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Viral subpopulation diversity in influenza virus isolates compared to clinical specimens☆

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ABSTRACT

Background: Influenza virus (IFV) isolates obtained from mammalian cell cultures are valuable reagents used for vaccine production, antigenic characterization, laboratory assays, and epidemiological and evolutionary studies. Complete genomic comparison of IFV isolates with their original clinical specimens provides insight into cell culture-driven genomic changes which may sequentially alter the virus phenotype.

Objectives: The genome of the viral isolates and of the viruses in the clinical specimens was examined by deep sequencing in order to determine nucleotide heterogeneity (measured number of variances or numbers of mixed bases) as a marker for IFV population diversity.

Study design: Clinical respiratory specimens were collected between July and October 2012 and identified by RT-PCR as positive for influenza A H3N2 or H1N1, or influenza B. The viruses in the clinical specimens were amplified using mammalian cell culture. Next generation sequencing (NGS) was used to investigate genomic differences between IFV isolates and their corresponding clinical specimens.

Results: There was less nucleotide heterogeneity in 5 of 6 viral isolates compared to the corresponding clinical specimens, especially for influenza B. A phylogenetic analysis of the hemagglutinin (HA) gene consensus sequences obtained from deep and Sanger sequencing showed that the viral isolates and their corresponding clinical specimens contained the same IFV strains with less than 5% pair-wise genetic distance.

Conclusion: The IFV sequence data analysis detected a substantial decrease in nucleotide heterogeneity from clinical specimens to viral cultures in 5 out of 6 investigated cases.

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1. Background

Influenza viruses (IFVs), members of the orthomyxoviridae family, circulate worldwide and are a major global health threat. Continuously evolving IFVs accumulate changes in the viral genome resulting in subpopulation diversity and emergence of new strains. This leads to annual epidemics and occasional pandemics. Host immunity, zoonotic vectors and other environmental and ecological factors can drive viral genetic variations and antigenic modification [1]. Genetic variation can be the result of point mutations leading to antigenic drift, gene reassortment leading to genetic shift, defective-interfering particles, and RNA recombination altering the IFV genome [2].

Currently, IFV isolates obtained from mammalian cell cultures are mainly utilized for genetic and antigenic characterization using sequencing and hemagglutination inhibition (HI) assays [3,4]. This information is crucial for monitoring the circulating strains in endemic regions and to assess the evolution of viruses and emergence of novel virulence and drug resistance traits. This information is also used to determine the composition of seasonal influenza vaccines. Because influenza vaccine production relies on viral

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amplification in fertilized chicken eggs or mammalian cell culture [5–9], it is critical to accurately measure the viral subpopulations in cell culture isolates and to understand how they differ from the original primary clinical specimens.

Several studies have documented how IFV isolates differ from the viruses found in clinical specimens [1,10,11]. Azzi et al. found that influenza H1N1 is antigenically different after cell culturing [10]. Zhirnov et al., specifically investigated the matrix (M), hemagglutinin (HA), neuromidase (NA) and non-structural (NS) genes and detected several alterations in the sequences of human/canine cell culture isolates compared to the original clinical specimens [1]. Roedig et al., reported changes in the glycosylation patterns after cultivation using Sanger sequencing [11]. However, a comparison of the complete genome sequences of influenza subpopulations between clinical respiratory specimens and viral isolates has not yet been reported. Deep sequencing has been shown to have the capacity to provide high quality and large volumes of genomic sequences. It has been shown to detect low frequency mutations and the presence of quasispecies [12]. Examination of the full-length genome of influenza virus A (13.6 kb) and B (14.6 kb), including all 8 genomic segments: RNA polymerase PB1 gene (2341 nt); RNA polymerase PB2 gene (2300 nt), RNA polymerase PA gene (2233 nt), Hemmaglutinin HA gene (1765 nt), matrix M gene (1027 nt), nonstructural NS protein coding gene including nuclear export protein NEP gene (890 nt), nucleoprotein NP gene (1565 nt) and neuraminidase NA gene (1413 nt) with additional NB protein (210-249) are essential for understanding the evolution of influenza B under cultivation.

2. Objectives

In this study, we utilized deep sequencing to compare nucleotide heterogeneity at genomic positions between influenza viral isolates and their original primary clinical specimens. Nucleotide heterogeneity and pair wise genetic distances were utilized to monitor the changes in viral genetic variability detected in samples. Measuring the genomic differences caused by the amplification of IFVs in mammalian cell cultures provides important information relevant for vaccine manufacturers and scientists in general.

3. Study design

3.1. Clinical data and demographics information of clinical samples

Nasopharyngeal swabs were collected in Universal Transport Media-UTM (UTM) (Copan Diagnostics Inc., Corona, USA) from 6 patients with acute influenza-like-illness (ILI) enrolled during surveillance studies from July to October 2012. One clinical specimen was collected in Nepal and the other five in Thailand. All 6 subjects (VIROAF 1–6) were found positive for influenza by QuickVue (Quidel, Inc., San Diego, USA) and equal volumes of RNA from all samples were later confirmed by influenza rRT-PCR and HI (27). The studies were approved by the Institutional Review Boards (IRBs) of Pramongkutlao (PMK) Hospital in Bangkok Thailand, the Nepal Health Research Council in Kathmandu, Nepal, and the Walter Reed Army Institute of Research (WRAIR) as appropriate. For each clinical specimen, IFV was amplified in Madin Darby canine as described previously and confirmed by influenza HI [14].

3.2. Sample preparation from viral isolates and corresponding clinical specimens for deep sequencing reactions

Sample preparation and nucleotide quantitation procedures for Illumina MiSeq Platform followed a previously described method [13], with the exception of an additional centrifugation step at $6200 \times g$ for 10 min at 4 °C and DNaseI (PreAnalytiX, QIAGEN, Franklin Lakes, USA) treatment at 37 °C for 15 min for the clinical specimens. For the control experiments testing the effects of the concentrations on nucleotide heterogeneity, 10^{-1} to 10^{-4} dilutions of the original viral isolates were used for the sample preparation. An internal control, 1% PhiX was utilized for the efficiency of DNA incorporation and error rate during DNA sequencing on cartridges of 2×150 and 2×250 according to the manufacturer's protocol (Illumina, Hayward, USA).

3.3. Sequence data analysis

Eighty-five percent to 90% of sequence reads analyzed by the Sequencing Analysis Viewer (SAV) (Illumina, Hayward, USA) had a quality control (QC) of base-calling score of ≥Q30. The ≥Q30 score indicates 99.9% accuracy of base calling at a particular sequence position. The identification of viruses was conducted by 2 procedures: de novo assembly by Trinity [15] and "resequencing" or read-mapping alignment of the obtained sequence reads to reference sequences using MiSeq Reporter program (MSR), which used Burrows–Wheeler Aligner (BWA) for sequence read alignment [16,17]. Contigs, consensus sequences obtained from de novo assembly, were identified as a particular organism based on the top hits with high identity from "blastn" against GENBANK database entitled nt20130708 [18]. GENBANK accession numbers for VIROAF 1-6 are KJ577146-KJ577193 as previously described in [13] for viral isolates and KJ848671-KJ848715 for clinical specimens, respectively. The SRA submission numbers are X–Y. The identification of each sample was used to compare with the results from the alignment with MSR.

The details of data analysis by MSR with the default settings were described in Rutvisuttinunt et al., 2013 (22): GATK program was used for variance calling [19,20] and BWA for alignment. Briefly, references used in alignment analysis from GenBank of IFVs include A/Brisbane/11/ 2010 (H3N2) (CY121792-CY121799), A/California/07/ 2009 (H1N1), (KC781781-KC781788), and B/Wisconsin/01/2010 (CY115183-CY115190). For phylogenetic tree analysis of HA gene, we used the following IFV references for influenza A: H3N2

A/Victoria/361/2011 vaccine 2012-2014 (KC306165), A/Perth/16/2009 vaccine 2009-2011 (GQ293081), A/Thailand/CU-B590/2009 (GQ902818), H1N1 A/California/07/2009 vaccine 2009-2014(GQ906804), A/Thailand/CU-H88/2009 (HM446345). For influenza B we used the influenza B Yamagata lineage: B/Massachusetts/02/2012 vaccine 2013-2014 (KC892118) and B/Brisbane/60/2008 vaccine 2013-2014 (CY115151).

Bam files and VCF files by MSR provided measurements of nucleotide heterogeneity. Nucleotide heterogeneity was measured by the genetic variances (as manifested by numbers of sequence sites contain variances or mixed bases) at single genomic positions from deep sequence reads. The default setting for calling the variances was 100. The Bam and VCF files were further analyzed for quality and variances by Integrative Genomics Viewer (IGV) [21]. Further analysis of the variances' derived synonymous and non-synonymous mutations in sequence reads found above limit of detection (LOD, sequence reads with depth of coverage equal to 10) required IGV [21], MySQL (http://dev.mysql.com), SNPer [22] and visualization. Statistical analysis of the data comparisons was conducted with R Statistical Analysis Environment (version 2.15.3). The correlation test used was 'Pearson Correlation' tool from the website http://www.vassarstats.net/rdiff.html

3.4. Sample preparation for Sanger sequencing and pyrosequencing reaction

A subset of samples was sequenced by Sanger sequencing [23] and pyrosequencing [24]. For pyrosequencing of a

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