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Analysis of the HA Gene of the Novel Influenza A (H1N1) Virus in Taiwan and Epidemic Strains in Other Countries

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Abstract

The first outbreak of novel H1N1 influenza virus appeared in North America in April 2009. Until July 1, a total of 77,201 cases have been reported in 103 countries in the world with mortality rate of 0.43%. Through the genetic sequence analysis of influenza A (H1N1) virus, it was a double reassortants with European swine flu virus and North American swine flu virus, so it was called swine flu initially. Later it was renamed as novel influenza A (H1N1). Until July 1, there have been 70 confirmed cases in Taiwan, with 4.3% positive rate. We selected 6 positive specimens for cell culture and compared the HA gene from virus isolates, the result revealed 99% similarity, which was similar to the published results in the world currently. Through phylogenetic analysis, the gene sequence of influenza A (H1N1) virus isolates in Taiwan could be categorized as the same group with the virus in New York, USA and Mainland China, but

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different from other strains in the world. Since the clinical symptoms of novel influenza A (H1N1) were quite similar to classical seasonal flu, only laboratory tests could distinguish the difference. Thus, for a suspected case, one can rely on effective and accurate identification of the case to achieve the goal of rapid prevention. In order to ascertain whether or not the virus isolated in Taiwan has mutated or changed antigenicity, and whether or not that may affect pathogenicity and transmissibility, further analyses with control antigens and antiserum are needed.

Keywords: influenza A (H1N1), sequence analysis, Taiwan

Introduction

In April 2009, novel influenza A (H1N1) outbreaks appeared in North America. Until July 1, a total of 77,201 cases have been reported in 103 countries, which included 332 death cases. The mortality was about 0.43%~2.71%, the distribution of global cases was shown in Figure 1 [1]. The first imported case was a 52-year-old male passenger, flew from New York on May 17, through Hong Kong and arrived at Taiwan Taoyuan International Airport on May 18. Respiratory specimen was taken because of fever and the passenger was sent to negative pressure isolation room. He was confirmed as a novel influenza A (H1N1) case on May 20. As of July 1, there were a total of 70 confirmed cases in Taiwan. The positive rate was 4.3% which included one cluster, and most patients had mild symptoms.

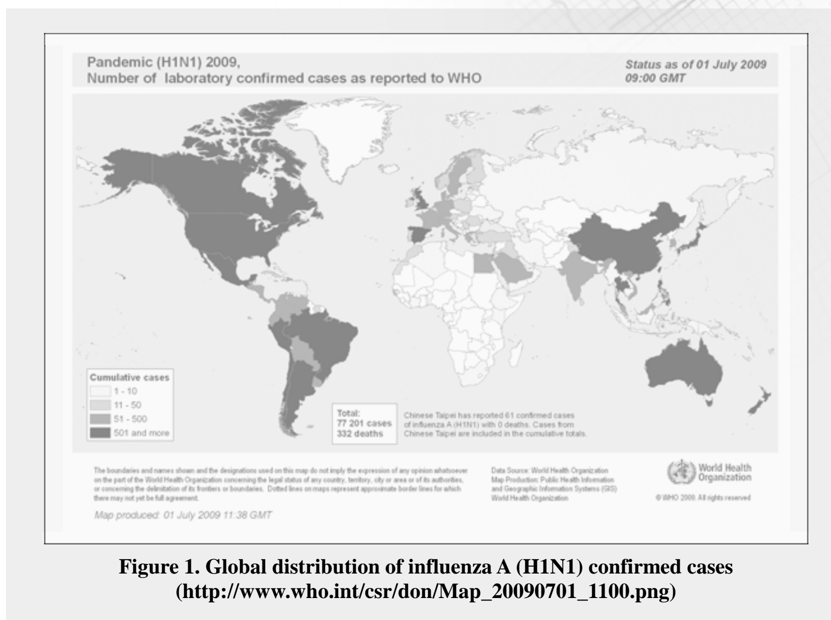

Figure 1. Global distribution of influenza A (H1N1) confirmed cases (http://www.who.int/csr/don/Map_20090701_1100.png)

Through the genetic sequence analysis of novel influenza A (H1N1) virus, it was a reassortants with Eurasian swine flu virus and North American swine flu virus, so it was called swine flu initially. The virus was renamed as influenza A (H1N1) by World Health Organization (WHO) on April 30, 2009. The Department of Health in Taiwan announced the new Chinese name as novel influenza A (H1N1) as well. According to the definition of novel influenza A (H1N1), the cases can be divided into confirmed cases, probable cases, and suspected cases. When suspected patients with acute respiratory symptoms and the results were positive for real-time RT-PCR or viral culture, the patients were categorized as confirmed cases. If a patient, with acute respiratory symptoms and travel history, whose specimen was positive for influenza A but the differential test for H1 and H3 were both negative by RT-PCR method, the patient was

diagnosed as probable case. Because the flu-like symptoms of novel influenza A (H1N1) was similar to seasonal flu, we could not diagnose by regular testing protocols. Specific testing system for the novel influenza A (H1N1) virus is necessary; this demonstrated the importance of differentiation methods for novel influenza A (H1N1) virus in a laboratory.

Material and method

1. Specimen collection

Specimen is collected from passenger who has foreign travel history and the body temperature is above 38℃ by fever screen station at the airports. Throat swabs were collected and delivered with refrigeration to influenza virus laboratory, Research and Diagnostic Center, Centers for Disease Control for the following tests.

2. Specimens pre-treatment

Vortex the throat swab within the 0.5 ml transfer medium completely to dislodge the virus from the swab. The transfer medium was preserved in the tubes for further RNA extraction and cell culture for virus isolation.

3. Nucleic acid extraction of influenza virus

Viral RNA was extracted with the QIAmp viral RNA Kit (QIAGEN, Hilden, Germany) Pipet 560µl of prepared Buffer AVL containing carrier RNA. Add 140µl specimens and mix by pulse-vortexing thoroughly. Incubate at room temperature (15-25℃) for 10 min. Add 560µl of 100% ethanol to the sample, remove the sample to spin column and centrifuge at 8000 rpm for 1 min. Place the column into a clean 2ml collection tube, and discard the tube containing the filtrate. Add 500µl of Buffer AW1 and centrifuge at 8000 rpm for 1 min. Place the column in a clean 2ml

collection tube, and discard the tube containing the filtrate. Add 500µl of Buffer AW2 and centrifuge at 14,000 rpm for 3min. Place the column in a clean 1.5ml microcentrifuge tube. Add 50µl of DEPC water. Incubate at room temperature for 1min. Centrifuge at 8000 rpm for 1min. Collect the eluate for further analysis. The residue of viral RNA was prepared at -20℃ for use.

4. Real-time RT-PCR identification

We use ABI 7500 in real-time RT-PCR assays for type A influenza virus identification. The CDC real-time RT-PCR protocol was suggested by WHO [2]. The reagents include 2X buffer (ABI One-step RT-PCR kit) 12.5µl, enzyme mix 0.67µl, both type A and type B primers 1µl (10µM), and probe 0.5µl (5µM) each, add DEPC water up to total volume 20µl. Finally, add viral RNA eluate extracted previously 5µl for Real-time RT-PCR assays to confirm if it is type A influenza virus. The primer and probes were processed from previous published reference [3]. The amplification conditions are 48℃ for 30 minutes and 95℃ for 10 minutes. The PCR amplification was reacted at 95℃ 15 sec, 60℃ 1min for 40 cycles.

The confirmed specimen with type A influenza virus was tested by real-time RT-PCR. The detection and characterization protocols followed WHO standard protocols for swine influenza A (H1N1) specific primer/probe sets [2]. The reagents include 2X buffer 12.5µl, enzyme mix 0.67µl, H1N1 virus reaction primers 1 µl (10µM), and probe 0.5µl (5µM) each, add DEPC water up to total volume 20µl. Add virus RNA 5µl for Real-time RT-PCR assay and confirmed by analysis software.

5. Influenza virus isolation

Filter the transfer medium with virus from the throat swab. Inoculate 100μl of the fluid to MDCK cell line. Use inverted microscope to evaluate cytopathic effect (CPE) every day. Centrifuged the cells after incubation for 7-10 days and collected the supernatant. The supernatant was used for indirect immunofluorescence assay with the Respiratory Panel 1 Viral Screening and Identification Kit (Chemicon, Cat no.3105) for identifying virus in infected cell cultures. The protocol followed the manufacturer's manual.

6. Sequencing and analysis of HA gene of influenza virus isolates

Extract nucleic acid from positive virus suspension with QIAamp Viral RNA Kit. Then perform nucleic acid amplification on HA gene fragment separately. The amplification length of HA fragment is 1115 bp which include full length of HA1 gene. The sequences of primers used in PCR amplification and sequencing followed WHO's suggestion [4]. After the PCR amplification, the products were purified with QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Then use sequencing primer with BigDye v3.1 Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) for amplification and sequencing by Applied Biosystems 3130XL Genetic Analyzer. To prevent cross contamination and affect the results, all the sequencing reactions were repeated at different time and used different lot of reagents separately.

All the sequences were assembled with Lasergene software. Finally, use MEGA 4 (Molecular Evolutionary Genetic Analysis, version 4.0) [5] for evolutionary genetic analysis.

Results

After the nucleic acid was extracted from specimens, we used a panel of specific primers and probes in real-time RT-PCR assays for novel influenza A (H1N1) detection and characterization. The results were shown in Figure 2. The ΔRn value elevation means the result was positive in PCR reaction for novel influenza A (H1N1) detection. When Ct value was higher, the concentration of virus was higher. Six specimens were selected from patients who had obvious clinical symptoms for virus isolation; these were all real-time RT-PCR positive samples. Among these, only case A/Taiwan/731/2009 had no travel history and was a contact, all other 5 isolates were imported cases. The history of the 6 isolates and the Ct values of real-time RT-PCR detection results were listed in Table 1.

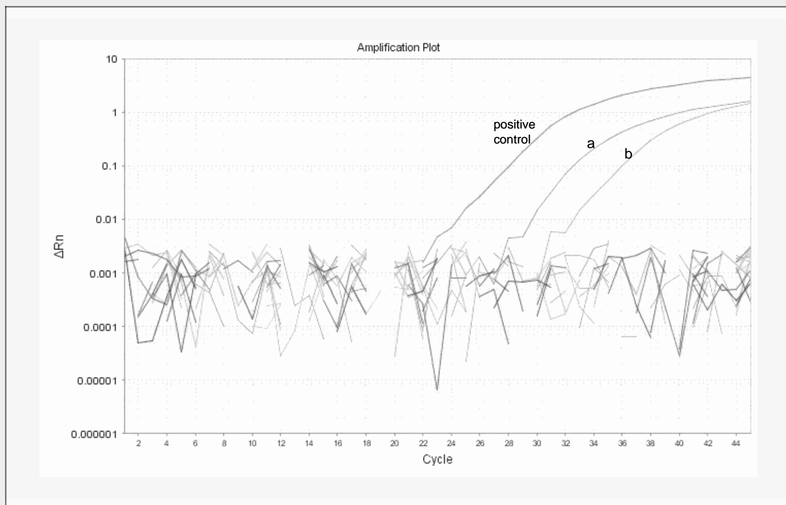

Figure 2. Real-Time RT-PCR results of novel influenza A (H1N1) with positive control and negative control. The result revealed 2 positive samples marked as a: A/Taiwan/732/2009 and b: A/Taiwan/731/2009.

Table1. The travel history and the Ct value of realtime RT-PCR results of novel influenza A (H1N1) positive cases

No.	Isolates	Sampling date	Age	Gender	Ct value	Travel History
1	A/Taiwan/727/2009	2009/5/21	22	Male	29	New York, USA
2	A/Taiwan/728/2009	2009/5/21	40	Female	32	Malina, the Philippines
3	A/Taiwan/729/2009	2009/5/22	25	Female	33	California, USA
4	A/Taiwan/730/2009	2009/5/22	5	Female	28	Malina, the Philippines
5	A/Taiwan/731/2009	2009/5/23	40	Female	35	No travel history
6	A/Taiwan/732/2009	2009/5/24	24	Female	32	California, USA

We used indirect fluorescence assay (IFA) for the CPE positive cell cultures. A positive reaction, bright apple-green fluorescence, can be seen with the aid of a fluorescence microscope. Since there is no control antiserum for testing influenza A (H1N1) virus, genetic sequencing would be the final confirm step as recommended by the WHO standard manual.

We analyzed HA gene sequence from the 6 isolates, which include HA1 full sequence (1101 bp, from 1-1101). After alignment of the 6 sequences, we found that the nucleotide similarity of the 6 isolates was 99.9%~100%, which was similar to the published results internationally [6]. The source of A/Taiwan/729/2009 and A/Taiwan/732/2009 were from California, USA and the sequences were similar, but slightly different to the other four isolates, as shown in Figure 3 part A. Besides, we collected the gene sequences of novel influenza A (H1N1) virus published globally [7], and compared with the 6 Taiwan isolates with alignment and phylogenetic analysis. The results revealed that there were two main groups in the novel influenza A (H1N1) in the world, which was shown in Figure 3 part B. Through sequence analysis, the 6 isolates in Taiwan was

similar to USA A/New York/3313/2009, A/California/14/2009 and some isolates from Mainland China such as A/Guangdong/2/2009 and A/Shanghai/71T/2009 (Figure 3, part B).

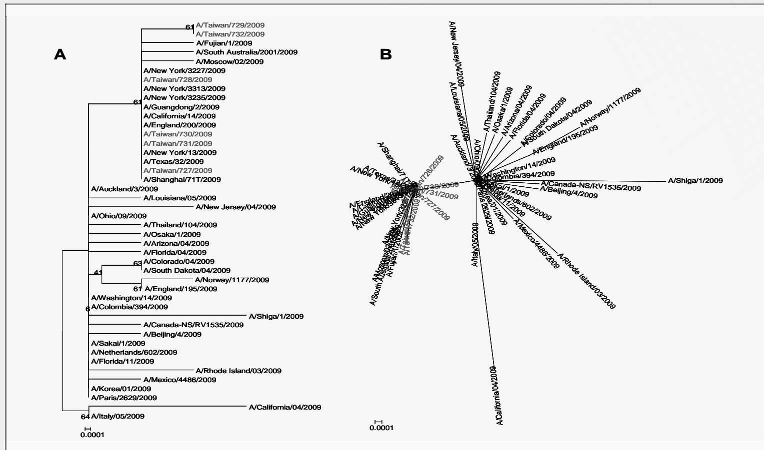

Figure 3. Phylogenetic trees for novel influenza A (H1N1) virus, the isolates from Taiwan were show as red color. The analyzed method was Neighbor-Joining Method and the boostrap was 1000. The 6 isolates from Taiwan were in the same group with isolates from California, New York, and some isolates from Mainland China.

Discussion

The pandemic novel influenza A (H1N1) virus was isolated in the United States and Mexico since April 2009. The WHO had raised the alert level to phase 6 since June 11, 2009 [8]. Especially in USA, form April to July, the novel influenza A (H1N1) had replaced general seasonal influenza and became the main epidemic flu type. The isolation rate was 60.2% of all the flu type [9]; it revealed the fast transmission of novel influenza A (H1N1) virus.

According to the alignment and phylogenetic analysis for the HA gene

sequences of the novel influenza A (H1N1) virus, the isolates could divide into two groups. The isolates in Taiwan were similar to those in New York, USA and Mainland China. At the site of amino acid sequence 203 is Tyrosine in this group, which is different from Serine in the other group. The amino acid site 203 of HA Ca1 was the antigenic site of the H1N1 virus. More alignment and analysis by using control antigen and antiserum are needed to explore if the mutation causes the change in virulence or antigenicity, or even affects on the pathogenicity and transmissibility.

In July, WHO announced some novel influenza A (H1N1) virus isolates resistant to Tamiflu in Denmark, Japan, Hong Kong and some other countries [10]. The MP and NA gene fragments from the 6 isolates in Taiwan were identified the same as isolates in the world that MP gene of the isolates in Taiwan had amino acid site S31N mutation, which cause them resistant to Amantadine, the antiviral medicine. However, amino acid site 274 of NA gene was Histidine, so the isolates were still susceptible to Tamiflu. Hence, for the purpose of prevention and treatment of novel influenza A (H1N1), Tamiflu is one of the top priorities for clinical prescription. Besides, according to the research from Research and Diagnostic Center at CDC Taiwan previously, the A (H1N1) seasonal flu virus at the last season and now mostly were resistant to Tamiflu. During the next flu season in fall, novel influenza A (H1N1) may cocirculate with the seasonal flu in populations and cause gene reassortment of viruses, which then become Tamiflu resistant. As the result, we have to keep monitoring the antigenicity and gene structure of the viruses to understand the viral activities.

Conclusion

Since April 2009, the first outbreak in Mexico, influenza A (H1N1) virus with swine influenza gene reassortment, has spread to 103 countries around the world within three months. WHO hence announced the pandemic alert level to the highest revealing that the novel virus could cause human-to-human transmission and global influenza A (H1N1) pandemic. WHO experts considered that the novel influenza A (H1N1) virus had no characteristics of high fatality such as the viruses in 1918, they predicted the viruses would not cause widespread human death in the world. However, monitoring the activities of seasonal flu viruses in fall and winter is necessary to observe if the usual seasonal flu viruses was reassorted with novel influenza A (H1N1) and caused reassortant virus exhibited high virulence and efficient transmissibility.

There have been 70 cases of novel influenza A (H1N1) in the country, include one community cluster, revealing that prevention from imported cases had limited effectiveness. Because of Taiwan's close relationship with other countries, novel influenza A (H1N1) could transmit to Taiwan through travelers. Besides the sentinel-doctors surveillance system to monitor infections in the community and national airport fever screening system to monitor international passengers, the capability of laboratory should be strengthen at the same time. Since novel influenza A (H1N1) and seasonal flu exhibit similar flu-like symptoms, laboratory confirmation is the only way to differentiate. As the result, improving the sensitivity and specificity for influenza A (H1N1) testing and rapid confirmation can achieve the goal of disease control.

References

1. Interim WHO guidance for the surveillance of human infection with swine influenza A (H1N1) virus 3 May 2009. Available at: http://www.who.int/csr/don/2009_07_01a/en/index.html
2. WHO. CDC protocol of realtime RTPCR for swine influenza A (H1N1). Available at: http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html
3. Ward CL, Dempsey MH, Ring CJA, et al. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J. Clin Virol 2004; 29:179-88.
4. WHO. Sequencing primers and protocol. Available at: http://www.who.int/csr/resources/publications/swineflu/sequencing_primers/en/index.html
5. Tamura K, Dudley J, Nai M, et al. MEGA4:Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24:1596-9.
6. Garten RJ, Davis CT, Russell CA, et al. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science 2009; 325:197-201.
7. Bao Y, Bolotov P, Dernovoy D, et al. The Influenza Virus Resource at the National Center for Biotechnology Information. J. Virol. 2008; 82:596-601.
8. WHO. WHO: Influenza Pandemic alert raise to level 6. Available at: http://www.who.int/pmnch/media/membernews/2009/20090611_who/en/
9. CDC. 2008-2009 Influenza Season Week 26 ending July 4, 2009. Available at: http://www.cdc.gov/flu/weekly/
10. WHO. Viruses resistant to oseltamivir (Tamiflu) identified. Available at: http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_resistance_20090708/en/index.html