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Genetic and antigenic characteristics of a human influenza C virus clinical isolate

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Abstract

Unlike influenza A and B viruses that infect humans and cause severe diseases in seasonal epidemics, influenza C virus (ICV) is a ubiquitous childhood pathogen typically causing mild respiratory symptoms. ICV infections are rarely diagnosed and less research has been performed on it despite the virus being capable of causing severe disease in infants. Here we report on the isolation of a human ICV from a child with acute respiratory disease, provisionally designated C/Victoria/2/2012 (C/Vic). The full-length genome sequence and phylogenetic analysis revealed that the hemagglutinin-esterase-fusion (HEF) gene of C/Vic was derived from C/Sao Paulo lineage, while its PB2 and P3 genes evolved separately from all characterized historical ICV isolates. Furthermore, antigenic analysis using the HI assay found that 1947 C/Taylor virus (C/Taylor lineage) was antigenically more divergent from 1966 C/Johannesburg (C/Aichi lineage) than from 2012 C/Vic. Structure modeling of the HEF protein identified two mutations in the 170-loop of the HEF protein around the receptor binding pocket as a possible antigenic determinant responsible for the discrepant HI results. Taken together, results of our studies reveal novel insights into the genetic and antigenic evolution of ICV and provide a framework for further investigation of its molecular determinants of antigenic property and replication.

Keywords

Influenza C virus; Genesis; Antigenic evolution; Phylogenetic evolution

1. Introduction

The *Orthomyxoviridae* family has four genera of influenza viruses, influenza A, influenza B, influenza C, and influenza D. Among them, influenza A, B, and C are known to cause moderate to severe respiratory diseases in humans. Specifically, human infections by influenza A (IAV) and B (IBV) viruses can lead to severe respiratory diseases, while influenza C virus (ICV) usually causes mild upper respiratory diseases in humans, although it has the ability in causing severe lower respiratory illness in children less than 2 years of age.^{1–3} ICV is distributed worldwide^{4–6} and multiple genetic lineages co-circulate globally.^{7–10} In addition to three genera of influenza viruses that all infect humans, a new group of influenza viruses with cattle as a primary reservoir has been recently described and these new viruses are classified into influenza D genus due to its distinctness from other influenza genera.^{11–14} Influenza D viruses are thought to primarily infect and cause respiratory diseases in cattle and to some extent in pigs. Nevertheless, IDV-specific antibodies had been found in humans, especially those who had a previous history of contact with cattle.

Among human influenza viruses, ICV infections are rarely diagnosed and less research has been performed on it despite the virus having the ability to cause severe diseases in newborn infants. Previous analyses of the hemagglutinin-esterase-fusion (HEF) gene, encoding the antigenic determinants of viruses, have divided ICVs into six genetic and antigenic lineages, designated C/Taylor, C/Mississippi, C/Aichi, C/Yamagata, C/Kanagawa, and C/Sao Paulo.^{9,15–19} ICV is thought to evolve relatively slow compared to IAV or IBV to a lesser extent.^{10,18,20,21} However, frequent reassortments among ICVs have always occurred in nature and most of the circulating ICV are generated due to various reassortments involving multiple ICV lineages.¹⁹ Co-circulation of several ICV lineages in humans has been well documented.⁹ Genetic diversity and the associated antigenic drift caused by reassortment play an important role in driving ICV's periodical recurrence in humans. To date, the majority of published research on human ICV utilized historic reference virus strains isolated between the 1950s and 1960s. As such, more studies of currently circulating strains of ICV are critically needed in order to better understand the epidemiology, genetic and antigenic evolution, and biology of this group of human influenza viruses, which can pose a significant risk to worldwide infant population.

Here we described the isolation of a contemporary influenza C virus C/Victoria/2/2012 (C/Vic) - from a diseased child with acute respiratory symptoms in 2012. Phylogenetic analysis indicated that viral HEF gene was derived from C/Sao Paulo lineage, which is consistent with the finding that the dominant antigenic group is C/Sao Paulo lineage from 2006 to 2016.^{22–25} However, its most internal genes (PB1, NP, M, and NS) were donated from multiple ICVs. Interestingly, we found that the PB2 and P3 genes of C/Vic diverged very early from all known historical ICV viruses. Furthermore, antigenic analysis using the HI assay found 1947 C/Taylor virus (C/Taylor lineage) was antigenically more divergent

from 1966 C/Johannesburg (C/Aichi lineage) than from C/Vic. Structure modeling of the HEF protein identified two mutations in the 170-loop of the HEF protein around the receptor binding pocket as a possible antigenic determinant responsible for the discrepant HI results. Information obtained through this study shall provide novel insights into genetic and antigenic evolution of human influenza C virus.

2. Materials and Methods

2.1 Cell and virus cultures

Madin-Darby canine kidney (MDCK) was grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. The infectious culture medium is serum-free DMEM containing a final concentration of 0.0005% trypsin (Sigma-Aldrich, St Louis, USA). Virus cultures were incubated at 33 °C for up to 7 days or development of cytopathic effect (CPE).

2.2 Genome sequencing and phylogenetic analysis

Viral RNA was isolated using a MagMAX™ Viral RNA Isolation kit. Full genome amplification was performed as previously described except that the primers were modified to match the non-coding regions of ICV: "ICV3'", 5'-ACGCGTGATCGCATAAGCAG-3' and "ICV5'", 5'-ACGCGTGATCAGCAGTAGCAAG-3'. Amplicons were used for library preparation using the NEBNext® Fast DNA Library Prep Set for Ion Torrent™. Libraries were sequenced using an Ion Torrent Personal Genome Machine with manufacturer's reagents and protocols. Contigs were assembled with C/Ann Arbor/1/50 (accession numbers NC_006306-NC_006312) as templates, which were analyzed by the SeqMan NGen module from DNASTar. Nucleotide sequences were edited and compiled using the Lasergene 8 software package (DNASTAR, Inc.). The genome sequence of C/Vic was submitted to GenBank under accession numbers KM504277-KM504283.

Sequences of each segment were aligned at amino acid level using Muscle (ref), while nucleotide sequences were used for phylogenetic tree construction through the MEGA7 program. The best substitution model for each segment was estimated using MEGA7. The best models were HKY+I for PB1, T92+I for P3, T92+G+I for HEF, TN93+G for NP, HKY+I for M, and HKY+G+I for NS. The phylogenetic trees were generated using Maximum-likelihood method of MEGA7 with 1000 bootstrap replicates to verify the topology. The trees were rooted using the ancestor branches of influenza D virus strains including D/Bovine/Ibaraki/7768/2016, D/Bovine/France/29862012, D/Bovine/Italy/46484/2015, D/Swine/Italy/199724-3/2015, D/swine/Oklahoma/1334/2011 and D/bovine/Oklahoma/660/2013.

2.3 Hemagglutination inhibition assay

Antibody cross-reactivity was determined using a panel of reference viruses and antisera in hemagglutination inhibition (HI) assays. Antiserum against C/Taylor/1233/47 (C/Taylor lineage) and C/Taylor/1233/47 virus were provided by NIAID biodefense and Emerging Infections Research Resources Repository (BEI Resources). Antiserum against C/Victoria/2/2012 (C/Sao Paulo lineage) was generated in rabbits, while C/Johannesburg/1/66 virus (C/

Aichi lineage) was provided by Peter Palese at Mt. Sinai Medical School, New York. All sera were heat inactivated at 56°C for 30 min prior to use. HI assays were performed following standard procedures. In brief, sera were treated with a receptor-destroying enzyme for 24 h at 37°C and then adsorbed with a 20% suspension of turkey erythrocytes in phosphate-buffered saline (PBS) for 30 min at room temperature. Virus suspensions containing 4 to 8 HA units of the virus were incubated for 1 h with serial 2-fold dilutions of antiserum, and the HI titer was determined as the reciprocal of the highest dilution that showed complete inhibition of hemagglutination using 0.5% washed turkey erythrocytes (Lampire biological laboratories, Pipersville, PA). All viruses were assayed in triplicate. Mean HI titers and standard deviations were calculated from triplicate data. Heterologous mean HI titers were normalized to mean homologous HI titers (mean heterologous titers/mean homologous titers). Relative HI titers were reported to account for differences in homologous HI titers between C/Taylor and C/Victoria/2/2012.

2.4 Structure modeling and sequence alignment

The viral HEF sequences (C/Vic and C/Taylor) were modeled with Modeller.²⁶ The structure of C/Johannesburg/1/66 (C/JHB/1/66) was used as template (sequence identity: 95–96%). Sequences were aligned with Muscle.²⁷ Domain boundaries and the trimeric interface were determined according to those of C/JHB/1/66.²⁸ Variant residues of the HEF protein among three viruses were colored pink.

3. Results and Discussion

3.1 Virus isolation and full-length genome analysis

In 2012, a nasopharyngeal swab from child patient exhibiting clinical symptoms of acute respiratory infection was submitted for virus isolation to Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne, Australia. After cultivation in MDCK cells at 33 °C together with RT-PCR diagnosis and sequence confirmation, human influenza C virus was isolated from the patient-derived nasopharyngeal swab sample, provisionally designated C/Victoria/2/2012. Total two passages were made in VIDRL, Australia. Following one more passage in MDCK cells in South Dakota State University, the virus was then sequenced on an Ion Torrent Personal Genome Machine and De novo genome assembly was employed to compile viral full-length genome. The full-length sequences of all segments were determined and used for phylogenetic analysis.

3.2 Phylogenetic analysis

To determine the evolutionary pathway of C/Vic, we performed phylogenetic analysis on all seven segments of C/Vic and reference ICVs. Nucleotide sequences of the complete coding regions of the M and NS segments and partial coding sequences of PB2, PB1, P3, HEF and NP were used for phylogenetic tree construction (Fig. 1). Partial gene sequences of several segments were used due to limited sequence database of ICVs. To date, only four ICV strains with the complete genome sequences were reported. Reference viruses including historic ICVs such as C/Taylor/1947 and C/Ann Arbor/50, as well as the recent isolates including C/Catalonia/2588/2010 and C/Eastern-India/1202/2011 were used in our analysis.

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Previous studies of the HEF segment, the primary determinant of host range and target of virus-neutralizing antibodies, have classified ICVs into six genetic and antigenic lineages, designated C/Taylor, C/Mississippi, C/Aichi, C/Yamagata, C/Kanagawa, and C/Sao Paulo. As shown in Fig. 1, the HEF-based tree recaptured the currently defined lineage classification for ICVs, thereby validating our analytical approach. Phylogenetic analysis of the HEF gene placed C/Vic in C/Sao Paulo lineage, which also includes C/Yamagata/1/93 and C/Miyagi/5/93. Interestingly two recent ICV isolates, C/Catalonia/2588/2010 and C/Eastern-India/1202/2011, belonged to C/Kanagawa lineage. This result supports the notion that multiple ICV lineages have co-circulated in global human populations in recent years.

As shown in Fig. 1, in contrast to a HEF-based tree, the topologies of internal branches of the phylogenetic trees of other segments have low statistical supports, which may be due to their high sequence similarities. This prevented us from drawing any precise lineage classification of ICVs. Nevertheless, a close examination of the PB1 tree revealed that C/Vic, C/Yamagata/10/89, and C/Eastern Indian/1202/2011 closely clustered together with C/pig/Beijing/115/81, a porcine ICV isolated from pigs with influenza-like symptoms in China in 1981. A similar finding was also observed in the M gene-derived tree in that C/Vic formed a subgroup with C/pig/Beijing/115/81 along with C/Hiroshima/251/2000 and C/Eastern Indian/1202/2011. These results indicated the potential that C/Vic may replicate and transmit among pigs. Phylogenetic analysis of the NP gene showed the C/Vic was closely related to two viruses isolated from Japan in the 1980s, forming a subgroup with C/Mississippi/80, C/Yamagata/5/92, and C/Nara/1/85 viruses. Analysis of NS gene-derive tree demonstrated that C/Vic was most closely related to C/Hiroshima/251/2000 with 99.0% identity. Interestingly, both PB2- and P3-based trees separated C/Vic from all characterized historical ICV isolates, indicating its novelty in PB2 and P3 evolutionary pathways. It should be noted that historical ICV isolates earlier than 1966 were not included in these two gene analyses due to unavailability of their PB2 and P3 sequences. Taken together, the results of our phylogenetic studies demonstrated that C/Vic is a reassortant virus composed of segments derived from multiple ICV lineages or strains, which evolved independently.

3.3 Antigenic evolution

To investigate the antigenic property of C/Vic, HI assays were performed using polyclonal antiserum generated against 1947 C/Taylor (C/Taylor lineage) and 2012 C/Vic (C/Sao Paulo lineage). Historical 1966 C/Johannesburg (C/JHB) (C/Aichi lineage) virus was also included together with 1947 C/Taylor and 2012 C/Vic in this antigenic study. Homologous HI titers for C/Taylor and C/Vic were 5120 and 1280, respectively. Heterologous mean HI titers against C/Taylor and C/Vic antisera were normalized to homologous C/Taylor and C/Vic titers. Interestingly, the three lineage viruses spanning over 60 years showed equivalent cross-reactivity with C/Vic antisera with relative HI titers 1.0 (Fig. 2). In contrast, cross-reactivity profile with C/Taylor antisera discriminated three viruses clearly. Specifically, C/Taylor virus reacted most strongly with homologous C/Taylor antisera (relative HI titer 1.0) followed by C/Vic (relative HI titer 0.5) and by C/JHB (relative HI titer 0.25) (Fig. 2). These data suggested that 1947 C/Taylor was more antigenically related to 2012 C/Vic than to 1966 C/JHB. The observed antigenic variations were in good agreement with HEF phylogeny of these ICV strains (Fig. 1).

3.4 Structure-basis of antigenic variation

To identify critical residues of the HEF protein among these three ICVs contributing most to the observed antigenic drift (i.e., HA titer change), pairwise comparisons of viral HEF structures were pursued. The resolved crystal structure of C/JHB HEF (PDB ID: 1FLC) was chosen as a model template to model the HEF structures of the C/Vic and C/Taylor strains. We focused on four secondary elements (the 170-loop, 230-helix, 270-loop, and 290-loop) constituting the HEF receptor-binding pocket (RBP) for our investigation (Fig. 3A). Structure modeling showed that three ICVs exhibited two amino acid substitutions in the 170-loop proximal to the RBP of the HEF, which likely have a decisive role in the degree of antigenic distance observed among three ICV strains (Fig. 3). Specifically, the more antigenically similar virus pair C/Vic-C/JHB or C/Vic-C/Taylor only had one mutation in the 170-loop. K172G or T170V substitution occurred between C/Vic and C/JHB (Fig. 3B) or C/Vic and C/Taylor (Fig. 3C) (listed in an arrangement as the C/Vic amino acid residue, HEF position, and C/JHB or C/Taylor amino acid), respectively. In contrast, the more antigenically divergent virus pair C/Taylor-C/JHB (Fig. 3D) acquired these two mutations V170T and K172G of the 170-loop. These structure-based analyses seemed to indicate a link between the level of antigenic variation among three strains of ICV and the number of the amino acid changes in the proximity of the HEF receptor-binding pocket. In addition, multiple amino acid substitutions were also identified among three viruses in various regions of the HEF including HEF1, HEF2, and the trimeric interface. Considering that all those mutations were distant from the RBP of the HEF protein, we speculate that these amino changes may not modulate directly antigenicity of influenza C viruses.

In summary, we have determined the antigenic and genetic characteristics of a contemporary human C/Vic virus and described its evolutionary pathway. In addition, structural modeling work presented here has pinpointed two critical residues in the 170-loop of the HEF protein that are likely responsible for the observed antigenic differences among three ICV strains. The information described on this recent ICV here, as a whole shall aid in the further investigation of biology, evolution, and pathogenesis of ICV.

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Figure 1. Phylogenetic trees of the seven genomic segments of influenza C virus.

Sequences of each segment of human influenza C viruses were aligned and then used for phylogenetic tree construction through the MEGA7 program. The best substitution model for each segment was estimated using MEGA7. The phylogenetic trees were generated using Maximum-likelihood method of MEGA7 with 1000 bootstrap replicates to verify the topology. The trees were rooted using the ancestor branches of influenza D virus strains including D/Bovine/Ibaraki/7768/2016, D/Bovine/France/29862012, D/Bovine/Italy/46484/2015, D/Swine/Italy/199724–3/2015, D/swine/Oklahoma/1334/2011 and D/bovine/Oklahoma/660/2013. Influenza D viruses are not shown in phylogenetic trees, while C/Victoria/2/2012 strain is marked as diamond (◆).

Figure 2. Relative mean hemagglutination inhibition titers for 3 influenza C viruses from triplicate data.

Relative mean HI titers were calculated by normalizing mean heterologous HI titers to mean homologous HI titers (mean heterologous HI titer/mean homologous HI titer). Error bars represent standard deviations.

Figure 3. Structure-basis of antigenic variation.

(A) Cartoon view of modeled HEF1 domain of C/Vic (colored Wheat), while HEF2 was colored light blue. Salic acids in receptor binding pocket (RBP) and esterase active site (EAS) were colored cyan. Four secondary elements (the 170-loop, 230-helix, 270-loop, and 290-loop) constituting the HEF receptor-binding pocket were indicated with different colors. (B) (C) (D) HEF1 residues different among three virus pairs were mapped onto the modeled HEF1 domain of C/Vic. Note the critical amino acid changes in the 170-loop of the HEF protein around the receptor-binding pocket were highlighted by bold and underlined words.