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Developments and current challenges in the process of cell culture-based seasonal influenza vaccine manufacture in Japan

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Abstract: Seasonal influenza is an acute respiratory infection primarily caused by influenza A and B viruses, which circulate annually and cause substantial morbidity and mortality worldwide. Annual influenza vaccination is currently the most effective measure for preventing influenza and greatly reduces the risk of disease severity and the incidence of complications and death. Annual seasonal influenza vaccines are traditionally produced in Japan and many other countries using viruses propagated in embryonated chicken eggs. However, at present, the effectiveness of the seasonal influenza vaccines has some significant limitations, partly because of egg-adaptive mutations in the antigenic sites of the influenza virus haemagglutinin, which are caused by the continued evolution of seasonal influenza viruses. To overcome the limitations of egg-based influenza vaccine production, a mammalian cell culture-based influenza vaccine production system has been developed in Japan in the past decade as an alternative to the current production method. In this review, I have summarised the progress in the development of cell-based seasonal influenza vaccines and discussed the technological challenges encountered in the development of influenza vaccines.

***Keywords*:** MDCK cell-based influenza vaccine, vaccine development, seasonal influenza candidate vaccine virus, mutation, cell culture techniques

Introduction

The World Health Organization (WHO) estimates that annual influenza epidemics worldwide cause one billion cases of influenza, three to five million cases of severe illness, and approximately 290,000-650,000 respiratory deaths (*1*). Influenza vaccines are the most effective preventive measures against influenza infection and generally reduce the risk of influenza disease in the general population by 40-60% during influenza season, particularly when the circulating viral strains are antigenically well matched to the vaccine strains (*2,3*).

However, every year, the influenza virus evolves rapidly worldwide; therefore, propagation of the influenza virus in eggs is most likely a significant cause of an antigenic mismatch between the circulating influenza virus strains and the vaccine strains, reducing vaccine effectiveness (*3*). A sustainable and continuous cell-based influenza vaccine production system was developed in Japan to produce and supply vaccines that are effective and sufficient for pandemics and seasonal influenza preparedness. Compared to the advantages of egg-derived influenza vaccines, cell-based influenza vaccines offer additional advantages, including: *i*) a large-scale continuous supply process; *ii*) low risk of microbial contamination; *iii*) no risk of severe allergic reactions; *iv*) enhanced vaccine immunogenicity; *v*) ease of manufacture, manipulation, and production; *vi*) manufacturing flexibility (*e.g.*, strengthening of manufacturing capacity and procurement of influenza vaccines during pandemics); and *vii*) a lack of egg-adaptive mutations in the antigenic sites of haemagglutinin (HA) (*4,5*).

Currently, two manufacturers produce cell-based influenza vaccines: Flucelvax Quadrivalent (Flucelvax Tetra in various markets; CSL Seqirus Ltd., Melbourne, Australia) and SKYCellflu trivalent and quadrivalent subunit inactivated vaccines (SK Bioscience Co., Ltd., Seongnam-si, South Korea) (*6*). Since the 2019/2020 influenza season, all four influenza strains (2009 H1N1 Pandemic [A{H1N1}pdm09], A[H3N2], B[Yamagata], and B[Victoria]) of the Flucelvax Quadrivalent formulation have been isolated and propagated exclusively in a Madin-Darby Canine Kidney (MDCK) 33016-PF cell line (*7*). A cell-based quadrivalent inactivated influenza vaccine has been approved in the European Union (Flucelvax Tetra) and the United States (Flucelvax Quadrivalent) for the prevention of influenza in adults and children (*8*).

In 2019, SKYCellflu trivalent and quadrivalent vaccines, produced in MDCK-Sky3851 cells, were accorded WHO prequalification status for being the

first cell-based influenza vaccines, which certifies the safety and the protective efficacy of the vaccines that were assessed by monitoring the manufacturing process, quality, and clinical trial results of the vaccines (*9,10*). Currently, the SKYCellflu trivalent and quadrivalent vaccines have been approved in 11 countries (*10*). Since the 2017/2018 influenza season, the WHO has provided strain recommendations for seasonal influenza candidate vaccine viruses (CVVs) derived from both eggs and cells (*11*). One of the major developments in cell-based influenza vaccines is the potential to use cell-derived seed viruses that can avoid egg-adaptive mutations and produce viruses that are antigenically similar to the circulating viruses, thereby improving vaccine effectiveness (*6*).

As part of the ongoing strategies to provide a continuous long-term supply of effective cell-based seasonal influenza vaccines, the following fundamental research studies have been undertaken: *i*) development of a cell line substrate for the isolation of influenza viruses from clinical specimens; *ii*) assessment of isolation rates, growth properties, and antigenic and genetic characteristics of influenza viruses in the cell lines; *iii*) establishment of an assay for detection and identification of viral adventitious agents in biological materials, including vaccines and clinical specimens; *iv*) assessment of the viral clearance capacity of the cell line during the process of the isolation of influenza viruses; and *v*) development of *in-vitro* methods for assessing the potency of cell-derived seasonal influenza vaccines. In this review, I have described the progress made until now and the current challenges in the practical use of cell-based seasonal influenza vaccines in Japan.

Efforts towards the practical application of cell-based influenza vaccines in Japan

Establishment of a certified National Institute of Infectious Diseases (NIID)-MDCK cell line

MDCK cells are the most suitable substrates for isolation and propagation of a broad range of influenza viruses (*12*); however, they are not suitable for propagating certain influenza viral strains. When seasonal influenza vaccines are manufactured using cell-based technology, effective cell-derived influenza viruses of the WHO-recommended strains with high growth capacity and antigenic consistency are selected (*3*) and distributed to vaccine manufacturers to establish vaccine seed viruses. To address these challenges, a novel adherent NIID-MDCK cell line was developed from the parental MDCK line (ATCC CCL-43) that can isolate influenza viruses from original clinical specimens. The viral growth medium must be free from extraneous agents at all stages of virus preparation for vaccine production (*13,14*). MDCK cells that were grown in serum-free medium in the presence of trypsin showed high titres of influenza virus (*15*). Therefore, the NIID-MDCK cell line was grown in both serum- and protein-free cell culture media. Because cell substrates used in the manufacturing process have an impact on the safety and the purity of vaccines (*13,16*), the biological characteristics of the NIID-MDCK cell line were assessed, and the quality was ensured in the context of cell-substrate use for producing cell-derived influenza vaccines by a global comprehensive biosafety testing company, BioReliance (Glasgow, UK).

Isolation efficiency of influenza viruses in the NIID-MDCK cell line

Following influenza virus isolation from clinical specimens using the NIID-MDCK cell line, appropriate NIID-MDCK cell-derived isolates were adapted and propagated in vaccine manufacturer-owned cells to establish seed viruses for cell-based seasonal influenza vaccine production. If the NIID-MDCK cell-derived isolates have low growth capacity and are antigenically mismatched to the WHO-recommended vaccine prototype viruses, they cannot be distributed to vaccine manufacturers as they may have a significant impact on manufacturing volume, leading to a risk of vaccine shortage. To evaluate the growth properties and the isolation rates of influenza viruses grown in the NIID-MDCK cell line, clinical specimens were directly inoculated into the cell line. Five clinical samples of each type/subtype/lineage with various Ct values were inoculated into MDCK cell lines. Following each passage, the growth characteristics of the influenza viruses isolated from the NIID-MDCK and the conventional MDCK cell lines were determined by HA tests. Influenza A viruses (H1N1pdm09 and H3N2) and influenza B viruses (Yamagata and Victoria lineages) were isolated when the HA titres were greater than two after serial passages. Similar to that in the conventional MDCK cell line, the NIID-MDCK cell line allowed for the successful propagation of A(H3N2) and influenza B viruses (Yamagata and Victoria lineages) at high HA titres (*17*) (Table 1). Although A(H1N1)pdm09 viruses showed limited propagation and lower HA titres in the NIID-MDCK cell line than that in the conventional MDCK cell line, it was possible to isolate A(H1N1) pdm09 viruses with high efficiency by selecting clinical specimens that contained high virus titres (*17*) (Table 2). The A(H1N1)pdm09 viruses were efficiently isolated from clinical specimens with cycle threshold (Ct) values of 24 or below, and the A(H3N2) viruses were isolated from clinical specimens with Ct values of 27 or below after multiple passages (*17*). All influenza B viruses (Victoria and Yamagata lineages) were successfully isolated from clinical specimens after two passages, with Ct values close to 35 (*17*). Therefore, the NIID-MDCK cell line is considered a suitable substrate for isolating influenza A and B viruses from clinical

Table 1. Virus isolation efficiency and haemagglutinin (HA) titres of the isolates in the National Institute of Infectious Diseases-Madin-Darby Canine Kidney (NIID-MDCK) and conventional cell lines

Type/Subtype	Cell line	Specimen ID	Passage number			
			1	2	3	4
A(H1N1)pdm09	NIID-MDCK	TA77	< 2	< 2	< 2	< 2
		TA78	8	32	64	ND
		TA79	< 2	< 2	< 2	< 2
		TA95	< 2	< 2	< 2	< 2
		TA108	< 2	< 2	< 2	< 2
	Conventional MDCK	TA77	< 2	4	16	ND
		TA78	32	32	16	ND
		TA79	< 2	8	4	2
		TA95	< 2	4	8	16
		TA108	2	16	4	4
A(H3N2)	NIID-MDCK	TA232	< 2	< 2	64	256
		TA233	< 2	< 2	8	256
		TA234	2	16	128	ND
		TA235	< 2	128	128	ND
		TA236	< 2	128	128	ND
	Conventional MDCK	TA232	< 2	64	512	ND
		TA233	< 2	< 2	256	ND
		TA234	8	16	256	ND
		TA235	4	128	256	ND
		TA236	< 2	128	512	ND
B(Victoria)	NIID-MDCK	TA318	256	512	256	ND
		TA322	128	512	256	ND
		TA327	128	512	256	ND
		TA330	< 2	512	256	ND
		TA331	64	1,024	512	ND
	Conventional MDCK	TA318	128	512	512	ND
		TA322	128	512	512	ND
		TA327	128	512	1,024	ND
		TA330	16	512	512	ND
		TA331	128	512	1,024	ND
B(Yamagata)	NIID-MDCK	TA336	4	512	512	ND
		TA338	2	512	512	ND
		TA351	64	512	512	ND
		TA376	512	512	256	ND
		TA381	128	512	512	ND
	Conventional MDCK	TA336	128	512	512	ND
		TA338	32	512	512	ND
		TA351	512	512	512	ND
		TA376	512	512	512	ND
		TA381	512	1024	512	ND

Data source: Ministry of Health, Labour and Welfare. The 8th Health Science Council Immunisation and Vaccine Sectorial Committee (5th September, 2014) (*17*). MDCK, Madin–Darby Canine Kidney; ND, Not Done; NIID, National Institute of Infectious Diseases.

Table 2. Virus isolation efficiency of A(H1N1)pdm09 isolates in the NIID-MDCK cell line

Specimen ID	Passage number				Ct value
	1	2	3	4	
TA71	4	4	16	32	19.77
TA72	2	2	16	16	19.71
TA73	< 2	8	16	16	21.86
TA75	< 2	< 2	32	32	23.44
TA84	< 2	< 2	4	64	24.81
TA95	< 2	< 2	< 2	< 2	25.16
TA87	< 2	< 2	< 2	< 2	26.03
TA85	< 2	< 2	< 2	< 2	27.73
TA83	< 2	< 2	< 2	< 2	30.13
TA79	< 2	< 2	< 2	< 2	35.15

Data source: Ministry of Health, Labour and Welfare. The 8th Health Science Council Immunisation and Vaccine Sectorial Committee (5th September, 2014) (*17*). Ct, cycle threshold.

specimens. The A(H1N1)pdm09 viruses isolated from the MDCK33016-PF cell line also had lower HA titres than those isolated from the conventional MDCK cell line (*18*).

Recently, clinical strains of influenza A(H3N2) viruses have been observed to replicate poorly in MDCK cells and rapidly acquire HA or neuraminidase (NA) mutations, leading to viral antigenic alternation (*19-21*). Due to the fact that these findings are prevalent in various MDCK cell lines, and that the NIID-MDCK cell line has similar isolation and propagation efficiency to other MDCK cell lines, these major issues of enhanced virus isolation and propagation are not necessarily improved by propagation in the NIID-MDCK cell line. Therefore, an alternative strategy should be utilised to isolate and propagate a wide range of influenza viral

strains in the NIID-MDCK cell line.

Antigenic analyses of NIID-MDCK cell isolates

Influenza vaccine strains may undergo genetic changes at antigenic and glycosylation sites during the process of egg-based seasonal influenza vaccine production, resulting in antigenic changes in the original vaccine strain. To assess genetic and antigenic variations in the influenza virus during repeated passages in the NIID-MDCK cell line, clinical specimens were inoculated onto confluent monolayers of the cell line, and the HA and NA genes of the NIID-MDCK cell isolates were sequenced and analysed. The impact of amino acid substitutions on the antigenic properties of influenza viruses was assessed using a haemagglutination inhibition (HI) assay with post-infection ferret antisera raised against the cell culture-propagated vaccine prototype viruses. NIID-MDCK cell isolates were considered antigenically similar to each other if their HI titres were within two-fold of the reference virus titre. Diverse amino acid substitutions were observed among NIID-MDCK cell isolates, similar to that in conventional MDCK cell isolates (*18*); however, most of the isolates were antigenicity equivalent with prototype viruses (*22*) (Table 3). After isolating and propagating the influenza viruses in the NIID-MDCK cell line, the suitability of the NIID-MDCK cell isolates for the establishment of a vaccine seed virus whose antigenicity matches that of the prototype viruses was assessed using the one-way HI or the viral neutralisation (VN) tests. If vaccine manufacturers develop their own vaccine seed viruses from appropriate NIID-MDCK cell isolates using their own cell lines, the antigenic similarity between these seed viruses and the prototype viruses can be confirmed by the two-way HI or VN tests.

Development of a multiplex real-time polymerase chain reaction (PCR) assay for the detection of adventitious viruses

Vaccine safety concerns regarding potential contamination with adventitious viruses have arisen from the incidental introduction of viruses into a vaccine through starting materials, such as cell substrates, porcine trypsin, bovine serum, or any other source materials of animal or human origin (*23,24*). The influenza virus was propagated and isolated using the qualified NIID-MDCK cell line that was free of serum and animal components. However, adventitious agents can be introduced into the production process *via* clinical specimens and human intervention. Real-time PCR is one of the most common laboratory methods for detecting pathogens as it is highly sensitive, even for low-level detection of pathogens. Therefore, a rapid and highly sensitive multiplex real-time PCR detection system was developed for the detection of 42 different viruses that are frequently found in clinical specimens and commonly grown in MDCK cells.

Using this assay, 34 NIID-MDCK isolates were tested to detect the presence of 27 different respiratory viruses. No adventitious viral genome was detected in the NIID-MDCK cell isolates, whereas the viral genome of human enterovirus D68 (HEV-D68) was detected when it was present at a concentration below the limit of detection (LOD) in two NIID-MDCK cell isolates (*25*). HEV-D68 did not proliferate in the NIID-MDCK cell line (*25*). Furthermore, the susceptibility of the NIID-MDCK cell line to eight representative human respiratory viruses, namely, human adenovirus serotype 4, human coronavirus OC43, HEV-D68, human metapneumovirus A, human respiratory syncytial virus A, human rhinovirus A, herpes simplex virus 1 (HSV-1), and severe acute respiratory syndrome coronavirus 2, which are frequently co-detected in influenza clinical specimens, was evaluated. The NIID-MDCK cell line was infected with individual viruses, and the viral genome copy number was determined using the assay after each passage. Similar to the findings of a previous study showing that the MDCK33016-PF cell line only supports the growth of a limited spectrum of viruses (*26*), the results of this analysis showed that all the tested respiratory viruses were effectively eliminated through passages in the NIID-MDCK cell line (*25*) (Table 4). A previous study demonstrated that HSV-1 could propagate in the MDCK33016-PF cell line (*27*), whereas

Table 3. Characterisation of viruses isolated in the NIID-MDCK cell line

Type A subtype; B lineage	Specimen ID	Passage number	HI test (one-way)	MDCK cell culture-propagated vaccine prototype viruses
A(H1N1)pdm09	TA73	5	equivalent	A/Wakayama/153/2013 (C1/C1)
	TA78	3	2-fold difference	
A(H3N2)	TA232	4	equivalent	A/Victoria/361/2011 (C2/C2)
	TA233	4	equivalent	
B(Victoria)	TA318	3	equivalent	B/Brisbane/60/2008 (CX/C1/C2)
	TA322	3	equivalent	
B(Yamagata)	TA336	3	equivalent	B/Wisconsin/1/2010 (C1/C1/C2)
	TA338	3	2-fold difference	

Data source: Ministry of Health, Labour and Welfare. The 11th Health Science Council Immunisation and Vaccine Sectoral Committee (19th February, 2016) (*22*). HA, haemagglutinin; HI, haemagglutination inhibition; MDCK, Madin-Darby Canine Kidney.

the number of HSV-1 genomes decreased with each passage and reduced to a level below the LOD at the third passage in the NIID-MDCK cell line, indicating the low susceptibility of the NIID-MDCK cell line to HSV-1. Overall, the NIID-MDCK cell line is less susceptible to most respiratory viral infections, suggesting that it is a suitable substrate for the isolation and propagation of influenza viruses from a safety perspective.

Development of a modified NIID-MDCK cell line with high productivity of influenza viruses

As described above, the isolation efficiency and the growth characteristics of influenza A and B viruses in the NIID-MDCK cell line did not differ significantly from those in the conventional MDCK cell isolates. A modified NIID-MDCK cell line, which could be used for isolating a wide range of high-yield influenza viruses, was further developed for cell-based seasonal influenza vaccine production. The human interferon regulatory factor 7 (IRF7) gene was identified using a small interfering RNA library targeting 78 human genes involved in the type I interferon signalling pathway (*28*), the first line of immune defence against influenza virus infection (*29*). The HA titres of both influenza A and B viruses in the NIID-MDCK-shIRF7 cell line, expressing short hairpin RNA (shRNA) against IRF7, displayed a two to eight-fold increase compared to that of the NIID-MDCK-control shRNA cell line at 48-96 h post-infection (hpi) (*28*) (Table 5). A recent study also demonstrated that influenza A and B viruses showed an approximately four to five-fold increase in numbers at 48 hpi in IRF7-knockout-MDCK cells, using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 technology (*30*). Based on these findings, the NIID-MDCK cell line with low IRF7 expression levels can be used to improve the production capacity of cell-based seasonal influenza vaccines.

Table 4. Detection of the virus genomes in the supernatant of the infected culture[1)]

Virus	Copy number[2)] of each viral genome at different passage in NIID-MDCK cell isolates			
	Input	P1	P2	P3
FluAV	2.5×10^{8}	2.5×10^{9}	5.0×10^{9}	
HAdV4	1.0×10^{9}	5.0×10^{5}	— [3)]	
FluAV	2.5×10^{8}	3.2×10^{9}	1.6×10^{9}	
HCoV-OC43	1.3×10^{9}	1.3×10^{5}	—	
FluAV	2.5×10^{8}	5.0×10^{9}	1.3×10^{9}	2.0×10^{9}
HEV-D68	1.0×10^{10}	1.6×10^{6}	1.3×10^{4}	—
FluAV	2.5×10^{8}	5.0×10^{9}	3.2×10^{9}	
HMPVA	7.9×10^{8}	1.0×10^{4}	—	
FluAV	2.5×10^{8}	4.0×10^{9}	3.2×10^{9}	
HRSVA	3.2×10^{8}	1.3×10^{8}	—	
FluAV	2.5×10^{8}	2.5×10^{9}	2.0×10^{9}	
HRVA	2.5×10^{8}	3.2×10^{4}	—	
FluAV	2.5×10^{8}	1.0×10^{10}	6.3×10^{9}	5.0×10^{9}
HSV-1	6.3×10^{8}	2.0×10^{6}	4.0×10^{3}	—
FluAV	2.8×10^{7}	3.1×10^{9}	2.2×10^{9}	
SARS-CoV-2	3.3×10^{9}	1.8×10^{5}	—	

[1)] NIID-MDCK cell isolates were co-infected with influenza A virus and one of the common respiratory viruses. The supernatant of the infected culture was used for the serial passages in NIID-MDCK cell isolates. [2)] Copy numbers were determined *via* the improved assay using viral nucleic acids extracted from the supernatant of the culture medium, as described in the text. Copy numbers were shown per 1 mL of the supernatant of the infected culture. [3)] The copy number was below the limit of detection. This table was modified from Hamamoto I, *et al*. Microbiol Immunol. 2022 (*25*). HAdV4, human adenovirus serotype 4; HCoV-OC43, human coronavirus OC43; HEV-D68, human enterovirus D68; HMPVA, human metapneumovirus A; HRSVA, human respiratory syncytial virus A; HRVA, human rhinovirus A; HSV-1, herpes simplex virus 1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; NIID-MDCK, National Institute of Infectious Diseases-Madin-Darby Canine Kidney.

Development of in-vitro potency testing methods for cell-based seasonal influenza vaccines

The single radial immunodiffusion (SRD) assay is the only internationally recognised quality control method for determining influenza vaccine potency and stability. The SRD assay requires strain-specific reference sera and calibrated standard reference antigens to determine the number of influenza HA antigens in the vaccine (*31,32*). In Japan, the SRD assay is routinely used to measure the HA content of egg-cultured seasonal influenza vaccines; however, no method has been established to measure the HA content of cell-cultured seasonal influenza vaccines. To establish a method for determining the HA content of a cell-culture-derived quadrivalent vaccine using the SRD assay, a reference serum and a purified primary liquid standard (PLS), which were used to calibrate the antigen reference reagent in SRD, were prepared from cell-derived influenza CVVs. The proportion of HA protein in the PLS was determined from a densitometric analysis of the viral proteins separated by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis. Although a small amount of cell debris, including nucleic acids,

Table 5. HA titres of influenza viruses produced from the NIID-MDCK cell line

Influenza virus	Subtype/Lineage	shRNA-Control	shRNA-IRF7	Input virus titre/well	Time after infection (hour)
A/Puerto Rico/8/1934	H1N1	8	16	100 pfu	48
A/Narita/1/2009	H1N1pdm09	4~8	16~32	1000 $TCID_{50}$	96
A/Victoria/361/2011	H3N2	4	16~32	100 $TCID_{50}$	72
B/Florida/4/2006	Yamagata	4~8	16~32	100 pfu	72

This table was modified from Hamamoto I, *et al*. PLoS One. 2013 (*28*). shRNA, short hairpin RNA.

was detected using electron micrographs, virtually pure PLS (less than 5% non-viral proteins) was successfully prepared (*33*). As the purity of PLS is important for the accurate determination of vaccine potency (*34*), it is necessary to devise a reliable alternative method for measuring the HA content by improving the technique for increasing the degree of HA purification. The antiserum is prepared by immunising sheep with HA prepared from prototype viruses or their derivatives, whereas the standard reference antigens, which are used to determine the HA content of the vaccine, must be derived from the strain used in the vaccine. The standard reference antigens containing a known amount of diluted HA (15 μg HA per 0.5 mL) and a cell-derived vaccine solution were placed in the wells of a serum-spiked agarose gel to determine the relative titres against the standard reference antigens. The HA content of each antigen in the quadrivalent vaccines that were isolated in the NIID-MDCK cell line, determined using SRD assay reagents derived entirely from cell-cultured vaccine viruses, were virtually equivalent to each other (approximately 15 μg HA per 0.5 mL) (*33*). Overall, the SRD assay can also be used for the determination of HA content in seasonal cell culture-derived influenza vaccines using cell-derived SRD standards.

Further challenges for the practical application of cell-based seasonal influenza vaccines

To establish a basis for producing seed viruses used in cell-based seasonal influenza vaccines, a certified NIID-MDCK cell line and its cell bank, a modified MDCK cell line with improved virus propagation, and an adventitious virus detection method were developed. The isolation rates and genetic and antigenic stability of the NIID-MDCK isolates were assessed. Moreover, the SRD method for the assessment of the vaccine quality was developed. Even though the cell-based production process has been established, there remains a technical challenge, including efficiently isolating a wide range of seasonal influenza viruses without introducing MDCK cell-adapted mutations. Beyond the technical issue, further challenges include: *i*) relatively high manufacturing cost versus egg-based influenza vaccines (*35*); *ii*) seasonal influenza is lower in priority than other health emergency risks; *iii*) limited function, human resources, and collaboration between industry and academia in research institutes capable of developing the vaccines; *iv*) insufficient strategic research funds for vaccine development; *v*) financial risk related to investment in vaccine manufacturing equipment; and *vi*) insufficient evidence demonstrating the benefits and effectiveness of developing cell-based influenza vaccines. It is essential to encourage participation by Japanese pharmaceutical companies, universities, and research institutions in vaccine development, as well as collaboration with overseas institutions, to make cell-based seasonal influenza vaccine development practical in Japan.

Conclusions

Over the last decade, several new platforms that do not use egg-based technologies have been developed to produce effective seasonal influenza vaccines in many countries worldwide, and efforts are being made to make cell-based seasonal influenza vaccines available in more regions. In Japan, effective cell-cultured seasonal influenza vaccine production strains have been developed, and a quality control system for cell-cultured seasonal influenza vaccines has been established in collaboration with vaccine manufacturers. Furthermore, Japan has substantially contributed to the field globally by not only originally establishing the certified NIID-MDCK cell line for vaccine production, but also by listing the cell line on the WHO-recommended list since the southern hemisphere influenza season of 2017 (*36*). Therefore, it can be used globally for human cell culture-derived CVV isolation for the development and production of annual seasonal influenza vaccines. In addition, Japan is the sole nation promoting cell-based seasonal influenza vaccine production as a national project, and its advances and achievements have been drawing international attention. Although there are theoretical benefits of producing cell-based influenza vaccines, some drawbacks remain in the practical use of cell-based seasonal influenza vaccines in Japan. Limited isolation rates, poor growth, and antigenic changes in seasonal influenza viruses grown in cell lines reduce the selection and availability of viruses for the establishment of seed viruses and vaccine manufacture. In addition, even if highly proliferative isolates were obtained after passages in the NIID-MDCK cell line, antigenic mutations were introduced in the isolates. Therefore, it is necessary to focus on more effective alternative strategies using cell lines to propagate primary influenza isolates and distribute effective influenza viruses to vaccine manufacturers in Japan.

Numerous clinical studies have shown that cell-based inactivated influenza vaccines are more effective than egg-based inactivated influenza vaccines (*37,38*). Hence, it is expected that the use of cell-based seasonal influenza vaccines will further improve the efficacy of the vaccine in future years.

One of the challenges identified in this study was that previous research has demonstrated that the antigenic changes that occur during egg acclimation can be avoided by growing cultured cells. A further challenge was avoiding mutations caused by egg culture, but it became clear that antigenic mutations occur even in MDCK cell lines due to cell adaptation. We need to demonstrate the advantages of this manufacturing strategy, such as the ability to avoid changes in antigenicity that would otherwise occur, and promote research and development

through industry-academia-government collaboration.

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