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Systematic evaluation of suspension MDCK cells, adherent MDCK cells, and LLC-MK2 cells for preparing influenza vaccine seed virus

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ABSTRACT

Suspension Madin–Darby canine kidney (MDCK) cells (MDCK-N), adherent MDCK cells (MDCK-C), and adherent rhesus monkey kidney LLC-MK2 cells (LLC-MK2D) were systematically evaluated for the preparation of influenza vaccine seed viruses for humans on the basis of primary virus isolation efficiency, growth ability, genetic stability of the hemagglutinin (HA) and neuraminidase (NA) genes, and antigenic properties in hemagglutination inhibition (HI) test of each virus isolate upon further passages. All the subtypes/lineages of influenza viruses (A(H1N1), A(H1N1)pdm09, A(H3N2), B-Victoria, and B-Yamagata) were successfully isolated from clinical specimens by using MDCK-N and MDCK-C, whereas LLC-MK2D did not support virus replication well. Serial passages of A(H1N1) viruses in MDCK-N and MDCK-C induced genetic mutations of HA that resulted in moderate antigenic changes in the HI test. All A(H1N1)pdm09 isolates from MDCK-C acquired amino acid substitutions at the site from K153 to N156 of the HA protein, which resulted in striking antigenic alteration. In contrast, only 30% of MDCK-N isolates showed amino acid changes at this site. The frequency of MDCK-N isolates with less than two-fold reduction in the HI titer was as high as 70%. A(H3N2) and B-Yamagata isolates showed high antigenic stability and no specific amino acid substitution during passages in MDCK-N and MDCK-C. B-Victoria isolates from MDCK-N and MDCK-C acquired genetic changes at HA glycosylation sites that greatly affected their antigenicity. When these cell isolates were applied to passages in hen eggs, A (H1N1), B-Victoria, and B-Yamagata viruses grew well in eggs, while none of the cell isolates of A (H3N2) viruses did. Thus, we demonstrate that MDCK-N might be useful for the preparation of influenza vaccine seed viruses.

1. Introduction

Influenza, a global public health problem, is highly contagious and causes mild to severe illness such as high fever, myalgia, headache, and pneumonia. In the United States alone, influenza virus is thought to infect 3–16% of the population, with an estimated economic burden of $47.2–$149.5 billion every year [1,2]. Annual influenza epidemics around the world result in about three to five million cases of severe illness and about 290,000 to 650,000 deaths each year [3]. Furthermore, influenza pandemics, like those in 1918, 1957, 1968, and 2009, can occur. The Spanish influenza pan-

Abbreviations: EID_{50}, 50% egg infectious dose; HA, hemagglutinin; HI, hemagglutination inhibition; LLC-MK2D, adherent LLC-MK2 cells; MDCK cells, Madin–Darby canine kidney cells; MDCK-C, adherent MDCK cells; MDCK-N, suspension MDCK cells; NA, neuraminidase.

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demic of 1918–1919 resulted in hundreds of millions of cases and led to approximately 20–50 million human deaths [4]. Human infection with the highly pathogenic A(H5N1) avian influenza virus was first detected in 1997, and the World Health Organization (WHO) has confirmed 860 human cases and 454 deaths as of January 21, 2019 [5,6]. Since March 2013, when the novel A(H7N9) influenza virus emerged in China, 1567 laboratory-confirmed human cases, including at least 615 deaths, have been reported [7,8]. Avian influenza viruses pose a threat of future influenza pandemics because the frequency of avian influenza cases in humans is increasing.

Vaccination is one of the most effective strategies to prevent influenza virus infection and its associated complications. For several decades, influenza vaccines have been principally produced in embryonated hens' eggs, but this system has two limitations: (i) it is difficult for the egg-based system to match the surge in demand for vaccines, especially during influenza pandemics and (ii) influenza viruses isolated and propagated in eggs will acquire antigenic alteration through host adaptation [9–15]. Antigenic alteration in isolates is a serious concern because it can reduce the effectiveness of the produced vaccines owing to the mismatch between circulating viruses and vaccine-induced antibodies.

Cell-based vaccines are considered to solve the problems of egg-based vaccines because manufacturers do not have to depend on the availability of eggs and can avoid antigenic changes associated with the adaptation to eggs. For the propagation of influenza viruses, some cell lines have been used, such as Madin–Darby canine kidney (MDCK) cells, Vero cells [16,17], LLC-MK2 cells, PER.C6 cells [18], EB66 cells [19], and AGE1.CR cells [20].

The MDCK cell line is an adherent cell line established from the kidney of a healthy cocker spaniel dog in 1958. This cell line has been utilized for influenza surveillance worldwide because of its high susceptibility to influenza viruses [21,22]. Hence, this conventional MDCK (MDCK-C) with adherent growth is one of the suitable candidates for the preparation of influenza vaccine seed viruses.

Recently, a suspension MDCK cell line MDCK33016PF (referred to as MDCK-N in this paper) has been established and used for the production of seasonal influenza vaccines [22–27]. Suspension cells have advantages such as simpler culture process without microcarrier beads, higher virus yield, and lower production cost compared to adherent cells; therefore, MDCK-N could be a good candidate for influenza vaccine seed preparation.

LLC-MK2, established from the kidney of a healthy rhesus monkey, is susceptible to a variety of viruses, including influenza virus [28–30]. We have confirmed that LLC-MK2D, a derivative cell line of LLC-MK2, is free of specific adventitious agents and has no tumorigenicity in nude mice. The qualified LLC-MK2D cell line was shown to be useful for the generation of seed viruses by using a reverse genetics technology [31]. If this qualified cell line is proved to have the ability to efficiently propagate influenza virus, it will be applicable in practical use soon. Hence, the evaluation of the LLC-MK2D cell line for vaccine seed preparation from clinical specimens is necessary.

Although some cell lines like MDCK have been in practical use for vaccine production, it remains unclear whether mammalian cell lines can amplify a wide variety of influenza viruses without changing their genetic and antigenic properties. Therefore, it is necessary to examine cell lines for the identification of the cell substrate most suitable for the development of vaccine seed viruses. Moreover, since many countries will continue to produce vaccines with hen eggs, it is desirable that cell-derived seed viruses could also be applied to egg-based vaccine production.

Thus, in this study, we systematically evaluated MDCK-N, MDCK-C, and LLC-MK2D cell lines for the preparation of influenza vaccine seed viruses on the basis of the observed isolation efficiency, growth properties, genetic and antigenic properties of the isolates, and characteristics of egg-passaged cell isolates.

2. Materials and methods

2.1. Cells

MDCK-N cells were cultivated in 500 mL disposable spinner flasks (Corning, Corning, NY, USA) with 100 mL of chemically defined medium (CDM) prepared by Lonza (Basel, Switzerland) at 100 rpm, 5% CO_2, and 37 °C on a shaking platform (MIR-S100C; Sanyo, Osaka, Japan). Cell passages were performed at intervals of 3–4 days at a seeding density of 1×10^5 cells/mL. Cells were monitored until the cell density reached 1.0–1.5 $\times$ 10^6 cells/mL 3–4 days after seeding.

MDCK-C cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) in a 75 cm^2 culture flask under 5% CO_2 at 37 °C. The confluent cells were trypsinized (0.05% trypsin-EDTA, 25300054; Gibco, Carlsbad, CA, USA), and 3.0 $\times$ 10^6 cells were seeded in a 75 cm^2 culture flask at each passage.

LLC-MK2D cells were maintained in MEM supplemented with 10% FBS, non-essential amino acids (Gibco), and 1 mM sodium pyruvate (Gibco) in a 75 cm^2 culture flask under 5% CO_2 at 37 °C. The confluent cells were trypsinized (0.05% trypsin-EDTA, Gibco), and 1.0 $\times$ 10^6 cells were seeded for further passaging.

2.2. Clinical specimens

Nasal or pharyngeal swabs were collected using a UTM 360C kit (Copan, Brescia, Italy) from patients diagnosed with influenza or influenza-like illness through the winter seasons of 2006/2007, 2008/2009, and 2010/2011. These specimens were subjected to virus isolation, and influenza virus-positive samples were further analyzed to determine type and subtype/lineage. Ten specimens each were selected from the following five groups: A(H1N1), A (H1N1)pdm09, A(H3N2), B-Yamagata, and B-Victoria viruses. The study protocol was approved by the ethics committee of the National Institute of Infectious Diseases, Japan.

2.3. Virus isolation and passage in cell culture

For virus isolation using MDCK-N, the infection medium was prepared at a 3:7 ratio of CDM and protein-free medium (prepared by Novartis). Neomycin (37.5 μg/mL) was added to prevent bacterial contamination from specimens, and 1 μg/mL of TrypZean (Sigma-Aldrich, St. Louis, MO, USA) was added to support viral growth in the infection medium. The density of MDCK-N was adjusted to 1×10^6 cells/mL in the infection medium, and 5 mL of cell suspension was distributed to 50 mL filter-capped tubes (TPP, Trasadingen, Switzerland). Fifty microliters of each clinical specimen was inoculated into each aliquot of MDCK-N, and tubes containing cells and specimens were incubated under 5% CO_2 at 34 °C on a shaker platform. MDCK-C and LLC-MK2D for virus isolation were prepared as follows: 1.5×10^6 (MDCK-C) and 3×10^5 (LLC-MK2D) cells were seeded in 60 mm diameter dishes 3 days before the inoculation and kept in MEM with 10% FBS until use. OPTI-PRO serum-free medium containing 4 mM L-Glu, 37.5 μg/mL neomycin, and 1 μg/mL TrypZean (MDCK-C) or 5 μg/mL Trypsin Acetylated (LLC-MK2D) was used to prepare the virus isolation medium. Fifty microliters of clinical specimens was inoculated, and the cultures were kept under 5% CO_2 at 34 °C. These cell lines were incubated for 72 h after the inoculation. Then, the supernatant was collected, and the hemagglutinin (HA) titer in the fluid was determined. If HA activity was not observed, the supernatant

was applied for blind passage. Samples showing HA activity after blind passage were judged as positive for isolation.

The isolates obtained successfully were applied to subsequent serial passages. The passaging was performed similarly to the procedure described above. Dilution factors of 1, 10, 100, and 1000 were adopted for the inocula showing HA titers of ≤8, 16–128, 256–512, and ≥1024, respectively. On and after the third passage, the volume of inoculum was increased to 500 μL with ten-fold dilution and filtered in a sterile manner instead of using neomycin. The isolates were passaged repeatedly until the passage number reached 10. The differences in virus isolation efficiency among the three cell lines were statistically analyzed by the *z*-test.

2.4. Hemagglutination tests

Hemagglutination tests were performed according to our laboratory's protocol. Briefly, the samples were serially diluted two-fold with PBS, and 50 μL of 0.75% guinea pig red blood cells (GRBC) in PBS was added to 50 μL of diluted samples. After 90 min incubation, the wells showing complete agglutination of GRBC were judged as HA positive. The HA titer was determined as the reciprocal value of the dilution with HA-positive samples.

2.5. Genetic analysis

Viral RNA in each sample was extracted by using the QIAamp Viral RNA Mini Kit (Qiagen K. K., Tokyo, Japan). Extracted RNA was used as the template for RT-PCR using PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio Inc., Shiga, Japan) with specific primers to amplify the HA and neuraminidase (NA) genes of the isolates. PCR amplicons were subjected to DNA sequencing using the BigDye Terminator Kit Ver.3.1 (Applied Biosystems Japan, Tokyo, Japan) with the specific primers according to the manufacturer's instructions. Sequence data were collected using an ABI3730xl automatic capillary sequencer (Applied Biosystems Japan) and analyzed by Sequencher ver. 4.9 software (Gene Codes Co., Ann Arbor, MI, USA). Multiple sequence alignment of HA and NA genes was performed by GENETYX-MAC ver. 15 (Genetyx Co., Tokyo, Japan). The differences in the frequency of amino acid substitution between MDCK-N and MDCK-C were statistically analyzed by Student's *t*-test.

2.6. Antigenic analysis

HA inhibition (HI) tests were performed using 0.75% GRBC with 4 HA units of virus according to standard methods. The HI value was determined as the reciprocal of the highest dilution of serum at which HA activity was completely inhibited. The differences in $\log_2$ unit of fold reduction of the HI titer between MDCK-N and MDCK-C isolates were statistically analyzed by Student's *t*-test.

2.7. Virus propagation in eggs

Isolates passaged 10 times in MDCK cells were applied for further serial passages in eggs. Ten-fold serially diluted isolates were prepared and inoculated into the allantoic cavity of 10-day-old embryonated hens' eggs. The eggs were incubated at 34 °C in a humidified atmosphere for 48 h and chilled at 4 °C overnight. After the collection of allantoic fluids, the endpoint of dilution with HA activity was determined. The 50% egg infectious dose (EID_{50}) titers of the isolates were calculated, and allantoic fluids from 10 to 1000 times lower dilution than the endpoint were pooled for the next passage in eggs. The viruses were passaged eight times in eggs to analyze the growth ability and genetic and antigenic stability of egg-passaged cell isolates.

3. Results

3.1. Isolation efficiency from clinical specimens and growth characteristics of the isolates

To evaluate the efficiency of virus isolation, 50 clinical samples (10 samples/subtype or lineage) were inoculated into MDCK-N and MDCK-C cells, and 20 clinical samples (four samples/subtype or lineage) into LLC-MK2D cells. The virus isolation efficiency was higher than 80% for each of the five subtypes/lineages in MDCK-N and MDCK-C cells (Table 1). However, LLC-MK2D showed significantly lower efficiency compared to the other two cell lines. No isolate of the A(H1N1), A(H3N2), or B-Victoria viruses was isolated, and only two isolates of B-Yamagata were obtained after a blind passage. Since the virus isolation efficiency in LLC-MK2D cells was poor from the first 20 samples (five samples each of A (H1N1), A(H3N2), B-Victoria, and B-Yamagata), inoculation of the remaining 30 samples was not done.

To evaluate the growth characteristics of the isolates, HA tests were performed for the viruses serially passaged in these cell lines. Both isolates from MDCK-N and MDCK-C showed high HA titers in A(H1N1), A(H3N2), B-Victoria, and B-Yamagata viruses (Fig. 1). The cell concentrations were approximately 5×10^6 cells/mL for both cell lines at the time of infection, and cell-specific yields (amounts of HA produced per cell) in MDCK-N and MDCK-C were also comparable. However, isolates of A(H1N1)pdm09 from MDCK-N showed significantly lower HA titers than those from MDCK-C (Fig. 1). The HA titers of two B-Yamagata isolates from LLC-MK2D cells, which ranged from 1 to 6 in $\log_2$ scale (data not shown), were not comparable to those from MDCK-N and MDCK-C (ranging from 7 to 9 in $\log_2$ scale). Overall, both MDCK-N and MDCK-C cell lines were considerably better suited than the LLC-MK2D cell line for the isolation and propagation of influenza A and B viruses.

3.2. Genetic and antigenic analyses of the cell-passaged isolates

We performed genetic and antigenic analyses to assess the stability of cell-passaged viruses. The frequency of amino acid changes in the HA of cell-passaged viruses compared to the clinical specimens was between 0.00 ± 0.00 and 2.10 ± 0.31 (mean ± standard error) per virus (Table 2). In the NA gene, cell-passaged viruses showed amino acid substitutions at a ratio of 0 ± 0.00–0.40 ± 0.16 per virus.

Table 1
Efficiency of virus isolation from clinical specimens using MDCK-N, MDCK-C, and LLC-MK2D cell lines.

Cell line	Isolation efficiency (number of isolates recovered/number of specimens tested)				
	A(H1N1)	A(H1N1)pdm09	A(H3N2)	B-Victoria	B-Yamagata
MDCK-N	90% (9/10)	100% (10/10)	100% (10/10)	80% (8/10)	80% (8/10)
MDCK-C	90% (9/10)	100% (10/10)	100% (10/10)	80% (8/10)	90% (9/10)
LLC-MK2D	0% (0/5)*	n.d.	0% (0/5)*	0% (0/5)*	40% (2/5)**

* Isolation efficiency in LLC-MK2D was significantly lower than that in MDCK-N and MDCK-C ($p < 0.01$).
** Isolation efficiency in LLC-MK2D was significantly lower than that in MDCK-C ($p < 0.05$).

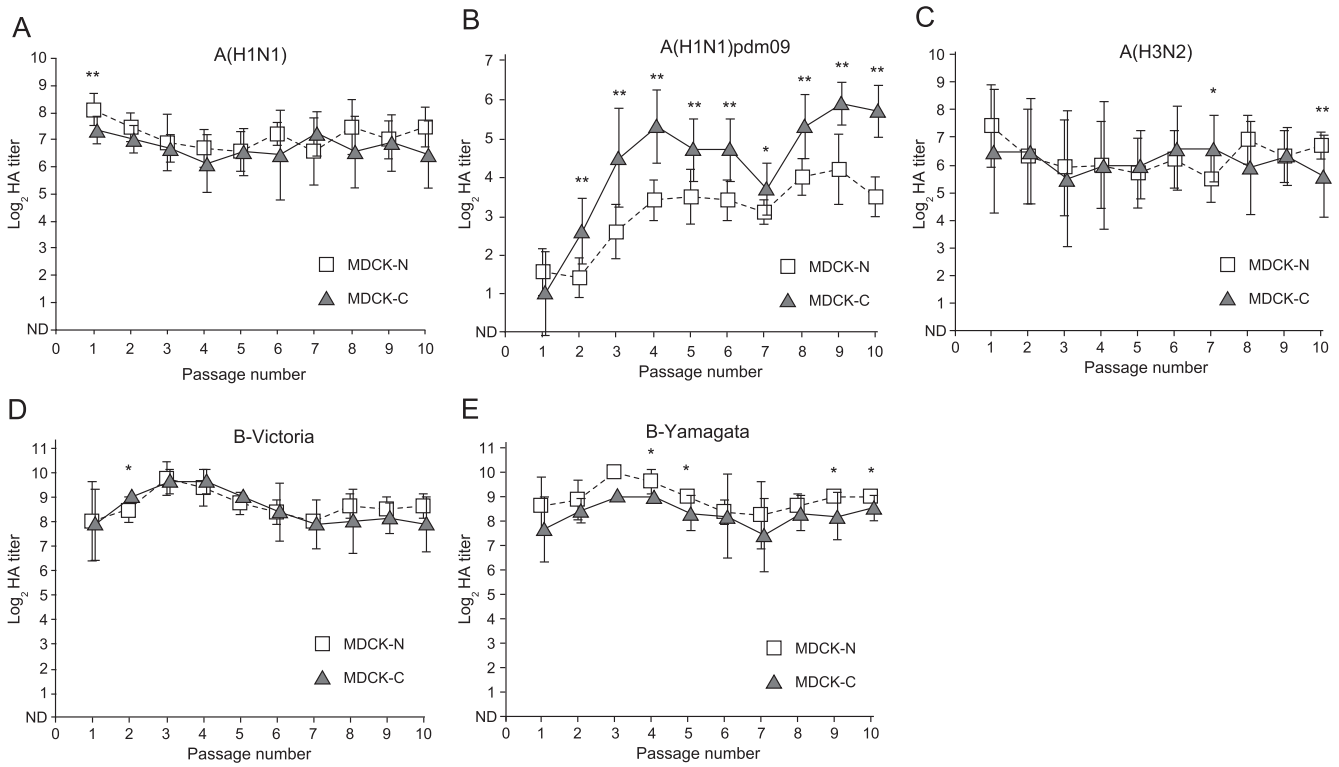

Fig. 1. Growth characteristics of influenza viruses in MDCK cell cultures. A(H1N1), A(H1N1)pdm09, A(H3N2), B-Victoria, and B-Yamagata viruses were isolated and passaged in MDCK-N (open square) and MDCK-C (closed triangle) cells. Each symbol represents the mean HA titers for the obtained isolates (eight to ten isolates for each subtype or lineage). The error bars represent the standard deviations. The differences in HA titers between MDCK-N and MDCK-C were statistically analyzed by Student's *t*-test (*$p < 0.05$, **$p < 0.01$). ND, not detectable.

Regarding A(H1N1) viruses, most isolates propagated in MDCK-N and MDCK-C cells showed the D186N or D186V amino acid substitution in HA (Table 3). These amino acid changes moderately affected the reactivity of the cell-passaged viruses with antiserum against A/Brisbane/59/2007_MDCK. The frequency of MDCK-N isolates with less than two-fold reduction in HI titer was 33.3%, and that of MDCK-C isolates was 88.9% (Table 4). The $\log_2$ fold reduction in the HI titer was 1.33 ± 0.33 for MDCK-N and 0.67 ± 0.24 for MDCK-C, with no significant difference (Table 5).

All A(H1N1)pdm09 isolates from MDCK-C cells acquired one or more amino acid substitutions at the site from K153 to N156 (Table 3). These amino acid changes resulted in striking antigenic alteration. All the MDCK-C isolates showed two-fold or higher reduction in HI titer (Table 4). In contrast, only 30% of MDCK-N isolates showed the amino acid changes at the site from K153 to N156 and more than 70% of the viruses retained the antigenicity of the reference antigen from cells. It was obvious that the reduction in HI titer for MDCK-N isolates was significantly lower than that for MDCK-C isolates (Table 5).

As for A(H3N2) isolates, the pattern of the amino acid changes was variable in each isolate, where only H183L and V223I were shared by MDCK-N and MDCK-C isolates (Table 3). For the antigenicity of the A(H3N2) virus, 90% of MDCK-N isolates and 70% of MDCK-C isolates remained within two-fold reduction in HI titer (Table 4) and there was no significant difference between MDCK-N and MDCK-C (Table 5).

With regard to B-Victoria viruses, six of eight MDCK-N-propagated viruses and five of eight MDCK-C-propagated viruses had amino acid substitutions at the N-linked glycosylation site from N197 to T199 in the HA protein (Table 3). These amino acid changes greatly affected the antigenicity, resulting in more than four-fold reduction for 87.5% of MDCK-N isolates and 75.0% of MDCK-C isolates (Table 4). No significant difference was found for the reduction in HI titer between MDCK-N and MDCK-C (Table 5).

In the experiments with B-Yamagata viruses, no genetic change was observed in isolates from MDCK-N or MDCK-C (Table 3), and 87.5% of MDCK-N isolates and MDCK-C isolates essentially retained the same antigenicity (Table 4). There was no significant difference in the reduction in HI titer between MDCK-N and MDCK-C (Table 5). One of the two B-Yamagata isolates from LLC-MK2D acquired the amino acid substitution of N196D at the N-linked glycosylation site (Table 3).

Table 2
Frequency of amino acid substitutions in HA and NA proteins of viruses recovered from three cell lines.

Cell line	Protein	Amino acid changes/virus (mean ± standard error)				
		A(H1N1)	A(H1N1)pdm09	A(H3N2)	B-Victoria	B-Yamagata
MDCK-N	HA	1.00 ± 0.00	2.10 ± 0.31	0.80 ± 0.13	1.13 ± 0.30	0.00 ± 0.00
	NA	0.11 ± 0.11	0.10 ± 0.11	0.30 ± 0.15	0.13 ± 0.13	0.00 ± 0.00
MDCK-C	HA	0.89 ± 0.11	1.60 ± 0.16	0.40 ± 0.16	0.75 ± 0.25	0.00 ± 0.00
	NA	0.11 ± 0.11	0.00 ± 0.00	0.40 ± 0.16	0.00 ± 0.00	0.00 ± 0.00
LLC-MK2D	HA	–	–	–	–	0.50 ± 0.50
	NA	–	–	–	–	0.00 ± 0.00

Table 3
Amino acid substitutions in HA and NA proteins of recovered viruses.

Cell line		Observed amino acid substitutions (number of observed amino acid substitutions/number of analyzed viruses)				
		A(H1N1)	A(H1N1)pdm09	A(H3N2)	B-Victoria	B-Yamagata
MDCK-N	HA	D186N (8/9) D186V (1/9)	K119E/K (1/10) A139D/A (1/10) K153H/K (1/10) G155E/G (2/10) S183P/S (7/10) L191I (3/10) L191I/L (3/10) E432G (1/10) K443E (1/10) V478I (1/10)	A138S (1/10) H183L (2/10) P221L (2/10) V223I (1/10) N225D (1/10) I227S (1/10)	N197N/K (3/8) T199T/I (4/8) T199N (1/8) T199A (1/8)	None (0/8)
	NA	S385N (1/9)	H297Q (1/10)	T148I (3/10)	D432D/G (1/8)	None (0/8)
MDCK-C	HA	G152R (1/9) D186N (5/9) D186V (2/9)	K119N (1/10) K153E/K (5/10) K154E (1/10) K154E/K (2/10) K154E/Q/K (1/10) G155E (4/10) N156D (1/10) V307I (1/10)	H183L (1/10) Y195H (2/10) V223I (1/10)	F95Y (1/8) N150N/A (1/8) T168I (1/8) T199T/I (2/8) T199T/A (1/8)	None (0/9)
	NA	V114I (1/9)	None (0/10)	T148P (2/10) T148A (1/10) T148I (1/10)	None (0/8)	None (0/9)
LLC-MK2D	HA	–	–	–	–	N196D (1/2)
	NA	–	–	–	–	None (0/2)

Table 4
Antigenic characterization of influenza viruses propagated in MDCK cell lines.

Cell line	HI titer reduction	Virus:	A(H1N1) (Jan 2008, Jan 2009)	A(H1N1)pdm09 (Jan 2011)	A(H3N2) (Jan 2007)	A(H3N2) (Jan 2009)	B-Victoria (Apr 2007)	B-Victoria (Dec 2008)	B-Yamagata (Jan 2008, Apr 2008)
		Ferret antiserum against:	A/Brisbane/ 59/07	A/Narita/ 1/09	A/Uruguay/ 716/07	A/Toyama/ 123/08	B/Hiroshima/ 1/05	B/Brisbane/ 60/08	B/Shizuoka/ 109/07
MDCK-N	None		3/9	4/10	0/5	5/5	0/3	0/5	4/8
	2-fold		0/9	3/10	4/5	0/5	1/3	0/5	3/8
	4-fold		6/9	2/10	1/5	0/5	2/3	2/5	1/8
	≥8-fold		0/9	1/10	0/5	0/5	0/3	3/5	0/8
MDCK-C	None		4/9	0/10	0/5	5/5	0/3	0/5	0/9
	2-fold		4/9	0/10	2/5	0/5	2/3	0/5	7/9
	4-fold		1/9	2/10	2/5	0/5	1/3	3/5	2/9
	≥8-fold		0/9	8/10	1/5	0/5	0/3	2/5	0/9

Table 5
Statistical analysis of the antigenicity of influenza viruses propagated in MDCK cell lines.

Cell line	Log_2 (fold reduction of HI titer)				
	A(H1N1)	A(H1N1)pdm09	A(H3N2)	B-Victoria	B-Yamagata
MDCK-N	1.33 ± 0.33	1.00 ± 0.26*	0.60 ± 0.17	2.25 ± 0.25	0.63 ± 0.26
MDCK-C	0.67 ± 0.24	2.80 ± 0.14	0.90 ± 0.27	2.00 ± 0.27	1.22 ± 0.15

The values are expressed as mean ± standard error.
* Log_2 unit of fold reduction of the HI titer in MDCK-N was significantly lower than that of MDCK-C ($p < 0.01$).

3.3. Propagation of the cell-derived isolates in eggs

Two cell isolates each from A(H1N1), B-Victoria, and B-Yamagata viruses, which were passaged 10 times in MDCK-N or MDCK-C, were applied for serial passages in eggs. Although we attempted to grow all A(H3N2) cell isolates in eggs, none of the isolates grew in the allantoic cavity. No viral genome was detected in the virus-inoculated eggs by quantitative RT-PCR [32].

The growth properties of the cell isolates in eggs are shown in Fig. 2. The A(H1N1) isolates showed high HA titers from the beginning of the egg passages, and the EID_{50} values reached 10^8–10^9 immediately during passages (Fig. 2A). The HA titers and EID_{50} values of A(H1N1) viruses seemed high enough for both MDCK-N and

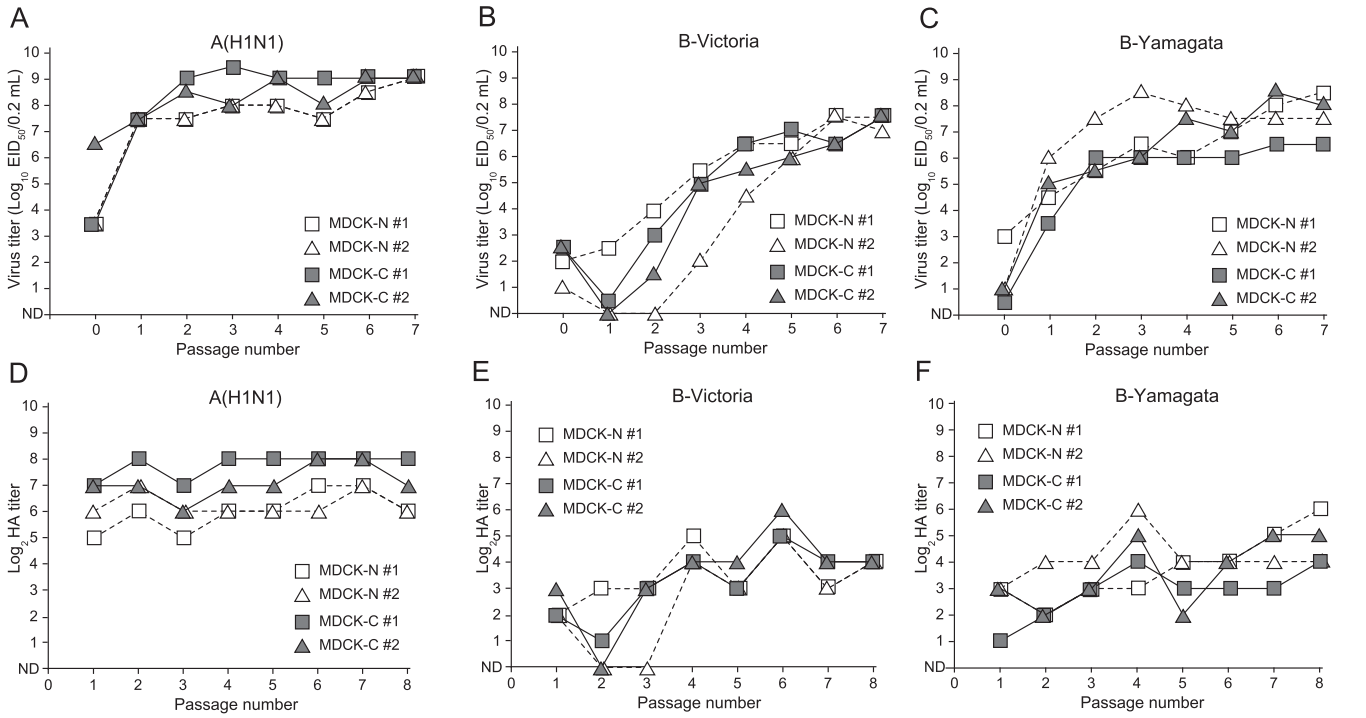

Fig. 2. Growth characteristics of cell isolates in embryonated hens' eggs. Isolates of A(H1N1), B-Victoria, and B-Yamagata viruses from MDCK-N (open square and triangle) and MDCK-C (closed square and triangle) were serially passaged in eggs. Each symbol represents the EID_{50} (A, B, and C) or HA titer (D, E, and F) of one passaged virus. ND, not detectable.

MDCK-C isolates, although the HA titers of the MDCK-N isolates were lower than those of MDCK-C (Fig. 2D).

The isolates of the B-Victoria lineage gradually acquired high infectivity for eggs, and EID_{50} values reached 10^6–10^7 after the fifth passage (Fig. 2B). The isolates of the Yamagata lineage grew well in eggs after the second passage, and EID_{50} values around 10^8 were observed for both MDCK-N and MDCK-C isolates (Fig. 2C). However, the HA titers of the B-Victoria and B-Yamagata viruses isolated in both cell lines remained lower even after extended passages in eggs; this finding is an important aspect warranting improvement (Fig. 2E and F).

3.4. Genetic and antigenic analyses of the egg-passaged cell isolates

All the A(H1N1) isolates from MDCK-N and one isolate from MDCK-C possessed the D186N substitution but had acquired no additional mutation during further passages in eggs (Table 6). For the other A(H1N1) isolates from MDCK-C, which had had no amino acid substitution as long as they were passaged in cells, the D186N substitution did occur after egg passages. It was shown that all the tested A(H1N1) viruses finally acquired the D186N change either after serial passages in cells or in eggs.

The HI assay of these A(H1N1) viruses revealed that the antigenicity of egg-passaged cell isolates (C10E8) was similar to that of cell isolates (C10) (Table 7). The C10 and C10E8 viruses reacted to antisera against both egg-derived and cell-derived reference antigens.

In B-Victoria viruses, both the MDCK-N isolates and one of two MDCK-C isolates already acquired genetic mutations at the glycosylation site from N197 to T199 before egg passages (Table 6). The loss of glycosylation by these amino acid changes resulted in decreased reactivity to antiserum raised against the cell-derived viral antigens and increased reactivity to antiserum against the egg-derived antigens (Table 7).

Regarding B-Yamagata viruses, passaging in MDCK-N and MDCK-C cells caused no amino acid substitution in HA, but egg passaging immediately induced amino acid changes at the receptor-binding site or the glycosylation site in HA (Table 6). The HI assay demonstrated that these amino acid changes during egg passages affected the antigenicity of B-Yamagata isolates. The C10E8 viruses lost their reactivity to the antiserum against the cell-derived antigen, and in contrast, their reactivity to antiserum against the egg-derived antigen increased (Table 7).

4. Discussion

In the present study, we showed that each cell line had a different ability to propagate influenza viruses, and in addition, each cell line induced specific genetic and antigenic changes in HA and NA proteins.

The isolation efficiency of viruses from clinical specimens was sufficiently high in MDCK-N and MDCK-C but very low in LLC-MK2D (Table 1). However, LLC-MK2, the parental cell line of LLC-MK2D, was reported to be susceptible to influenza viruses [28–30]. The low ability of LLC-MK2D to propagate influenza viruses might have resulted from the specific nature of a particular clone in the original cell line.

Both MDCK cell lines enabled the propagation of A(H1N1), A (H3N2), B-Victoria, and B-Yamagata viruses to high HA titers, but A(H1N1)pdm09 viruses from MDCK-N showed significantly lower HA titers than those from MDCK-C (Fig. 1B). The replication efficiency of A(H1N1)pdm09 viruses in MDCK-N cells should be, therefore, improved by strategies such as the development of high-growth vaccine strains by classical reassorting or reverse genetics [33], or the modification of MDCK-N cells [34].

The genetic and antigenic properties of MDCK-N and MDCK-C isolates were similar in A(H1N1), B-Victoria, and B-Yamagata, but different in A(H1N1)pdm09. A(H3N2) and B-Yamagata viruses from both MDCK-N and MDCK-C exhibited no specific amino acid substitution and maintained their antigenicity similar to those of cell-derived reference antigens (Tables 3 and 4). These results imply that both MDCK-N and MDCK-C cells would be suitable for

Table 6
Genetic changes observed in HA of the cell isolates after serial passages in eggs.

Clinical specimen	Cell line	Amino acid substitution at C10 (compared with clinical specimen)	Amino acid substitution at C10E8 (compared with C10)	Significance of the site
A(H1N1) #13	MDCK-N	D186N	No change	Antigenic/receptor binding site
	MDCK-C	D186N	No change	Antigenic/receptor binding site
A(H1N1) #15	MDCK-N	D186N	No change	Antigenic/receptor binding site
	MDCK-C	No change	D186N	Antigenic/receptor binding site
B-Victoria #11	MDCK-N	T199A	No change	Antigenic/glycosylation site
	MDCK-C	N150N/A	N/A150N	Antigenic site
		T199T/A	T/A199A	Antigenic/glycosylation site
B-Victoria #14	MDCK-N	N197N/K	N/K197N	Antigenic/glycosylation site
		T199T/I	T/I199I	Antigenic/glycosylation site
	MDCK-C	No change	N197N/S	Antigenic/glycosylation site
			T199T/I	Antigenic/glycosylation site
B-Yamagata #11	MDCK-N	No change	G141E	Receptor-binding site
			Q199P	Receptor-binding site
	MDCK-C	No change	Q199P	Receptor-binding site
			T371S	Unknown
B-Yamagata #12	MDCK-N	No change	N196D	Antigenic/glycosylation site
	MDCK-C	No change	G141G/E	Receptor binding site
			T198T/I	Antigenic/glycosylation site

Table 7
Antigenic characterization of cell-isolated viruses with or without serial passages in eggs.

Cell line	HI titer reduction	Ferret antiserum against:	A(H1N1)				B-Victoria				B-Yamagata			
			A/Brisbane/59/2007 MDCK		A/Brisbane/59/2007 Egg		B/Brisbane/6020/08 MDCK		B/Brisbane/6020/08 Egg		B/Shizuoka/109/2007 MDCK		B/Sendai-H/114/2007 Egg	
		Isolates:	C10	C10E8	C10	C10E8	C10	C10E8	C10	C10E8	C10	C10E8	C10	C10E8
MDCK-N	None		0/2	0/2	0/2	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	2/2
	2-fold		0/2	1/2	2/2	0/2	0/2	0/2	1/2	2/2	2/2	0/2	0/2	0/2
	4-fold		2/2	1/2	0/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2	1/2	0/2
	≥8-fold		0/2	0/2	0/2	0/2	0/2	2/2	0/2	0/2	0/2	2/2	1/2	0/2
MDCK-C	None		1/2	0/2	2/2	2/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	1/2
	2-fold		1/2	2/2	0/2	0/2	0/2	0/2	0/2	2/2	1/2	0/2	0/2	0/2
	4-fold		0/2	0/2	0/2	0/2	1/2	0/2	1/2	0/2	1/2	0/2	0/2	0/2
	≥8-fold		0/2	0/2	0/2	0/2	1/2	2/2	0/2	0/2	0/2	2/2	2/2	1/2

the isolation and propagation of A(H3N2) and B-Yamagata vaccine seed viruses.

Regarding B-Victoria viruses, it was unexpected that the passaging of these viruses in MDCK cells induced the same amino acid substitutions typical of egg adaptation. These results indicate that selecting highly stable isolates or modifying MDCK cells might be necessary for the prevention of antigenic alteration in B-Victoria viruses.

As for A(H1N1)pdm09 viruses, all the MDCK-C isolates acquired the specific amino acid changes at the site from K153 to N156 and showed more than four-fold reduction in HI titer compared with the cell-derived reference antigen. These amino acid changes might be associated with a higher replication efficiency of the MDCK-C isolates than that of the MDCK-N isolates. In contrast to the MDCK-C isolates, the majority of the MDCK-N isolates did not gain amino acid substitutions at this site and retained the antigenicity similar to that of the reference antigen from cells. These results suggest that the MDCK-N cell line is superior to MDCK-C cells for the isolation and propagation of A(H1N1)pdm09 viruses.

Donis *et al.* reported that the majority of viruses propagated in the three MDCK cell lines (MDCK-1, MDCK-2, and MIDCK-3) were within two-fold reduction in HI titer compared with reference antigens [22]. Our results from two MDCK cell lines were similar to theirs for A(H3N2) and B-Yamagata viruses but different for A(H1N1) and B-Victoria viruses, with the present study showing higher frequencies of antigenic alteration compared to the previous one. This might be explained by the difference in passage numbers. We passaged viruses ten times in each cell line, while Donis *et al.* did it thrice. In addition to the four subtypes/lineages (A(H1N1), A(H3N2), B-Victoria, and B-Yamagata) they examined, A(H1N1)pdm09 viruses from MDCK-N and MDCK-C were analyzed in our study. It was surprising that the pattern of amino acid substitutions and the antigenic properties were quite different between MDCK-N and MDCK-C isolates, although both MDCK-N and MDCK-C cells originated from the same MDCK cell line. The distinct mutation patterns between both cell isolates might have resulted from different patterns of protein expression and glycosylation on the surface of both cell lines. It was reported that the adaptation of adherent cells to suspension growth significantly influences N-glycosylation of HA in MDCK-propagated influenza virus [35]. Adaptation to different culture conditions (adherent versus suspension growth) might cause the different mutation patterns in MDCK-N isolates and MDCK-C isolates, but another possibility is that differences between the two MDCK cell lines characterized in this study might just reflect cell isolate-to-cell isolate variability and not necessarily properties related to adherent versus suspension growth.

Flucelvax was licensed in 2012 by the Food and Drug Administration (FDA) as the first cell-based vaccine in the United States. This vaccine was produced using egg-derived seed viruses in the beginning. Recently, an influenza vaccine using the cell-derived seed virus was approved by the US-FDA [36]. Two WHO collaborat-

ing centers (Centers of Disease Control and Prevention in the US and the Victorian Infectious Diseases Reference Laboratory in Australia) isolated and propagated seed viruses in MDCK33016PF cells (identical to MDCK-N). This made it possible to produce a completely cell-derived vaccine, which is expected to have higher vaccine effectiveness than that of egg-based vaccines. A(H3N2) viruses have two serious limitations: (i) they hardly replicate in embryonated hens' eggs, and (ii) critical antigenic alterations occur very frequently during egg adaptation [37–39]. It is hoped that a completely cell-based A(H3N2) vaccine will solve these problems in the egg-based one.

While A(H1N1), B-Victoria, and B-Yamagata viruses from MDCK-N and MDCK-C cells could replicate efficiently in embryonated hens' eggs, cell-passaged A(H3N2) viruses were not amplified by egg passaging. These results reflect the nature of A(H3N2) viruses, that is, the inability to replicate well in eggs [37–39].

For A(H1N1) and B-Victoria viruses, the genetic and antigenic properties of egg-passaged cell isolates were similar to those of the original cell isolates (Tables 5 and 6). This would be explained partly by the fact that the cell isolates had already acquired the same amino acid changes as seen in egg adaptation prior to the subsequent egg passages. In contrast, B-Yamagata viruses passaged in MDCK-N and MDCK-C had no mutation, but egg passages induced amino acid changes that resulted in the loss of a glycosylation side chain and antigenic alteration. These results would support the idea that cell isolates of A(H1N1), B-Victoria, and B-Yamagata viruses could be used as seed viruses for egg-based vaccine manufacture, even though some amino acid changes typical of egg adaptation would be present in the vaccine products.

In this study, we systematically evaluated MDCK-N, MDCK-C, and LLC-MK2D. Both MDCK cell lines were found to be useful for developing vaccine seed viruses because of their high susceptibility to influenza viruses. MDCK-N was assessed as the best of the three cell lines examined here based on its higher antigenic stability for A(H1N1)pdm09 viruses compared to MDCK-C. However, it should be noted that some A(H1N1) and B-Victoria viruses derived from MDCK cells showed genetic and antigenic changes. New viruses that easily acquire mutations during MDCK passage may emerge in the future. It would be therefore necessary to prepare several cell lines and use them in combination for covering a wide variety of influenza viruses. Cell-based influenza vaccines using MDCK and other cell lines would contribute critically to the protection against influenza virus infection and thus to the improvement of global public health.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: H. Trusheim is an employee of IDT Biologika GmbH. B.R. is an employee of GSK Vaccines GmbH. The other authors declare no conflict of interests.

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Author contributions

K.N, Y.H., H. Takahashi, and N.Y. designed the study, analyzed the data, and wrote the paper. H. Trusheim and B.R. contributed to the implementation of MDCK-N to NIID, the study design, and review of the paper. I.H. and H.A. performed genetic and antigenic analyses. K.M., A.H.S., and T.Ogane, N.K., and Y.K. performed laboratory tests. M.T., T.Odagiri, and N.Y. led the project and wrote the paper.

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