Case Report: The Laboratory Examination and Viral Sequencing Analysis of the First Novel Influenza A (H1N1) Patient with Sever Complication in Taiwan

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Abstract

On July 17, 2009, the Centers for Disease Control in Taiwan (Taiwan CDC) confirmed the first case of severe patient infected by novel influenza A(H1N1) virus. The patient is a 34-year-old male without underlying cardiopulmonary disease or travel history. He came to hospital because of cough, sore throat and dyspnea. His symptoms became worse despite treatment and led into multiple organ failure. Then he was sent to intensive care unit for further treatment. The Respiratory Virus Laboratory received the information and tested the throat swab and sputum from this patient. Both specimens revealed positive for novel influenza A(H1N1) virus by real time RT-PCR. Sequencing analysis of HA, NA, and M gene of this virus isolate, discovered S31N mutation in HA gene. This strain was resistant to amatadine and sensitive to oseltamivir because the 274th amino acid of NA gene was still Histidine. After comparing with the virus

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gene sequences from other countries, this virus contained D225G change. The evolutionary analysis showed all novel influenza isolates at Taiwan came from the same origin. Because this virus might come from community, correct and rapid test with virus surveillance and anti-viral medication in time would allow monitoring the changes of novel influenza virus in the future, furthermore, prevent the severe case and mortality from happening.

Keywords: H1N1 pandemic influenza, real-time RT-PCR, antiviral drug resistance, phylogenetic analysis

Introduction

The emergence of novel influenza was found from USA and Mexico between March and April this year. These patients presented with influenza-like symptoms including fever, headache, myalgia, cough and running nose. The severe case may have severe respiratory diseases such as pneumonia and cause death. According to the report from World Health Organization (WHO), 103 countries have reported more than 70000 cases and the mortality rate was around 0.1-0.5%. The novel influenza A (H1N1) virus spread in a moderate and rapid pattern globally. The novel (H1N1) influenza virus, derived from triple reassortment of human, bird, and swine influenza virus, had been found in 1998. But the case number of human infection was few; there were only 10 sporadic cases in USA between 2005 and 2009.

With the rapid increasing number of confirmed novel influenza infections internationally, WHO raised the global pandemic alert level to Phase 5 on April 29 then Phase 6 on June 11. The virus has caused pandemic in the world. Initially, Taiwan CDC classified this disease into Category I Notifiable Diseases. The suspected cases should be notified in

24 hours and the confirmed case should be sent to hospital for isolation and treatment. The first novel influenza case was confirmed on May 20 from a 52-year-old alien who just presented with mild symptoms. After that, the number of confirmed cases increased rapidly. There were 10 confirmed cases in a week. WHO announced that the global pandemic influenza is moderate in severity as seasonal flu on June 11 and it is impossible to restrict the disease to certain area. The Department of Health in Taiwan then announced the withdraw of novel influenza A(H1N1) from Category I Notifiable Disease according to the Act of Infectious Diseases Control. The severe cases of influenza should be reported as Category IV Notifiable Disease. The prevention strategy was also emphasized on surveillance in community. According to the surveillance data, the confirmed novel influenza A(H1N1) case numbers were 93 as of July 17. The virus surveillance also revealed 15 positive cases. The proportion of novel H1N1 virus was near 88.2%. The symptoms of patients with novel influenza A(H1N1) were mild and most cases were fully recovered.

WHO has defined that the confirmed case of novel influenza is determined by (1) RT-PCR or other derived method, (2) direct isolation of novel influenza A(H1N1) virus, and (3) A four-fold or greater rise in specific novel influenza A(H1N1) virus antibody titers.

This article reported the confirmation of the first case of severe novel influenza infection by the Respiratory Virus Laboratory of Taiwan CDC. The genomic sequence of the virus isolate was analyzed, checked the susceptibility of amantadine and oseltamivir, and also compared with the strains isolated by our laboratory and other countries.

Materials and Methods

The definition of severe influenza case

According to the definition by Taiwan CDC, the patient presented with influenza-like-illness and developed (1) pulmonary complications and hospitalized, (2) neurological complications, (3) myocarditis or pericarditis, (4) Invasive bacterial infection, or (5) not fit in above criteria but admitted to ICU or death, within 4 weeks. (Website available: http://www.cdc.gov.tw). After notification, patient's throat swab should be collected then send to the Respiratory Virus Laboratory for examination.

The case history

The first confirmed severe novel influenza A(H1N1) patient is a 34-year-old male; he presented with fever, cough, dyspnea and sore throat from July 2. He came to hospital for help because of difficulty breathing on July 9. The chest X-ray revealed pneumonia. After treatment, the symptoms did not resolve and progressed into multiple organ failure. He was sent to ICU for treatment. The patient did not have underlying cardiopulmonary disease or travel history, possibly the infection came from community.

Specimen processing and viral RNA extraction

Add 0.5mL DMEM (Invitrogen) medium into throat swab, vortex then squeeze the cotton buds by finger to mix the virus with medium. Take 140uL of medium to extract the viral RNA by QIAamp viral RNA Kit (QIAGEN). The rest of medium was kept frozen in -80℃ freezer or filtered for virus culture later.

Molecular diagnostics for novel influenza A (H1N1) virus

The molecular diagnostics for novel influenza A(H1N1) virus was done with One-step real-time RT-PCR by Roche LightCycler 480. The

sequence of primer and Taqman probe followed the recommendation from WHO. The target included: (1) type A influenza matrix gene, can detect all influenza A virus. (2) Swine Influenza A NP (Nucleoprotein) gene, can detect novel influenza A virus, and (3) Swine Influenza H1 HA (Hemagglutinin) gene, can detect novel influenza A virus H1. To save time, all 3 reactions were performed simultaneously. The reagent was prepared with LightCycler 480 RNA Master Hydrolysis Probes. The contents included DEPC Water 3.8ul, probe and primer 0.5ul, Activator 1.3ul、Enzyme Master Mix 7.4uL, Enhancer 1ul and virus RNA template 5ulL. The reaction was 63℃ for 3 minutes, 95℃ for 30 seconds, then 15 seconds at 95℃, 30 seconds at 55℃, 3 seconds at 72℃, run for 45 cycles. It takes about 1hour and 20 minutes.

The culture of novel influenza virus

Specimen processed as mentioned above was filtered by 0.45μm membrane, inoculate 100~200μL specimen into MDCK cell and monitor for cytopathic effect (CPE). After CPE presented (7-10 days culture), centrifuge the culture tube then identify the virus by immunofluorescence stain.

The HA, NA, and M gene sequence analysis of novel influenza virus

To amplify the HA, NA, M gene fragment, viral RNA extraction from clinical specimen is prepared with QIAamp Viral RNA Mini Kit, then RT-PCR protocol suggested by WHO is followed [4], then the sequence of amplified fragment is determined. Sequence fragment was analyzed by Sequencher 4.5 software first, then sequence comparison and phylogenetic analysis was done by MEGA 4.0 and Bioedit software. Every gene fragment was sequenced by at least 3 sets of primer to assure the accuracy. The amantadine resistant is determined by the 31st amino acid position at

M gene fragment. If it is Serine, then it is amantadine sensitive. If the amino acid is Asparagine, then it is amantadine resistant. The determination position of oseltamivir resistant is at the 274th amino acid of NA gene fragment. If Histidine, it is oseltamivir sensitive. If Tyrosine, then it is oseltamivir resistant.

Results

The results of real-time RT-PCR

The patient's throat swab was tested at Tri-Service General Hospital (TSGH) laboratory on July 11. Because the result was weakly positive for the novel influenza A(H1N1) virus, so the specimen was referred to the Respiratory Virus Laboratory at Taiwan CDC. After received the original specimen, the laboratory performed one-step real-time RT-PCR aimed at Influenza A, novel influenza A and novel influenza A subtype H1 immediately. The result was weakly positive for Influenza A and novel influenza A, matched with the result at TSGH laboratory.

Because of the weakly positive results, the laboratory took another sputum specimen at the same day and repeated the real-time RT-PCR for novel influenza A. The result showed strong positive for Influenza A, novel influenza A and novel influenza A subtype H1 (Figure 1). The Ct value was 26.2, 26.9 and 27.7, respectively. The patient was confirmed as the first severe case caused by novel influenza A(H1N1) virus in Taiwan on July 17. A virus culture was prepared immediately for further analysis.

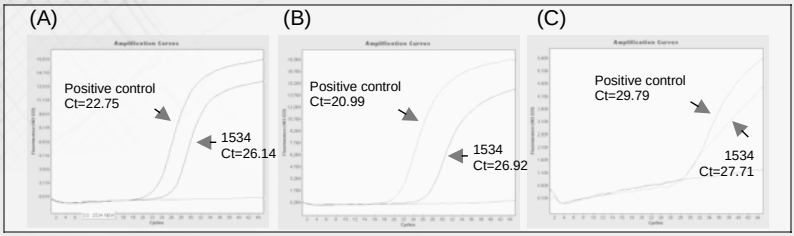

Figure1. The real-time RT-PCR results of the first novel influenza A(H1N1) patient with sever complication
(A) test for influenza A (B) test for novel influenza A (C) test for novel influenza A subtype H1. The positive control and the patient specimen (1534) fluorescence signal were signaled by arrow. According to above result, the 1534 specimen was positive for novel influenza A (H1N1) virus.

The HA, NA and M gene sequencing of novel influenza A(H1N1) virus

To compare the virus strain of patient and the novel influenza A(H1N1) virus strain isolated by other countries and checked the susceptibility to amantadine and oseltamivir, the laboratory sequenced the HA, NA and M gene fragment by conventional RT-PCR and analyzed the nucleotide and amino acid sequence. According to the M gene sequence, the 31st amino acid was Asparagine, showed the virus strain was resistant to amantadine. According to the NA gene sequence, the 274th amino acid point was Histidine, showed the virus was still susceptible to oseltamivir. The HA gene dendrogram showed the novel influenza virus strain (A/Taiwan/1534/2009) was in the same clade (clade Ia, Figure 2) with the strains isolated at USA, Mexico, Holland and Israel. The strain has nucleotide substitution of T658A (with amino acid S203T change) when compared to the vaccine strain A/California/07/2009. Besides, the 225th amino acid of HA gene of A/Taiwan/1534/2009 strain was Glycine. Other strain was Aspartic acid.

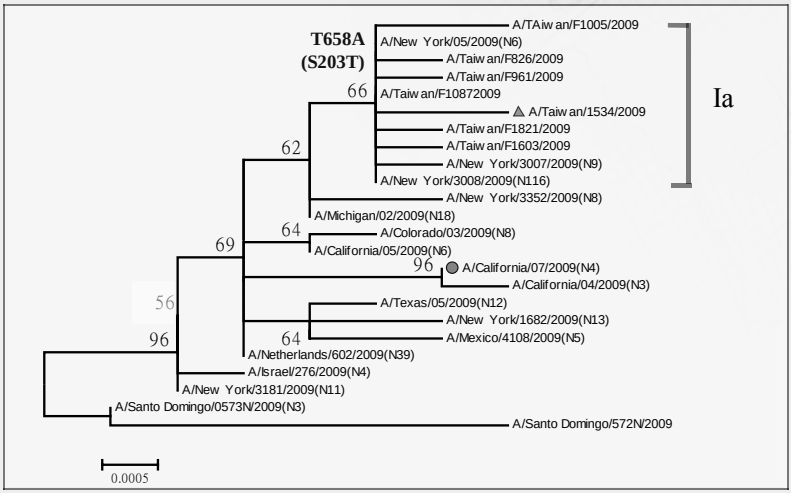

Figure 2. The phylogenetic dendrogram of HA gene of A/Taiwan/1534/2009 strain with other isolates obtained from GenBank.

Compare the virus strain A/Taiwan/1534/2009 (red triangle) with the H1N1 virus strain isolated by other country by MEGA 4.0 software, then build the evolutional tree by Neighbor-Joining Method (the branch boostrap value was calculated with 1000 times replicates). All isolates by Taiwan CDC including A/Taiwan/1534/2009 located in family Ia. The family has change at T658A (S203T) compared to vaccine strain A/California/07/2009 (Blue circle).

Discussion

There are 93 confirmed novel influenza A(H1N1) cases in Taiwan since April 26, with no mortality (as of July 17). The novel influenza virus had replaced the seasonal influenza virus; the virus proportion increased to

88.2% and became the most influenza strain detected. Hong Kong also reported the proportion of novel influenza detection of 70%, and USA was 63.4%. This shows the high transmission by this virus. In the strategy of infection control, Taiwan CDC begins with border quarantine, then blocking the outbreak in community and monitoring the severe cases, just like other countries. Now only the severe case of novel influenza was classified as Category IV Notifiable Disease, and the confirmed cases did not require being isolated. But the surveillance of novel influenza virus was fully enforced by Taiwan CDC. According to the published literatures, the severity of novel influenza was not worse than the seasonal influenza. But the variation of virus keeps changing. It is an important task to stop the next outbreak of novel influenza, not only for our country but also for the world.

The specimens from different sites were tested. The virus can be detected from deeper sputum specimen, and the viral load was higher than the throat swab collected from upper respiratory tract. It shows that the virus can infect human on upper and lower respiratory system at the same time. The result is compatible with previous report [8]. It also explains the change on 225th amino acid of HA gene sequence (i.e., from Aspartic acid to Glycine). Similar change has been reported in avian influenza virus such as H5N1 strain. The virus strain can combine with α2,3 type of sialic acid, which most located in lower respiratory tract. So the virus can be detected in deeper sputum specimen. The sputum from deeper site can be the suitable specimen to collect in the severe case of novel influenza.

Tamiflu (oseltamivir) resistant novel influenza A(H1N1) virus has been reported continuously from the world. Analysis of NA gene fragment

of the virus revealed the 274th amino acid is Histidine and indicates the virus is susceptible to Tamiflu. At present stage, Tamiflu remains the first choice to treat novel influenza A(H1N1). Besides, the virus strain from this severe case is in the same cluster with our previous isolates in HA phylogenetic analysis, but it has amino acid D225G change. It needs further analysis on other 5 gene fragments to elucidate the virus virulence change.

At present, Taiwan CDC has been preparing the novel influenza A(H1N1) vaccine and stocking up medication (Tamiflu) in response to the future epidemic in fall and winter. For the patients with influenza-like symptoms, novel influenza A(H1N1) virus infection will be our first consideration and early treated with Tamiflu to prevent severe cases and mortality. Because the first severe case did not have travel history, the infection may come from community. So it is important to monitor the virus in community. Furthermore, rapid and correct examination will play an important role in the future and with other preventive measures, Taiwan CDC can effectively monitor the virus activity.

References

1. WHO. Pandemic (H1N1) 2009 - update 58. 6 July 2009. Available at: http://www.who.int/csr/don/2009_07_06/en/index.html
2. Olsen, CW. The emergence of novel swine influenza viruses in North America. Virus Res 2002; 85:199-210.
3. WHO. CDC protocol of realtime RT-PCR for swine influenza A(H1N1) revision 1. 30 April 2009. Available at: http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html

4. WHO. Sequencing primers and protocol. 12 May 2009. Available at: http://www.who.int/csr/resources/publications/swineflu/sequencing_primers/en/index.html
5. Centre for Health Protection, HK. Flu express for the 2008/09 flu season (Volume 5, Number 23-28).
6. CDC. 2008-2009 Influenza Season Week 27 ending July 11, 2009. Available at: http://www.cdc.gov/flu/weekly/
7. Novel swine-origin influenza A (H1N1) virus investigation team. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009; 360: 2605-15.
8. Munster VJ, de Wit E, Fouchier RA, et al. Pathogenesis and transmission of swine-origin 2009 A (H1N1) influenza virus in ferrets. Science 2009; 325: 481-3.