

Laboratory and Epidemiology Communications

Virological Analysis of a Case of Dual Infection by Influenza A (H3N2) and B Viruses

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Communicated by Ichiro Kurane

(Accepted January 6, 2006)

The influenza epidemic in the 2004/2005 season was relatively large compared to those in other seasons in Saitama Prefecture, Japan. Thirty-seven influenza A (H3N2) and 38 influenza B virus isolates were obtained at Saitama Institute of Public Health (SIPH). Influenza A (H1N1) virus, a minor subtype in this season, was isolated from only one specimen at the inception of the epidemic, December 2004 (1).

Influenza A (H3N2) and B viruses were simultaneously isolated from one individual, a 2-year-old boy. This boy developed slight influenza symptoms on March 2, 2005. A rapid diagnostic test (ESPLINE influenza A & B-N; FUJIREBIO Inc., Tokyo, Japan) was positive for both A and B influenza antigens at the clinic. At the same time, his mother and sister were diagnosed with influenza A and B, respectively, by the same test kit. A nasal specimen was collected only from the boy for further virological examination and sent to SIPH a week later.

The specimen was positive only for influenza A by the rapid diagnosis test (ESPLINE influenza A & B-N) at SIPH. Subsequently, the specimen was inoculated onto several cell lines. An obvious influenza-like cytopathic effect was observed 3 days after inoculation to MDCK and CaCo-2 cells. The culture supernatant (the crude isolate) from inoculated MDCK cells was examined by a hemagglutination inhibition (HI) test using standard antisera (Table 1). The sample was not neutralized by any of the used antisera. Influenza A and B antigens were detected by the rapid diagnosis test kit (ESPLINE influenza A & B-N), influenza A antigen being much stronger than influenza B antigen. On the other hand, only influenza A antigen was detected in CaCo-2 supernatant.

In the next series of experiments, the hemagglutinin (HA) and neuraminidase (NA) genes were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Viral RNA was extracted from the culture supernatant using the QIAamp Viral RNA Mini Kit (QIAGEN, Germantown, Md., USA), according to the manufacturer's instructions. RT and PCR were performed consecutively in one tube by the Access RT-PCR system (Promega, Madison, Wis., USA), according to the manufacturer's recommendations. Influenza AH3-HA, AN2-NA and B-HA genes were detected, whereas influenza

AH1-HA, AN1-NA and AH5-HA genes were not. The results suggested that the patient was infected simultaneously by both influenza A (H3N2) and B viruses.

The levels of AH3-HA and B-HA genes in the original specimen were assessed by real-time quantitative PCR. Briefly, viral RNA was extracted from the specimen as described above and 50 μ l of complementary DNA (cDNA) was synthesized using SuperScriptTM II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA) from 20 μ l of RNA solution. Real-time PCR was performed at Tokyo Metropolitan Institute of Public Health as reported previously (2) with some modifications on primers and probes. The numbers of copies of influenza AH3-HA and B-HA genes in 10 μ l of cDNA were estimated to be 1.2×10^5 and 1.2×10^1 , respectively. This result suggested that the amount of influenza AH3 virus was 10,000 times higher than that of influenza B virus in the original specimen.

Viral isolation was attempted again using MDCK cells in Eagle's MEM with or without trypsin, in order to separate A (H3N2) and B viruses (3,4). The subtype of proliferating virus in each medium was determined by the rapid diagnosis test (ESPLINE influenza A & B-N). In the presence of trypsin, only influenza A virus was isolated, whereas in the absence of trypsin both influenza A and B viruses were isolated. They were passaged twice at a restricted dilution, and named A/Saitama/26/2005 and B/Saitama/37/2005, respectively. They reacted to antisera to A/Wyoming/03/2003(H3N2) and B/Johannesburg/5/99, respectively, in an HI test (Table 1).

In recent years, influenza epidemics were caused by both influenza A (H1N1 or/and H3N2) and B viruses, but the predominant virus differed year by year. There have been reports of simultaneous infection by different subtypes of influenza viruses (4-6). In addition, we sometimes encountered simultaneous infections by influenza virus and adenovirus or enterovirus. It is possible that differences in the sensitivity of cell lines to different influenza type or subtype viruses lead to the isolation of only predominant type/subtype viruses. Although CaCo-2 cells were previously reported to be sensitive to influenza viruses (7,8), they supported the propagation of influenza A virus only in the present study. In the analysis of a dual infection in Chiba Prefecture, Japan, only influenza A (H3N2) virus was isolated in MDCK cells, while only influenza B virus was isolated in HeLa cells, which were also reported to be sensitive to influenza viruses (9) (personal communication, T. Ogawa, Chiba Prefectural Institute of

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Table 1. Results of hemagglutination inhibition (HI) assay for the isolated viruses

Standard antigens or isolates	HI titer against ferret antisera ¹⁾				
	A/New Caledonia/20/99 (H1N1)	A/Moscow/13/98 (H1N1)	A/Wyoming/03/2003 (H3N2)	B/Johannesburg/5/99	B/Brisbane/32/2002
A/New Caledonia/20/99	160	– ²⁾	–	–	–
A/Moscow/13/98	–	1,280	–	–	–
A/Wyoming/03/2003	–	–	640	–	–
B/Johannesburg/5/99	–	–	–	1,280	–
B/Brisbane/32/2002	–	–	–	–	1,280
The crude isolate	<10	<10	<10	<10	<10
A/Saitama/26/2005	<10	<10	160	<10	<10
B/Saitama/37/2005	<10	<10	<10	640	<10

Standard antigens and ferret antisera were provided by National Influenza Surveillance Center, National Institute of Infectious Diseases.

¹⁾: 0.5% turkey red blood cell was used.

²⁾: Not done.

Public Health). The factors that are responsible for dual infection with two types of influenza viruses have not been determined; they may include the immune system of the host, virus properties, or other factors. Interestingly, in the present study, the level of influenza A virus was 10,000 times higher than that of influenza B virus in the clinical specimen. However, similar levels of influenza A (H3N2) and B viruses were detected in the clinical specimen from a case of dual infection by the direct plaque formation culture method in Yokohama City, Japan (personal communication, C. Kawakami, Yokohama City Institute of Health). Further study is needed to elucidate the mechanism of dual influenza virus infection.

In conclusion, a case of dual infection by influenza A (H3N2) and B viruses was described. The presence of dual infection should always be kept in mind in the virological analysis of influenza cases.

This article appeared in the Infectious Agents Surveillance Report (IASR), vol. 26, p. 152-153, 2005 in Japanese.

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