



Growth capability of epidemic influenza viruses in Japan since the 2009 H1N1 pandemic

Akeno Tsuneki-Tokunaga^{1,2} · Kyosuke Kanai^{1,2} · Asao Itagaki^{1,2} · Hideaki Tsuchie^{2,3} · Takayoshi Okada^{2,4} · Masaaki Kasagi^{2,5} · Kiyoshi Tanaka^{2,6} · Miho Aoki¹ · Alfredo Jr. A. Hinay¹ · Seiji Kageyama^{1,2}

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Abstract

The correlation of viral growth capability ($n = 156$) with the viral load in nasopharyngeal swabs ($n = 76$) was assessed. Epidemic influenza A/H1N1, A/H3N2, and B viruses showed a wide range of growth capability (10^4 – 10^{11} copies/mL) in Madin-Darby canine kidney cells. The growth was correlated with the nasopharyngeal viral load ($r = 0.53$). Six selected strains showed growth-dependent cell death ($r = 0.96$) in a growth kinetics assay. Epidemic influenza viruses exhibit a wide range of growth capability. Growth capability should be considered one of the key factors in disease prognosis.

Influenza A virus subtypes H1N1 (A/H1N1) and H3N2 (A/H3N2) and influenza B viruses circulate in the human population every winter in temperate countries. They account for 3–5 million cases of severe illness and about 290,000–650,000 respiratory deaths annually ([http://www.who.int/en/news-room/fact-sheets/detail/influenza-\(seasonal\)](http://www.who.int/en/news-room/fact-sheets/detail/influenza-(seasonal))). In addition to the social burden, disease severity is a serious concern, measured by hospitalization and fatality rates [22].

The viral load plays a key role in assessing the prognosis of influenza and the probability of viral transmission. Viral loads typically peak with maximal symptoms approximately 48 hours after inoculation, after which pathological damage, such as lung injury, can occur as a result of immune reactions [6, 18]. Viral growth capability may be one of the

factors contributing to the viral load. However, few studies have been conducted on the viral growth capability of epidemic strains, even though several host factors are known to be required for replication of influenza viruses [7].

Our previous study showed that emerging pandemic viruses consisted of only highly replicative strains, but more-slowly replicating strains were observed soon in the following 2010–2011 seasons [21]. This demonstrated that strain-dependent differences play a role in replication capability. However, further observation is needed to evaluate the clinical significance of these strain-dependent differences. Therefore, we performed a study examining the growth capability of influenza viruses, including another subtype A/H3N2 isolate and type B isolates from six consecutive seasons, and the correlation between growth kinetics and the nasopharyngeal viral load was investigated.

Nasal swabs and nasopharyngeal aspirates were collected from influenza A and B virus antigen-positive patients and those with suspected influenza who were admitted to a hospital and three clinics for medical consultation in Tottori prefecture of Japan during the six consecutive winter seasons of 2009–2015 ($n = 2,746$). The procedures for influenza virus isolation were based on a manual (<https://apps.who.int/iris/handle/10665/68026>) with slight modifications. One of the frozen aliquots was used as an inoculum to minimize bias in experiments.

Total RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN K.K., Tokyo, Japan). The eight RNA segments of the influenza A and B viruses were reverse transcribed and simultaneously amplified using a SuperScript™

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✉ Seiji Kageyama
skageyama@tottori-u.ac.jp

- ¹ Division of Virology, Department of Microbiology and Immunology, Tottori University Faculty of Medicine, Yonago, Japan
- ² Tottori Infectious Diseases Forum, Tottori prefecture, Japan
- ³ Tsuchie Internal Medicine and Pediatric Clinic, Sakaiminato, Japan
- ⁴ Department of Pediatrics, Tottori Prefectural Kousei Hospital, Kurayoshi, Japan
- ⁵ Kasagi Children's Clinic for Health Service, Yonago, Japan
- ⁶ Tanaka Pediatric Clinic, Tottori, Japan

III One Step RT-Polymerase Chain Reaction (PCR) System with Platinum[®] Taq DNA Polymerase (Thermo Fisher Scientific K.K., Tokyo, Japan) and the primer pairs MBTuni-12/MBTuni-13 and MBTuni-12b/MBTuni-13b [11, 23]. Two primer pairs, PH1F/PH1R for A/H1N1 and SH3F/SH3R for A/H3N2, were used in second-round PCR for typing and subtyping of influenza A viruses [13]. Similarly, the influenza B virus type-specific primer pair BHAB-5/BHACII [15] was used for second-round PCR.

To synthesize control RNA for generating a standard curve in real-time RT-PCR, a portion of the matrix gene of the influenza A and B viruses was targeted as described previously [1, 9]. A SuperScript[®] III Platinum[®] SYBR[®] Green One-Step qRT-PCR Kit (Thermo Fisher Scientific) was used for the one-step real-time RT-PCR reaction in accordance with the manufacturer's instructions with slight modifications. Briefly, either serially diluted control RNA (10^8 to 10^2 copies in 5 μ L) or sample RNA (5 μ L) was mixed with 20 μ L of buffer solution containing the primers FLUA-MAT-F/FLUA-MAT-R [1] or BMLF/BMLR [9] (250 nM each), a reverse transcriptase/Taq polymerase mixture, and ROX reference dye. The reaction was initiated from the first reverse transcription step at 50°C for 30 minutes and was followed by 40 cycles of denaturing (95°C for 15 seconds) and annealing/elongation (60°C for 30 seconds) steps.

The growth capability of seasonal influenza viruses was assessed by measuring virus production in cultures of MDCK cells and A549 human lung epithelial cells inoculated with the seasonal isolates at 3,300 and 10^6 copies/mL and culturing for 72 hours.

The correlation between growth capability and another factor was analyzed using Pearson's correlation coefficient. A *P*-value less than 0.05 was considered significant.

In the A/H1N1 pandemic season (2009–2010), all 21 A/H1N1 isolates exhibited high growth capability and reached more than 10^8 copies/mL in the cultures. Influenza A/H3N2 and B viruses were not detected in this season at all. In the next season (2010–2011), A/H1N1 retained its high replication capability ($n = 17$, more than 10^8 copies/mL), and strains with lower replication capability (10^6 – 10^8 copies/mL) were also recognized. In the following four consecutive epidemic seasons of 2011–2012, 2012–2013, 2013–2014, and 2014–2015 in Japan, all detected strains of A/H1N1, A/H3N2, and type B showed a wide range of growth in MDCK cell cultures *in vitro* (10^4 – 10^{11} , Fig. 1).

One reference strain (A/Puerto Rico/8/1934(H1N1), hereinafter referred to as PR8) and five epidemic strains (A/Tottori/ST215/2009(H1N1), ST215; A/Tottori/ST777/2011(H3N2), ST777; A/Tottori/ST1349/2014(H3N2), ST1349; A/Tottori/ST1705/2014(H3N2), ST1705; A/Tottori/ST1890/2015(H3N2), ST1890) were selected for growth kinetics analysis in MDCK cell culture. A difference in viral

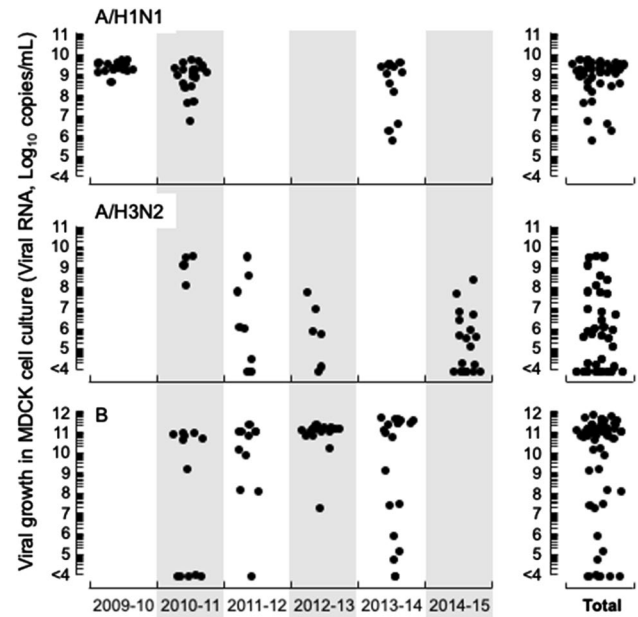


Fig. 1 Growth capability of epidemic influenza viruses from Japan, 2009–2015. Viral RNA copies produced in MDCK cell cultures were measured to assess the growth capability of epidemic influenza A/H1N1 (2009–2010, $n = 21$; 2010–2011, $n = 20$; 2013–2014, $n = 11$; total, $n = 52$), A/H3N2 (2010–11, $n = 4$; 2011–2012, $n = 10$; 2012–2013, $n = 7$; 2014–2015, $n = 21$; total, $n = 42$), and B (2010–2011, $n = 12$; 2011–2012, $n = 11$; 2012–2013, $n = 17$; 2013–2014, $n = 22$; total, $n = 62$) viruses circulating in Tottori prefecture, Japan, during the six consecutive seasons of 2009–2015

growth levels was clearly recognizable at 24 hours post-inoculation and became more obvious at 48 and 72 hours. Although highly replicative strains (PR8 and ST215) produced progeny viruses at levels of 10^8 and 10^{10} copies/mL in MDCK cell cultures, no virus production was observed after inoculation with the strain ST777 at the 24- and 48-hour time points. Cell viability decreased to different levels in a strain-specific manner. The largest decrease was observed with the strain PR8, which had the highest growth capability, and the least was observed with ST777, which had the lowest growth capability. The cell viability was related inversely to the viral growth in MDCK cell culture ($r = 0.96$, Fig. 2). In A549 cell cultures, strain-specific differences in progeny virus production were less obvious than in MDCK cell cultures. However, there was still 10^5 and 10^8 copies/mL of difference between the levels of progeny viruses of PR8 and ST777 (Fig. 2).

There was a correlation between viral growth capability and nasopharyngeal viral load, although the Pearson's correlation coefficient value was not high ($r = 0.53$, Fig. 3).

The viral load has been reported to be an important surrogate marker for assessing the prognosis of influenza [2, 4, 5, 10, 14, 19, 20]. Patients with a high viral load more often show abnormal findings in chest X-rays. However,

Fig. 2 Viral growth and cell viability. The growth of five selected strains (● ST1349, △ ST215, ▲ ST1890, □ ST777, ■ ST1705) and a laboratory strain (○PR8) was examined in MDCK and A549 cells after inoculation with 3,000 and 10^6 copies/mL. Viable cells were quantitated using a WST-8 assay with a linear standard curve. Cell viability decreased to different levels in a strain-specific manner (MDCK cell culture, $r = 0.96$, $P < 0.05$)

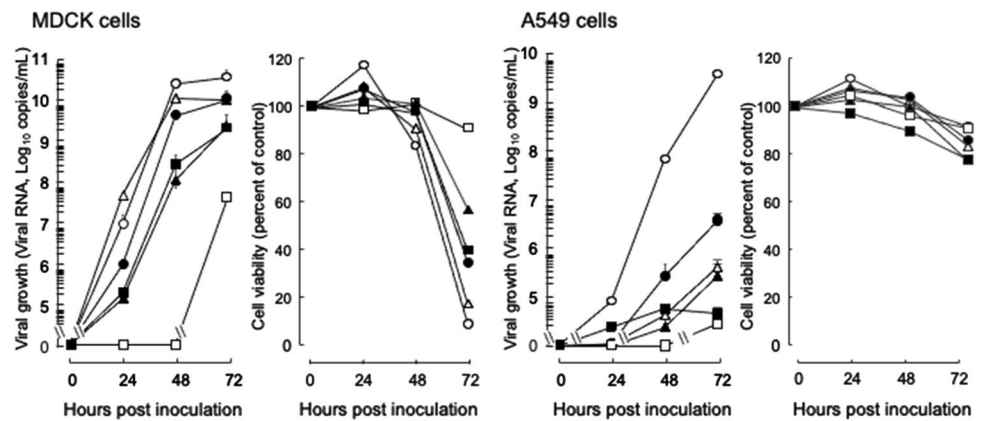
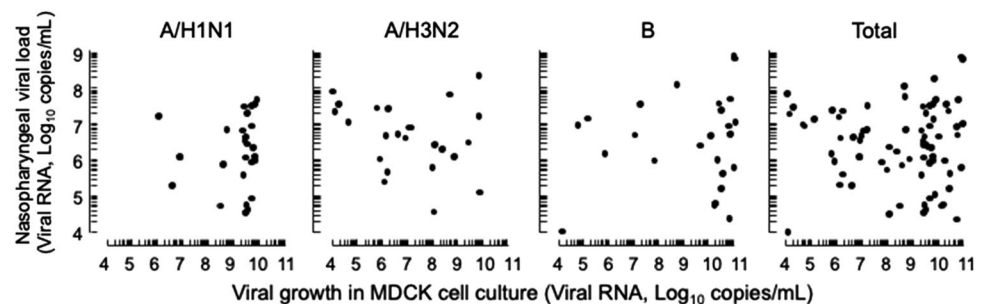


Fig. 3 Correlation between viral growth in MDCK cell culture and nasopharyngeal viral load. A/H1N1, $n = 27$, $r = 0.40$, $P < 0.05$; A/H3N2, $n = 24$, $r = 0.45$, $P < 0.05$; B, $n = 25$, $r = 0.51$, $P < 0.05$; total, $n = 76$, $r = 0.53$, $P < 0.05$



these findings are not always associated with a significantly worse prognosis [8, 19]. Although relationships were observed between viral growth and the cell death rate, virus-induced cell death was obvious only in MDCK cell culture, with a significant amount of virus production, but it was rather mild in A549 cell culture, with relatively modest virus production in the present study. The growth levels of 10^9 – 10^{10} copies/mL observed in MDCK cell culture may not be common in immunocompetent patients and occur only in cases of immunodeficiency, such as AIDS, with low CD4 cell counts [16]. Indeed, the maximum viral loads in viral transport medium have been reported to be 10^4 – 10^6 copies/mL in cases with immunocompetent hosts [3, 4, 10, 12].

Although a strong relationship of viral load levels to the severity of influenza has been recognized [12], wide variations in viral load are frequently observed [17]. The variations in viral load appear to be due to the difficulties associated with sample collection procedures, particularly with obtaining precise sample volumes from nasal and pharyngeal mucous membranes and the absence of a suitable internal control for estimating the original sample volume. Because the values for growth capability and viral load of epidemic influenza viruses correlated with each other in the present study, the growth capability appears to be one of the major factors contributing to the prognosis, apart from the viral load. However, the current methodology for measuring growth capability takes time and cannot give an immediate

result after specimen collection. Therefore, additional surrogate markers are required for high-throughput detection of strains with high growth capability.

The present study demonstrates that epidemic influenza viruses can have a wide range of growth capacity. The combination of nasopharyngeal viral load, growth capability, and immunological factors may be useful for establishing a prognosis for influenza illness.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval The present study was conducted with the approval and under the control of the Institutional Review Board of the Faculty of Medicine, Tottori University, Japan (no. 1981). All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Sample collection was conducted after obtaining informed consent from patients in collaborating medical facilities consisting of a hospital and three clinics. Samples were then shipped to the laboratory without patient identification.

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