-reaction Method for Whole Genome Sequencing of Influenza A Virus using Next Generation Sequencing*

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Abstract

Objective To evaluate a single-reaction genome amplification method, the multisegment reverse transcription-PCR (M-RTPCR), for its sensitivity to full genome sequencing of influenza A virus, and the ability to differentiate mix-subtype virus, using the next generation sequencing (NGS) platform.

Methods Virus genome copy was quantified and serially diluted to different titers, followed by amplification with the M-RTPCR method and sequencing on the NGS platform. Furthermore, we manually mixed two subtype viruses to different titer rate and amplified the mixed virus with the M-RTPCR protocol, followed by whole genome sequencing on the NGS platform. We also used clinical samples to test the method performance.

Results The M-RTPCR method obtained complete genome of testing virus at 125 copies/reaction and determined the virus subtype at titer of 25 copies/reaction. Moreover, the two subtypes in the mixed virus could be discriminated, even though these two virus copies differed by 200-fold using this amplification protocol. The sensitivity of this protocol we detected using virus RNA was also confirmed with clinical samples containing low-titer virus.

Conclusion The M-RTPCR is a robust and sensitive amplification method for whole genome sequencing of influenza A virus using NGS platform.

Key words: Influenza A virus; Whole genome sequencing; NGS

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INTRODUCTION

Influenza virus is one of the most common agents causing pervasive infections worldwide. While seasonal influenza virus usually causes mild respiratory illnesses, novel influenza viruses of zoonotic origin (mainly avian influenza virus) sporadically cross host barrier and cause severe human infections[1]. The high mutation rate of the viral RNA polymerase makes influenza virus genotype highly variable in the natural ecosystem. Influenza virus has also evolved

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through genetic reassortment, in which gene segments are exchanged among distinct viruses. Consequently, a highly variable composition of influenza genome has been produced in nature and further leads to different phenotypes. Therefore, characterization of the whole genome is critical for risk evaluation of certain influenza virus to public health.

The first step of influenza virus whole genome sequencing (WGS) is to reverse-transcribe and amplify the RNA genome. So far several protocols have been developed for this purpose. Most of these methods took advantage of the conserved 12 nucleotides at the 3' terminus and 13 nucleotides at the 5' terminus of the vRNA. Hoffmann[2] first introduced a universal primer set for the full-length amplification of all influenza A viruses, which used several segment-specific primers to generate full-length cDNAs. Zhou et al.[3] and Bourret et al.[4] further applied a single reaction to amplify the eight segments of influenza A virus. Hoper[5] developed a different strategy for full-length genome sequencing of influenza A virus using the next generation sequencing (NGS) platform 454FLX. With this method, every genome segment was divided into two amplicons covering its full length and subjected to further sequencing.

The advance in NGS technology has greatly improved our ability to launch a large scale of WGS of influenza virus. With more full-genome data, we can conduct more intensive and comprehensive phylogenetic analysis and achieve a deeper understanding in the epidemiology of influenza virus. In fact, NGS has already been applied in WGS of large amounts of influenza A virus[6]. Currently, a simple and robust amplification of the entire genome of influenza A virus is the prerequisite for successful WGS using NGS. Thus, the method developed by Zhou[3], a multisegment reverse transcription-PCR (M-RTPCR) approach that simultaneously amplified eight genomic RNA segments of influenza A virus, is very suitable to apply for its simplicity (single reaction) and universality (full-subtype amplification).

In this study, we evaluated the sensitivity of M-RTPCR for WGS of influenza A virus and its ability to differentiate mix-subtype virus with biased titer. We also verified the performance of M-RTPCR by directly sequencing the influenza A genome in clinical samples. Our study provided metrics for other researchers to conduct WGS of influenza A virus using NGS.

MATERIALS AND METHODS

Virus and Virus Quantification

The H1N1 influenza virus A/Puerto Rico/8/34 (PR8) and A/Quail/Hong Kong/G1/97 (H9N2, G1) used in this study were reverse-genetically rescued with PHW-2000 plasmid system to assure their purity[7]. Virus genome was extracted with a RNeasy mini kit (QIAGEN, Hilden, Germany) and separated into aliquots saved in -80 °C. The quantification of virus copy was performed using a standard curve generated by cycle threshold values obtained from serial 10-fold dilutions of *in vitro* transcripts containing 10^3-10^8 copies of the full-length M genes from influenza A virus.

Virus RNA mixing and M-RTPCR

In order to evaluate the method sensitivity of WGS for single subtype virus, we serially diluted PR8 RNA to 5, 25, and 125 copies/μL, from which 5 μL was subjected to M-RTPCR[8]. For evaluation of its ability to differentiate mixed virus with biased copies, four libraries were established. Each library contained 125 copies/μL of PR8 RNA, to which an equal volume of 125,000, 62,500, 25,000, and 12,500 copies/μL of G1 RNA were spiked in, respectively. Therefore, the low-titer virus (PR8) was expected to be detected in the background of the predominant virus (G1) with titers 1000-, 500-, 200-, and 100-fold higher than PR8, respectively. The mixed RNAs were reverse-transcribed and amplified with the same M-RTPCR method. The obtained cDNA was quantified with a Qubit® dsDNA HS Assay Kit (Life Technologies, New York, USA). All cDNA products were stored at -20 °C until use.

Next Generation Sequencing

All of the cDNA obtained from PR8 and G1 was subjected to NGS on the Ion Torrent PGM platform. Briefly, 100 ng cDNA was sheared with an Ion Shear kit and ligated with P1 and Xpress barcode adaptor using the IonXpress™ Plus gDNA Fragment Library kit. Fragment distribution was checked with the Agilent 2100 Bioanalyzer using a High Sensitivity Chip (Agilent Technologies, Santa Clara, USA). For sensitivity evaluation of single subtype virus, libraries from three PR8 RNA dilutions were pooled at equal mass and subject to emulsion-PCR using the Ion PGM™ Template OT2 200 Kit. For sensitivity of differentiating mixed virus, libraries from four RNA mixtures were pooled and emulsion-PCR as forward.

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