Genetic evidence for containment of viruses in the first outbreak of influenza A pandemic (H1N1) 2009 in Kobe, Japan

Ai Ninomiya-Mori, Souichi Nukuzuma, Tomoko Suga, Kyoko Akiyoshi, Masafumi Nukina, Toshitsugu Tanaka

Kobe Institute of Health, Hyogo, Japan.

Correspondence: Ai Ninomiya-Mori, Kobe Institute of Health, Minatojima-Nakamachi 4-6, Chuo-ku, Kobe, Hyogo 650-0046, Japan.
E-mail: ai_mori@office.city.kobe.lg.jp

Accepted 4 October 2010. Published Online 4 November 2010.

Background On 16 May 2009, a high school student in Kobe with no history of overseas travel was reported as the first case of influenza A pandemic (H1N1) 2009 virus infection in Japan. Subsequently, it was revealed that the infection had spread to some cities in the Kansai region and most patients were high school students. The number of patients decreased rapidly within a week; however, it began to increase in the middle of July.

Methods We phylogenetically analyzed viral characteristics using 27 viruses isolated from patients living in Kobe.

Results and conclusions We demonstrated that viruses isolated in the early phase of the outbreak were distinguishable from those after the reappearance of patients. These findings provide genetic evidence for the effectiveness of public health containment measures in the Kansai region in preventing the progression of the outbreak.

Keywords Genetic analysis, pandemic (H1N1) 2009, public health containment measures.

Introduction

On 16 May 2009, in Kobe, capital city of Hyogo prefecture, a high school student with no history of overseas travel was identified as being infected with influenza A pandemic (H1N1) 2009 virus as the first case of domestic transmission in Japan. Subsequent investigation revealed that the epidemic had spread among students of several high schools in Hyogo and the neighboring prefecture, Osaka. As of May 17, there were more than 40 patients in Kobe, the large majority of which were high school students. In Hyogo and Osaka, from May 16, all public schools were closed for one week, and most private schools, universities, colleges, and day-care facilities were also closed according to requests from the local governments. In Kobe, schools that had patients continued to be closed for an extra one or two weeks. In addition, the local government of Kobe decided to postpone the Kobe Festival, which is an annual festival where hundreds of thousands of people gather, and had been arranged for May 16 and 17. Within a week, the number of cases dropped sharply in both prefectures; however, some cases were still confirmed sporadically in June and the beginning of July, when closure was implemented at the class level. The number of patients gradually increased from the middle of July through August, although it was summer vacation at school, and rose steadily in September. In Kobe, weekly reported cases per sentinel clinic peaked in week 44, October.

Kobe Institute of Health (KIH), which is one of the local public health institutes in Japan, is responsible for laboratory confirmation of pandemic (H1N1) 2009 virus infection in patients consulting in Kobe. In compliance with the Infectious Diseases Control Law, the Ministry of Health, Labour and Welfare (MHLW) required to test all suspected cases and to report all diagnosed patients until July 23. By the end of August, 2183 cases were tested by KIH, of which 690 were positive. In this study, we analyzed full-length sequences of the coding region of hemagglutinin (HA) and partial sequences of neuraminidase (NA), PB2, PB1, and NS of 27 viruses; we demonstrated that isolates in the early phase of the outbreak were distinguishable from those after the widespread of infection. These results suggest that the public health containment measures, both pharmaceutical and non-pharmaceutical, implemented in this area might have been effective in preventing further local spread of the infection in the first stage of the epidemic.
Materials and methods

Laboratory confirmation of suspected cases

Nasal or throat swabs were collected from individuals, who included not only patients but also their contact cases without symptoms, and transferred to KIH. We performed tests using a protocol of real-time RT-PCR for pandemic (H1N1) 2009 that was provided for local public health institutes and quarantine stations by the National Institute of Infectious Diseases (NIID, Tokyo, Japan) on May 1. Specimens were then stored at ~80°C until they were used for virus isolation.

Viruses

Virus isolation was attempted on a total of 27 real-time RT-PCR positive samples. Viruses were isolated from nasal or throat swabs using MDCK cells in a 24-well microplate. Viruses analyzed in this study are listed in Table 1.

Sequence and phylogenetic analysis

We extracted viral RNA using a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and amplified it using a One Step RNA PCR kit (AMV) (Takara Bio Inc., Otsu, Japan). PCR products were purified with a SUPREC-PCR (Takara Bio Inc., Otsu, Japan), and nucleotide sequencing reactions were performed with a BigDye v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). We performed DNA sequencing using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). Sequences of primers for HA and NA were kindly provided by NIID. Those for PB2, PB1, and NS are available upon request. Analyzed regions of each gene segment are listed

Table 1. Pandemic (H1N1) 2009 viruses isolated from patients in Kobe

| Virus      | Patient       | Week of onset | Accession no* (HA, NA, PB2, PB1, NS) |
|------------|---------------|---------------|--------------------------------------|
| A/Kobe/9015/2009 | Student | 20 | A8568123, A8568124, A8568125, A8568126, A8568127 |
| A/Kobe/9019/2009 | Student | 20 | A8568128, A8568129, A8568130, A8568131, A8568132 |
| A/Kobe/9154/2009 | Student | 20 | A8568133, A8568134, A8568135, A8568136, A8568137 |
| A/Kobe/9185/2009 | Student | 20 | A8568138, A8568139, A8568140, A8568141, A8568142 |
| A/Kobe/9336/2009 | Student | 19 | A8568143, A8568144, A8568145, A8568146, A8568147 |
| A/Kobe/9356/2009 | Student | 21 | A8568148, A8568149, A8568150, A8568151, A8568152 |
| A/Kobe/9124A/2009 | Student | 22 | A8568153, A8568154, A8568155, A8568156, A8568157 |
| A/Kobe/9150A/2009 | Student | 26 | A8568158, A8568159, A8568160, A8568161, A8568162 |
| A/Kobe/9152A/2009 | Overseas traveler | 26 | A8568163, A8568164, A8568165, A8568166, A8568167 |
| A/Kobe/9153A/2009 | Student | 27 | A8568168, A8568169, A8568170, A8568171, A8568172 |
| A/Kobe/9157A/2009 | Student | 27 | A8568173, A8568174, A8568175, A8568176, A8568177 |
| A/Kobe/9155A/2009 | Student | 27 | A8568178, A8568179, A8568180, A8568181, A8568182 |
| A/Kobe/9156A/2009 | Overseas traveler | 28 | A8568183, A8568184, A8568185, A8568186, A8568187 |
| A/Kobe/9157A/2009 | Student | 28 | A8568188, A8568189, A8568190, A8568191, A8568192 |
| A/Kobe/9158A/2009 | Overseas traveler | 28 | A8568193, A8568194, A8568195, A8568196, A8568197 |
| A/Kobe/9156A/2009 | Student | 29 | A8568198, A8568199, A8568200, A8568201, A8568202 |
| A/Kobe/9161A/2009 | Overseas traveler | 29 | A8568203, A8568204, A8568205, A8568206, A8568207 |
| A/Kobe/9160A/2009 | Overseas traveler | 31 | A8568208, A8568209, A8568210, A8568211, A8568212 |
| A/Kobe/9170A/2009 | Student | 31 | A8568213, A8568214, A8568215, A8568216, A8568217 |
| A/Kobe/9173A/2009 | Student | 31 | A8568218, A8568219, A8568220, A8568221, A8568222 |
| A/Kobe/9178A/2009 | Student | 31 | A8568223, A8568224, A8568225, A8568226, A8568227 |
| A/Kobe/9179A/2009 | Student | 32 | A8568228, A8568229, A8568230, A8568231, A8568232 |
| A/Kobe/9182A/2009 | Student | 32 | A8568233, A8568234, A8568235, A8568236, A8568237 |
| A/Kobe/9183A/2009 | Student | 32 | A8568238, A8568239, A8568240, A8568241, A8568242 |
| A/Kobe/9185A/2009 | Student | 32 | A8568243, A8568244, A8568245, A8568246, A8568247 |
| A/Kobe/9187A/2009 | Student | 32 | A8568248, A8568249, A8568250, A8568251, A8568252 |
| A/Kobe/9189A/2009 | Student | 32 | A8568253, A8568254, A8568255, A8568256, A8568257 |
| Virus below was isolated and sequenced by NIID A/Kobe/1/2009 | Student | 20 | GQ219577, GQ220773, GQ222054, GQ222045, GQ223429 |

*Five consecutive accession numbers correspond to HA, NA, PB2, PB1 and NS of each virus, respectively.†Isolated from the first specimen collected in Kobe.‡The first patient in the school was a contact case of an overseas traveler.
Table 2. Analyzed regions in this study

| Segment | Analyzed regions of nucleotides* | Length of nucleotides |
|---------|----------------------------------|-----------------------|
| HA      | 1–1701                           | 1701                  |
| NA      | 727–1058                         | 332                   |
| PB2     | 1613–2069                        | 457                   |
| PB1     | 95–483                           | 389                   |
| NS      | 259–788                          | 530                   |

HA, hemagglutinin; NA, neuraminidase.
*Count from start codon.

in Table 2. Obtained data were compared with available sequence data from the National Center for Biotechnology Information (NCBI) Influenza Virus Resource database.\(^7\) Nucleotide sequences were aligned with ClustalW, and neighbor-joining trees were constructed with NJplot.\(^8\) The accession numbers are listed in Table 1.

Results

Laboratory confirmation of suspected cases

Almost all individuals with influenza-like symptoms who consulted their doctors in Kobe by July 23 were laboratory-confirmed in KIH. We show the numbers of tested and positive cases by the date of confirmation from May 15 through August 31, because limited information about onset date was available (Figure 1). In the first week of the epidemic, the positive rate was low although many cases were tested. The number of positive cases decreased rapidly and, from June 11 through June 22, no positive cases were confirmed with the exception of two imported cases. The first cluster after this period appeared in one school, which started with a contact case of an out-of-town patient who had returned from abroad. Surveillance that involved testing all suspected cases was replaced by routine sentinel surveillance, pathogen surveillance, school absentee surveillance, cluster surveillance, and hospital admission surveillance for severe cases after a notice from MHLW on July 24.\(^9\) This notice indicated the need to perform laboratory confirmation of the first case of a cluster and severe cases that needed hospitalization by real-time RT-PCR. However, in Kobe, an original surveillance system containing more than 300 sentinel clinics had been established to detect patients by laboratory confirmation; therefore, samples of consulting individuals were actually tested for a while as before the notice from MHLW, and we tested 2183 samples by the end of August, of which 690 were positive.

Genetic analysis of viruses isolated from patients

To analyze the phylogenetic relationship among 27 isolates, we compared nucleotide sequences of the full-length coding region of HA and partial sequences of NA, PB2, PB1, and NS (Table 2). Schools with patients were found in every ward in Kobe; therefore, we chose two or three viruses isolated from patients belonging to one school from each ward and also those from overseas travelers. Then, we analyzed the 27 isolates together with sequences available from public databases that included data of A/Kobe/1/2009 isolated by NIID from a patient in Kobe. Phylogenetic analyses of PB2 (Figure 2B) and NS (Figure 2C) showed that all viruses isolated from patients confirmed before June 11 in Kobe (designated as earlier viruses) clustered in one group.

Figure 1. Number of tested (bars) and positive (line) cases from May 15 through August 31 in Kobe. From May 16 through 24 (A), all public schools and most private schools, universities, colleges, and day-care facilities were closed in Hyogo and Osaka. From May 25 through June 2 (B), schools that had patients were closed. From June 3 through 8 (C), all classes in one grade in one school that had patients were closed. *Only dates when laboratory confirmation was performed are shown.
Figure 2. Phylogenetic analyses of hemagglutinin (HA) (A), PB2 (B) and NS (C) genes of influenza A pandemic (H1N1) 2009 viruses. Analyses were based on nucleotide sequences of each gene listed in Table 2. Numbers after strain names (e.g. 20 w) show the week of onset (viruses in Kobe) or sample collection (viruses from databases). Red indicates viruses isolated from patients confirmed before June 11 in Kobe. Green indicates those confirmed after June 22. Blue indicates those of imported cases. All trees were rooted to A/swine/Indiana/99(H1N2). Scale bars indicate nucleotide substitutions per site.
We also analyzed sequences of PB2 and NS of the virus isolated from a patient tested on June 10 and confirmed that it was from the same group (data not shown). This group only consisted of isolates in the Kansai region at the beginning of the epidemic (Amagasaki, Himeji, Sakai and Shiga are the names of cities or prefectures in the Kansai region), and no viruses containing the same sequences as earlier viruses were found in databases except for these. Interestingly, one strain isolated in Shiga in May, A/Shiga/1/2009 (marked with two asterisks in figures), was located in a different cluster from that of earlier viruses. Meanwhile, viruses isolated from patients confirmed after June 22 (designated as later viruses) were separated into other clusters (shown in blue or green).

Unlike for PB2 and NS, earlier viruses did not form an independent cluster in the phylogenetic tree of the HA gene (Figure 2A). However, two nucleotide changes were detected at positions 1408 and 658, and earlier and later viruses were distinguished by these changes. All earlier viruses contained C1408 and T658, while all later viruses contained T1408 and 16 of them contained A658. In contrast, there was no obvious difference between earlier and later viruses in the NA and PB1 genes (Figure 3).

We analyzed nucleotides including regions associating with resistance to oseltamivir and pathogenicity of influenza A virus as follows: amino acids at positions 275 of NA and 627 of PB2 and PB1-F2, and C-terminal residues of NS1. All 27 viruses contained H275 of NA and E627 of PB2 did not express PB1-F2 and lacked C-terminal residues of NS1. These features were the same as other pandemic (H1N1) 2009 viruses in public databases, which meant that there was no noticeable mutation responsible for the change of characteristics in these 27 viruses.

**Discussion**

Little is known about the initial outbreak of influenza A pandemic (H1N1) 2009 in Kobe, Japan. In this study, we analyzed this epidemic by genetic analysis. Phylogenetic analyses of PB2, NS, and HA revealed that the origin of earlier viruses was different from that of the later viruses. This indicates that the reappearance and increase in the number of patients after June 22 were not caused by viruses directly derived from those that circulated among high school students in May but by a variety of viruses that entered from outside the city. Furthermore, although pandemic (H1N1) 2009 began to spread throughout Japan from the middle of June, and all prefectures were affected by July 16, we could not find viruses with the same PB2, NS, and HA as earlier viruses in public databases, with the exception of those isolated from patients in the Kansai region in May. These results provide strong support that
Figure 3. Phylogenetic analyses of neuraminidase (NA) (A) and PB1 (B) genes of influenza A pandemic (H1N1) 2009 viruses. Analyses were based on nucleotide sequences of each gene listed in Table 2. Numbers after strain names (e.g. 20 w) show the week of sample collection. Red indicates viruses isolated from patients in Kobe confirmed before June 11. Green indicates those confirmed after June 22. Blue indicates those of imported cases. Trees of NA and PB1 were rooted to A/swine/Spain/53207/2004(H1N1) and A/swine/Indiana/9035/99(H1N2), respectively. Scale bars indicate nucleotide substitutions per site.
earlier viruses in the Kansai region were contained there and could not be direct predecessors of viruses that caused epidemics in other prefectures in Japan. One exception, an isolate in Shiga, was thought to have entered Japan via a different route around the same time as the first outbreak. However, viruses considered as descendants of it were not found in databases, and this suggests that it might also have been contained, as were other earlier viruses, by simultaneous implementation of public health measures in the Kansai region. Epidemiological studies show that the first outbreak in the Kansai region was contained, and our results are consistent with these. We confirmed two imported cases from June 11 to 23, and the first cluster after this period was derived from an imported case. In addition, phylogenetic trees of later viruses indicate that some viruses with different origins were circulating in Kobe simultaneously. These suggest that, as pandemic (H1N1) 2009 continued to spread worldwide, various viruses probably entered Japan, in both unaffected and affected areas, by international and domestic human migration. Consequently, the number of sporadic cases increased all over Japan, and they seemed to be the cause of the epidemic in each area.

Infection control of influenza among school-aged children has been considered one of the most effective strategies to mitigate the social burdens caused by influenza. Thus, school closures are included in the guidelines for pandemic influenza as a strict containment measure in many countries, including Japan and United States. Although data on the efficacy of school closures are still limited, results from epidemiological studies based on simulation modeling have been accumulated recently. In the first outbreak in the Kansai region, most patients were high school students, and some epidemiological studies suggest that school closures contributed to reduce the number of cases. In Kobe, we confirmed a few cases of students congregating in other places even though they were advised to stay at home during school closures. This might indicate that it was necessary and successful to implement measures to keep students away from one another to prevent transmission among them and mitigation of the first outbreak in the Kansai region. On the other hand, during school closures, some non-pharmaceutical measures including canceling events and encouraging personal and community hygiene were also implemented in parallel. Moreover, in Japan, people can easily access medical care; therefore, families and health-care workers received antiviral prophylaxis at the beginning of the epidemic, while the effects of this prophylaxis in Kobe have not been proved yet. After the reappearance of patients, large-scale school closures were no longer practical, so that closure was implemented at the class level. In addition, as pandemic spread globally, it seemed that people paid less attention to hygiene than the beginning of outbreak. As a result, it is difficult to isolate and characterize the effectiveness of each measure separately; however, the disappearance of viruses is evidence that the combination of these measures was effective in slowing the spread of pandemic (H1N1) 2009 in the early stage of the epidemic. In the next pandemic, if an outbreak occurs under similar conditions, in a limited area or population, the measures performed during this outbreak in Japan may be valid to contain viruses and mitigate the impact. At the same time, implementation of social distancing measures such as school closures and canceling events is still controversial because of their economic and social burdens; therefore, we should consider how to lighten them by flexible planning according to the characteristics of the pandemic virus.

As of May 2010, pandemic influenza activity remains low in many countries and this is an opportunity for us to improve programs for influenza pandemic on the basis of our experiences obtained in 2009 so that we can take action more appropriately depending on the situation. In addition, it is essential to maintain a continuous and steady surveillance system for influenza even in interpandemic periods, because this enabled us to detect the first case in Kobe, to respond rapidly to an outbreak and detect antigenic and pathogenetic changes in viruses. This will be very useful in a future pandemic as well as seasonal influenza.

Acknowledgements

We thank all the staff members of Kobe Institute of Health and the Public Health Center of Kobe. We also thank Tsutomu Kageyama for providing information about primers and other valuable advice. These studies were supported by Kobe city.

References

1 Shimada T, Gu Y, Kamiya H et al. Epidemiology of influenza A(H1N1) virus infection in Japan, May-June 2009. Euro Surveill 2009; 14: pii:19244.
2 Nishiura H, Castillo-Chavez C, Safan M, Chowell G. Transmission potential of the new influenza A(H1N1) virus and its age-specificity in Japan. Euro Surveill 2009; 14: pii:19227.
3 Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo. Novel Influenza A(H1N1) Map (Cases in Japan). Available at: http://idsc.nih.go.jp/disease/swine_influenza_e/2009epi_e090722epi_e.html (Accessed 28 May 2010).
4 Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo. Influenza cases reported per sentinel weekly. Available at: http://idsc.nih.go.jp/idwr/kanja/weeklygraph/01flu_e.html (Accessed 28 May 2010).
5 Public Health Center of Kobe. Kobe infectious diseases weekly report. 2010 Jan 6 [in Japanese]. Available at http://www.city.kobe.lg.jp/life/health/infection/trend/img/sho9_53.pdf (Accessed 28 May 2010).
World Health Organization. WHO information for laboratory diagnosis of pandemic (H1N1) 2009 virus in humans – revised. 2009 Nov 23. Available at http://www.who.int/csr/resources/publications/swineflu/diagnostic_recommendations/en/index.html (Accessed 28 May 2010).

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 Perriere G, Gouy M. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie 1996; 78:364–369.

9 National institute of infectious diseases and tuberculosis and infectious disease control division, Ministry of Health, Labour and Welfare. Pandemic (H1N1) 2009 in Japan, May-September 2009. Available at http://idsc.nih.go.jp/iasr/30/356/tpc356.html (Accessed 28 May 2010).

10 Yasuda H, Suzuki K. Measures against transmission of pandemic H1N1 influenza in Japan in 2009: simulation model. Euro Surveill 2009; 14: pii:19385.

11 Neuzil KM, Hohlbien C, Zhu Y. Illness among schoolchildren during influenza season: effect on school absenteeism, parental absenteeism from work, and secondary illness in families. Arch Pediatr Adolesc Med 2002; 156:986–991.

12 Ministry of Health, Labour and Welfare. Guideline for Rapid Response Strategies during the Early Stages of Pandemic Influenza. Available at http://www.mhlw.go.jp/bunya/kenkoukenkatsuryou/fukkou.pdf (Accessed 28 May 2010).

13 Centers for Disease Control and Prevention. Interim pre-pandemic planning guidance: community strategy for pandemic influenza mitigation in the United States – early, targeted, layered use of non-pharmaceutical interventions. 2007 Feb. Available at http://pandemicflu.gov/professional/community/commitigation.html (Accessed 28 May 2010).

14 Bell DM. Non-pharmaceutical interventions for pandemic influenza, national and community measures. Emerg Infect Dis 2006; 12: 88–94.

15 Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. Strategies for mitigating an influenza pandemic. Nature 2006; 442:448–452.

16 Halloran ME, Ferguson NM, Eubank S et al. Modeling targeted layered containment of an influenza pandemic in the United States. Proc Natl Acad Sci USA 2008; 105:4639–4644.

17 Germann TC, Kadau K, Longini IM Jr, Macken CA. Mitigation strategies for pandemic influenza in the United States. Proc Natl Acad Sci USA 2006; 103:5935–5940.

18 Carrat F, Luong J, Lao H, Salle AV, Lajaunie C, Wackernagel H. A ‘small-world-like’ model for comparing interventions aimed at preventing and controlling influenza pandemics. BMC Med 2006; 4:26.

19 Glass RJ, Glass LM, Beyeler WE, Min HJ. Targeted social distancing design for pandemic influenza. Emerg Infect Dis 2006; 12:1671–1681.

20 Odaira F, Takahashi H, Toyokawa T et al. Assessment of secondary attack rate and effectiveness of antiviral prophylaxis among household contacts in an influenza A(H1N1)v outbreak in Kobe, Japan, May-June 2009. Euro Surveill 2009; 14: pii:19320.

21 World Health Organization. Pandemic (H1N1) 2009 – update 102. Available at http://www.who.int/csr/dor/2010_05_28/en/index.html (Accessed 28 May 2010).